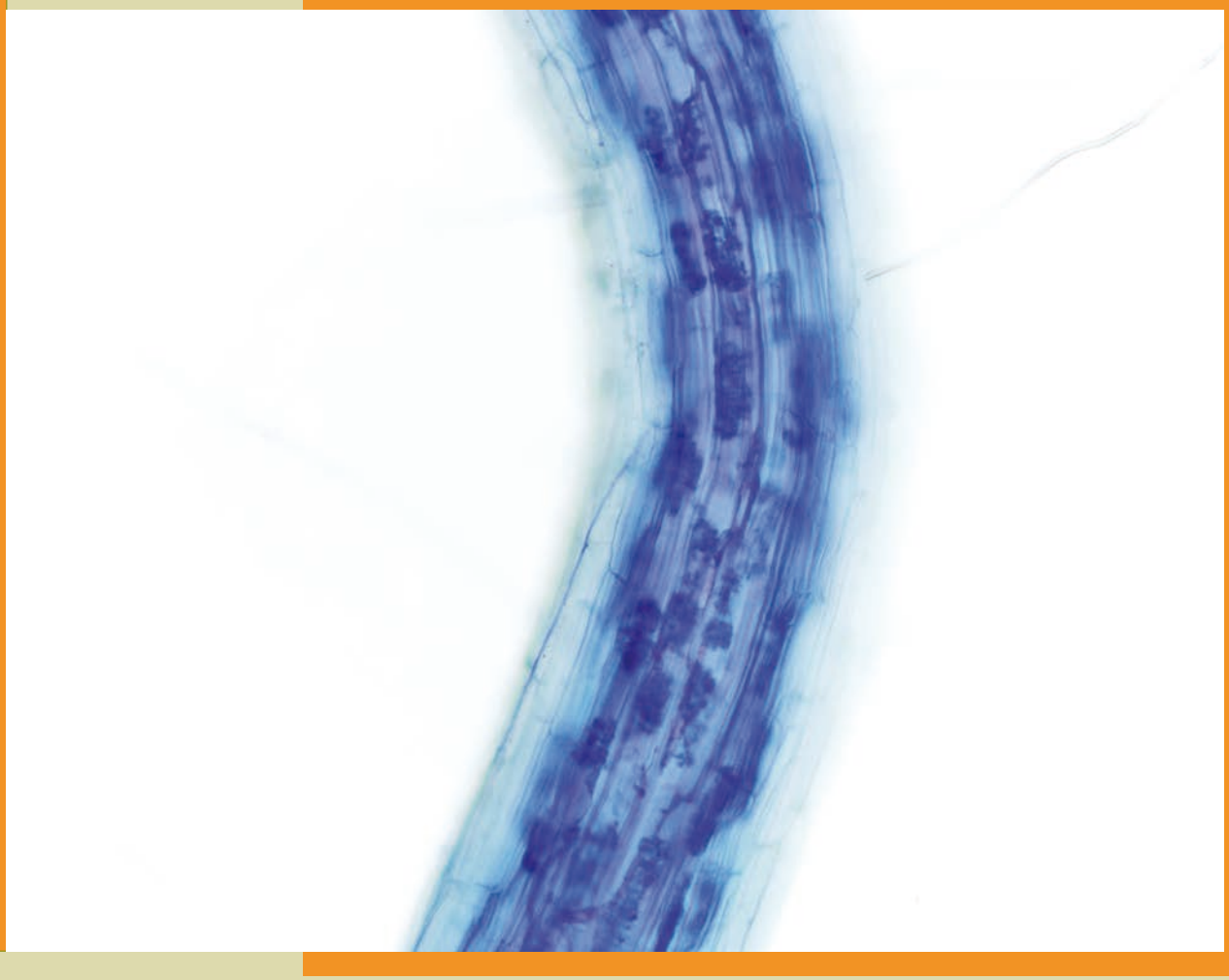


118 • 3
2022

ACTA AGRICULTURAE SLOVENICA

Acta agriculturae Slovenica • eISSN 1854-1941 • 118 – 3 • Ljubljana, oktober 2022

Univerza *v Ljubljani*
Biotehniška fakulteta

Acta agriculturae Slovenica **eISSN 1854-1941**
Letnik / Volume 118 · Številka / Number 3 · 2022

Ovitek: Arbuskularna mikoriza v koreninah koruze (*Zea mays* L.). Temno modro obarvane zgostitve so arbuskuli, ki so pomembni za izmenjavo hranil med arbuskularnimi mikoriznimi (AM) glivami in rastlinami (Foto: Irena Maček, 1–13)
*Cover: Arbuscular mycorrhiza in roots of maize (*Zea mays *L.). In dark blue are arbuscules, which are important for nutrient exchange between arbuscular mycorrhizal (AM) fungi and plants (Photo: Irena Maček, 1–13)*

Acta agriculturae Slovenica
Volume / Letnik **118** · Number / Številka **3** · **2022**

Table of Contents / Kazalo

Original Scientific Article / Izvirni znanstveni članek

Review Article / Pregledni znanstveni članek

Short Scientific Article / Kratki znanstveni prispevek

doi:10.14720/aas.2022.118.3.2344 Original research article / izvirni znanstveni članek

Genetic characterization of maize (*Zea mays* L.) landraces grown in Kosovo assessed by MITE-Hbr markers

Barbara PIPAN [1], Sali ALIU [2, 3], Dukagjin ZEKA [2], Vladimir MEGLIČ [1]

Received September 15, 2021; accepted June 29, 2022.
Delo je prispelo 15. septembra 2021, sprejeto 29. junija 2022

Genetic characterization of maize *(Zea mays* L.) landraces grown in Kosovo assessed by MITE-Hbr markers

Abstract: The aim of this study was to examine and describe genetic structure on autochthonous maize germplasm (flint types) from different localities in Kosovo using Hbr markers. The genetic characterization of 6-8 individual seedlings per each of 20 landraces was conducted by Hbr display calculated per selective base, the most efficient genetic diversity estimator to distinguish between landraces was primer combination Hbr-Int5-F/MseI+T. The strongest genetic relatedness (r = 55.57) had landrace ACC4 having orange colored seeds, showing the highest genetic uniformity when compared to other accessions. Clustering analysis using the Bayesian approach generated two genetic clusters for observed landraces. As a measure of population structure influenced by genetic drift and migration, Fst values for each genetic cluster were obtained. Higher Fst (0.4027) was calculated within the first genetic group comparing to the second one (0.2001), reflecting a higher levels of out-crossing and conservation between landraces from the first genetic cluster. A similar distribution of genetic linkages was observed from dendrogram, constructed using Dice coefficient and neighbour-joining (NJ) algorithm with minor deviations for landraces ACC6 and ACC28. Genotypes of ACCmk landrace reveal the highest genetic distinction compared to other genotypes, reflecting the highest number of bands (241) and the highest number of private bands (10) as the number of bands unique to a single population, respectively.

Key words: genetic variability; heartbreaker family markers; maize

Genetska karakterizacija lokalnih sort koruze (*Zea mays* L.) gojenih na Kosovu ovrednotena z MITE-Hbr označevalci

Izvleček: Namen raziskave je bil preučiti in opisati genetsko strukturo avtohtone dednine trdinke iz različnih lokalitet Kosova s Hbr označevalci. Genetska karakterizacija 6-8 sejank od vsake lokalne sorte je bila izvedena s prikazom nabora Hbr profilov, izračunanega na osnovi selektivne baze. Najučinkovitejši določitelj genetske raznovrstnosti za razločevanje med lokalnimi sortami je bila kombinacija primerjev Hbr-Int5-F/MseI+T. Najmočnejšo genetsko povezanost med genotipi (r = 55.57) je imela lokalna sorta ACC4 z oranžnimi zrni, ki je izkazovala največjo genetsko izenačenost v primerjavi z drugimi akcesijami. Klasterska analiza z uporabo modela aposteriorne verjetnosti (Bayesian approach) je za vključene lokalne sorte oblikovala dve genetski skupini. Kot merilo za analizo populacijske structure, na katero vplivata genetski zdrs in migracija, so bile izračunane vrednosti Fst za obe genetski skupini. Večja vrednost Fst (0,4027) je bila izračunana znotraj prve genetske skupine v primerjavi z drugo (0,2001), kar kaže na večji delež navskrižnega križanja in ohranjanja raznolikosti med lokalnimi sortami prve genetske skupine grozda. Podobna porazdelitev genetskih povezav je bila določena na dendrogramu, izdelanem z uporabo Dice-ovega koeficienta podobnosti in algoritma razvrščanja po metodi združevanja najbližjih sosedov (NJ) z manjšim odstopanjem za akcesiji ACC6 in ACC28. Genotipi akcesije ACCmk so se genetsko najbolj razlikovali od drugih na osnovi največjega števila prisotnih namnožkov (241) in največjega števila prisotnih "privatnih namnožkov" (10) in v številu namnožkov, ki so bili omejeni samo na eno populacijo.

Ključne besede: genetska raznolikost; družina "Heartbreaker" označevalcev; koruza

1 Crop Science Department, Agricultural Institute of Slovenia, Hacquetova ulica 17, SI-1000 Ljubljana, Slovenia
2 Department of Crop Science, Faculty of Agriculture and Veterinary, Prishtina, University of Prishtina, Kosovo
3 Corresponding author,e-mail: sali.aliu@uni-pr.edu

1 INTRODUCTION

Maize (Zea mays L.) is one of the most genetically diverse and widespread crops in South-East Europe (Đalović et al., 2015; Ignjatović-Micić et al., 2015). Maize landraces have been largely replaced by commercial maize hybrids in Kosovo. The share of hybrids was 4 % in the 1960's and already at 90 % in the early 2000's due to their high yield potential. Studies of specific combining abilities of inbred lines and physiological traits of some hybrids were already performed in agroecological conditions of Kosovo (Aliu et al., 2008, 2010). Maize became widely investigated plant species regarding different applications; its water use efficiency (Wang et al., 2013), morphological, physiological and biochemical response to SiO_2 nanoparticles (Sharifi-Rad et al., 2016), different irrigation regimes and planting methods (Singh Brar *et al.*, 2016). There are also different marker systems, applied to assess genetic characterization of maize germplasm, including RAPD (Random Amplified Polymorphic DNA) (Srdić et al., 2007), SSR (Simple Sequence Repeats) (Ignjatović-Micić et al., 2015) and MITE-Hbr (Miniature Inverted Repeat Transposable Element from the family Heartbreaker) (Casa et al. (2000; 2002) and Kavar et al. (2007)). MITE-Hbr markers have been firstly exploited and developed as a new marker system (modification of the AFLP-Amplified Fragment Length Polymorphism procedure) for evaluation of the maize genome (Casa et al. (2000; 2002)). Kavar et al. (2007) used Hbr markers to evaluate the genetic diversity of Slovenian maize germplasm, originating from Western Balkan (former Yugoslavia). Related to those three reports, MITE-Hbr markers were proven to be stable, highly polymorphic, cost-effective, easily mapped and evenly distributed throughout the maize genome.

SSRs are highly applicable markers in our genetic studies of different plant species, among other grapevines (Rusjan etal., 2012, 2015) sweet potato (Pipan et al., 2016), brassicas (Pipan et al., 2011, 2013), and beans (Maras et al., 2015). In a study by Ignjatović-Micić et al. (2015), using SSR markers, they reported that higher genetic variation was observed among flint genotypes, comparing to dent ones. They also suggest that landraces from Western Balkans are highly adapted to specific environmental conditions and uses and therefore could be a valuable source of genetic variability. The adaptation to diverse agro-ecological conditions is a result of natural and selection by farmers.

The aim of this study was to examine and describe genetic structure of autochthonous maize germplasm from Kosovo using Hbr marker system and to employ Hbr display to show genetic differences and associations between and within observed flint landraces of maize collected in different localities of Kosovo, with a possibility to detect conservation of gene flow into the maize genome. The knowledge of genetic characteristics and landrace-specific background of maize germplasm would be of a benefit for future breeding and germplasm improvement programmes in Kosovo.

2 MATERIALS AND METHODS

Twenty maize landraces, collected from different locations in Kosovo (Table 1), were screened using MITE-Hbr markers. DNA from 6 - 8 individuals of each landrace was extracted from each individual seedling using BioSprint 15 DNA Plant Kit (Qiagen) and MagMax Express Magnetic Particle Processor (Life Technologies, Grand Island, NY) following manufacturer's instructions. Hbr display with some modifications was performed as described by Casa et al. (2000, 2002) and Kavar et al. (2007). 600 ng of genomic DNA was digested for 3 h at 65 °C in 20 µl of 10x Tango buffer containing MseI (Fermentas). Adaptors (5'gacgatgagtcctgag and 5'tactcaggactcat) to the digested DNAs and aliquots of the restriction/ligation reactions were visualized on 0.9 % agarose gels to check the quality of DNA digestion. Pre-selective amplification was performed using primers Hbr-Int5-E (5'gattctccccacagccagattc) and MseI+0 (5'gacgatgagtcctgagtaa). Selective amplification was performed with each of the three selective primer combinations (MseI+C, MseI+G and MseI+T) with a fluorescently labeled Hbr internal primer (5'-6FAM-agccagattttcagaaaagctg). Fragment analysis was performed on the 3130XL Genetic Analyzer (Applied Biosystems), and sizing of fluorescent fragments/bands was determined by comparison with size standard GeneScan-500 ROX (Applied Biosystems) using GeneMapper 4.0 (Applied Biosystems). A binary matrix was constructed by scoring fragments as either present (1) or absent (0) in each DNA sample.

Principal Coordinate Analysis (PCoA), Analysis of Molecular Variance (AMOVA), number of different alleles (Na), number of effective alleles (Ne) and Shannon's information index (I) across landraces for each selective base was calculated in GenAlEx v.6.4 (Peakal and Smousse, 2006). Genetic similarities were calculated on the basis of a binary matrix using the Dice similarity index (Dice, 1945). These coefficients were used to construct the clustering using the neighbur-joining (NJ) algorithm by 100 bootstraps in FreeTree (Pavliček et al., 1999) and visualized using TreeView (Page, 1996) software. Genetic diversity parameters between and within landraces including AMOVA, band patterns (number of bands, number of private bands, number of locally common bands alleles occurring in 50 % or fewer

landraces, expected heterozygosity) and mean within landrace pairwise values (r) were conducted using GenAlEx v.6.4 (Peakal and Smousse, 2006). Structure 2.3.3 software (Pritchard et al., 2009) was employed for inferring landrace structure using a Bayesian approach. Ten independent runs for each K (from1 to 7) in the case of admixture model were performed and burning period of 10,000 followed by 100,000 Markov Chain Monte Carlo repeats was used. The ideal K-value was selected based on the increases in likelihood ratios between runs using Evanno's delta K statistic (Evanno et al., 2005) implemented in a Structure Harvester (Earl and von Holdt, 2011). The estimation of flowering time was made in the same year at different locations, which are presented in Table 1.

3 RESULTS

Kosovo landraces included in our study are all of a flint type with white kernel (fruit) color, except for a landrace ACC4, which kernels are orange (Table 1).

Genetic characterization of 6-8 individual seedlings per each landrace was conducted using Hbr display. A total of 498 markers, ranging in size from 60-500 bp, were generated using three primer combinations: Hbr-Int5-F/MseI+T, Hbr-Int5-F/MseI+C, and Hbr-Int5-F/MseI+G. Regarding genetic diversity estimators (PCoA, AMOVA, Na, Ne and I) calculated per selective base (T, C, G), the most efficient primer combination to distinguish between landraces from Kosovo, was Hbr-Int5-F/MseI+T, and Hbr-Int5-F/MseI+C respectively (Table 2). First three axes in PCoA (via covariance distance matrix) cumulatively explained 70 % of genetic variability for Hbr-Int5-F/MseI+T; 67 % for Hbr-Int5-F/MseI+C and 59 % for Hbr-Int5-F/MseI+G (Table 2). Percent of molecular variability among landraces in Hbr screening varied from 10 (Hbr-Int5-F/MseI+C) to 18 (Hbr-Int5-F/MseI+T), depending on selective primer applied (Table 2). Additionally, the highest values of Na (7.400), Ne (1.067) and I (0.074) were calculated for Hbr-Int5-F/MseI+T (Table 2).

Landrace-specific genetic diversity was estimated by applying different algorithms to compare a genetic composition among and within autochthonous landraces from Kosovo. The summary of mean within landrace pairwise values, based on genetic distance, is presented in Figure 1. The lowest mean r value was calculated

Table 1: Characteristics of maize landraces from Kosovo

Landrace label	Locality	Latitude [° ‘ ‘’]	Longitude [° ‘ ‘’]	Altitude [m]	Vernacular name	Landscape	Kernel type	Kernel Color	Kernel shape	Flowering time [days]
ACC2	Ferizaj	42.25.21	21.09.06	555	Bardhosh	Flat	Flint	White	Oval	69
ACC4	Shtime	42.26.40	21.42.96	642	Kolomboq	Mountain	Flint	Orange	Oval	72
ACC6	Skenderaj	42.44.39	20.48.04	603	Miser	Valley	Flint	White	Oval	71
ACC8	Skenderaj	42.44.39	20.47.39	597	Miser	Valley	Flint	White	Oval	75
ACC12	Skenderaj	42.45.00	20.48.23	623	Miser	Flat	Flint	White	Oval	76
ACC14	Drenas	42.39.30	20.42.46	565	Kolomboq	Flat	Flint	White	Oval	76
ACC16	Drenas	42.39.21	20.42.32	586	Kolomboq	Flat	Flint	White	Oval	75
ACC26	Vushtrri	42.48.38	20.58.30	518	Kolomboq	Flat	Flint	White	Oval	72
ACC28	Suharekë	42.21.45	20.49.02	388	Miser	Valley	Flint	White	Oval	77
ACC30	Vushtrri	42.50.46	20.59.26	557	Kolomboq	Flat	Flint	White	Oval	71
ACC32	Drenas	42.34.50	20.54.06	585	Kolomboq	Flat	Flint	White	Oval	71
ACC34	Podujevë	42.53.39	21.12.12	598	Kolomboq	Mountain	Flint	White	Oval	75
ACCmk	Lipjan	42.31.45	21.07.20	551	Miser	Flat	Flint	White	Oval	69
ACC38	Kamenicë	42.33.56	21.31.32	812	Kolomboq	Mountain	Flint	White	Longi	67
ACC40	Kamenicë	42.34.16	21.31.32	766	Kolomboq	Mountain	Flint	White	Oval	72
ACC42	Prishtinë	42.35.35	21.20.40	824	Kolomboq	Mountain	Flint	White	Oval	65
ACC44	Drenas	42.41.21	20.45.31	691	Kolomboq	Flat	Flint	White	Oval	71
ACC46	Malisheve	42.27.56	20.43.22	576	Miser	Mountain	Flint	White	Oval	72
ACC48	Malisheve	42.28.01	20.44.04	562	Miser	Mountain	Flint	White	Oval	70
ACC50	Drenas	42.41.50	20.44.43	567	Kolomboq	Flat	Flint	White	Oval	76

Table 2: Analysis of genetic diversity among landraces by selective bases

	Cumulative % in PCoA (first 3 axes)	AMOVA (% among landraces)	Na	Ne	I
Hbr-Int5-F/MseI+T	70	18	7.400	1.067	0.074
Hbr-Int5-F/MseI+C	67	10	6.850	1.066	0.072
Hbr-Int5-F/MseI+G	59	16	7.100	1.065	0.069

Notes: Na-The number of alleles; Ne- The number of detected effective alleles; I-Shannon's information index

across ACC50 (39.03) where r was also outside U and L limits (Figure 1) reflecting the weakest genetic relatedness of genotypes within ACC50 landrace. The strongest genetic relatedness (r = 55.57) was reached within orange colored seeds of ACC4 landrace (Figure 1) showing the highest genetic uniformity of included genotypes within ACC4 compared to other landraces. The genetic structure of observed landraces, described by Bayesian clustering approach in Figure 2 shows higher uniformity of ACC4, with 98.1 % probability that landrace ACC4 belongs to the first (red) genetic cluster and lower genetic uniformity within ACC50 with 79.9 % probability that genotypes from ACC50 belong to the second genetic cluster (green) which is also confirmed by r value (Figure 1).

In general, clustering analysis using the Bayesian method generated two genetic clusters (ideal K, conducted using Structure Harvester) for observed landraces (Figure 2).

Similary colorored segments represents the estimated membership to the genetic cluster. The first genetic cluster (red) posses 0.0778 of expected heterozygosity between genotypes and 0.0925 was calculated for the second one (green), respectively. Regarding their genetic structure, landraces ACC6, ACC12, ACC14, ACC34,

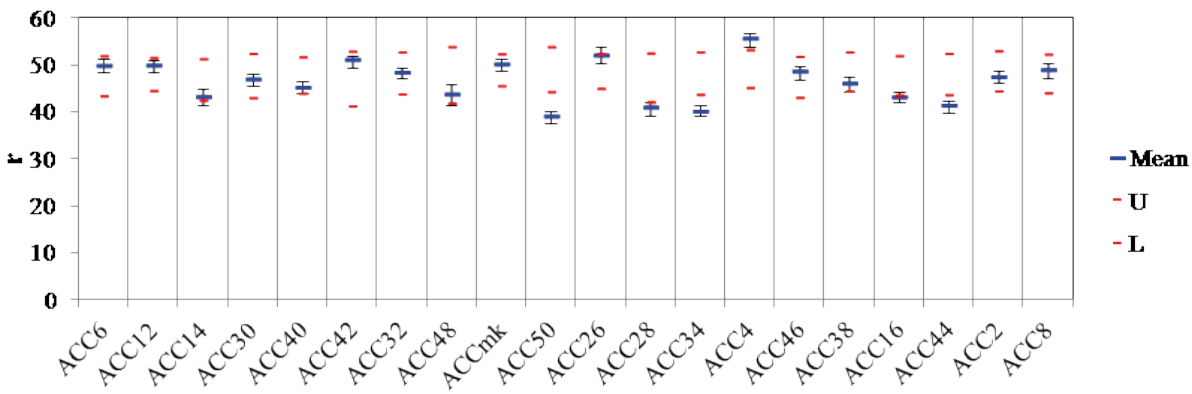


Figure 1: Mean within landrace pairwise values (r) according to genetic distance. Upper (U) and lower (L) confidence limits bound the 95 % confidence interval about the null hypothesis of 'No difference ' across the landraces as determined by permutation (99)

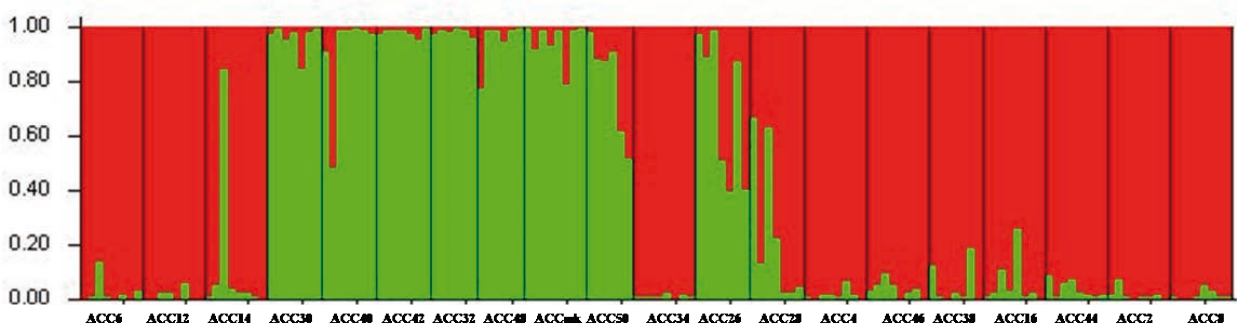


Figure 2: Structure plot of maize landraces from Kosovo

ACC28, ACC4, ACC46, ACC38, ACC16, ACC44, ACC2, and ACC8 belong to the first cluster of landraces (red); meanwhile landraces ACC30, ACC40, ACC42, ACC32, ACC48, ACCmk, ACC50, and ACC26 comprise the second genetic cluster (green) (Figure 2). A similar distribution of genetic relations between landraces was observed in a dendrogram, constructed using Dice coefficient (Dice, 1945) and the NJ algorithm with minor deviations for landraces ACC6 and ACC28 (Figure 3).

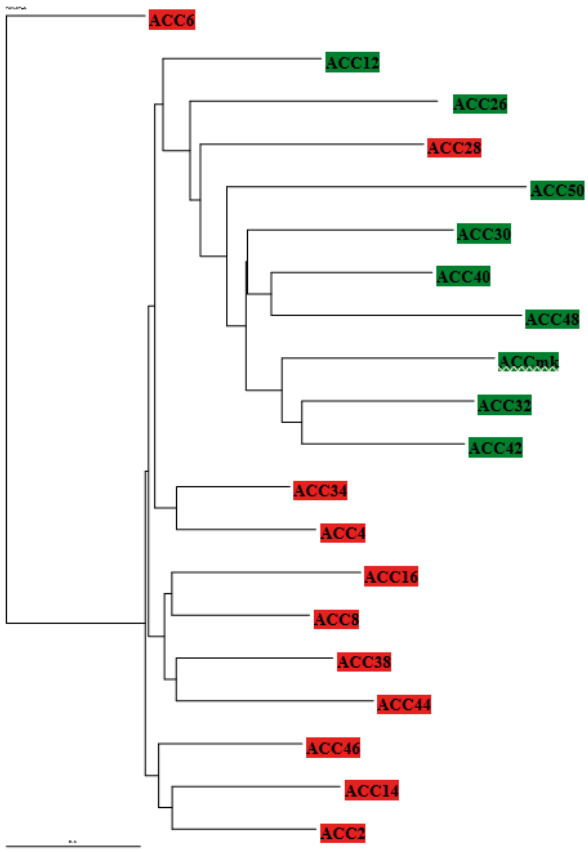


Figure 3: Genetic linkages of observed landraces from Kosovo using Dice coefficient and neighbour-joining (NJ) algorithm. (Green and red colours assign cluster colours from structure plot on Figure 2)

We have calculated landrace-specific parameters of genetic diversity (Figure 4) to compare genetic characteristics between all maize landraces collected, applying Hbr display. Genotypes of landrace ACCmk reveal the highest genetic distinction compared to genotypes within other landraces, reflecting the highest number of bands (241) and the highest number of private bands (10) (Figure 4), respectively.

A number of common bands with a frequency of > 5 %, which are found in the 50 % assessed landraces, reached the highest values for ACC40 (115), ACC32 (114) and ACCmk (111), respectively (Figure 4).

Evaluation of genetic differentiation within and between landraces, applying Hbr markers, provided useful information about genetic the structure, relatedness and genetic diversity of autochthonous maize germplasm from Kosovo. Genetic uniformity of genotypes within landraces is high, regardless to low values of expected heterozygosity (max He = 0.067, data not shown) as a measure of genetic diversity within landraces. Genotypes within the second (green) cluster reveal lower genetic diversity (Fst = 0.2001) compared to the first (red) cluster (Fst = 0.4027).

4 DISCUSSION

Results presented in Table 2 indicate that Hbr-Int5-F/MseI+T is the most informative selective primer provided by Hbr display to distinguish twenty maize landraces collected from different locations in Kosovo. In the study by Kavar et al. (2007) evaluating Slovenian maize landraces, the most informative primer combination was Hbr-Int5-F/MseI+G, revealing the highest number of loci (103) scored using Hbr display. On the other hand, calculated genetic diversity values for maize landraces from Kosovo are similar using Hbr display. This was as well the case evaluating Slovenian accessions by Kavar et al. (2007), where similar values of scored loci (73-103) were obtained applying different selective primers. Cluster analysis using Bayesian approach revealed no genetic relatedness (regarding their genetic

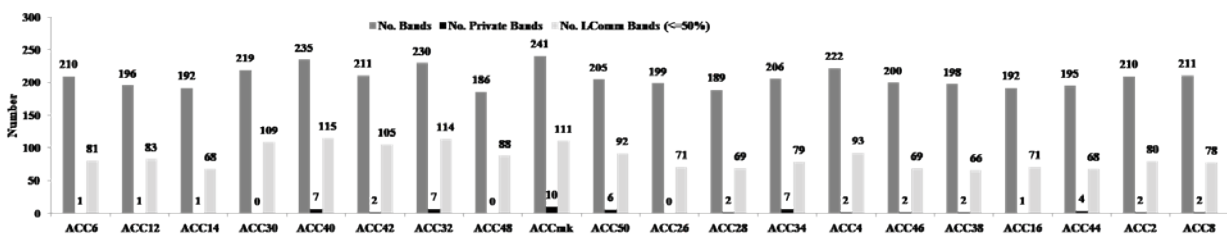


Figure 4: Band patterns across maize landraces from Kosovo

structure) between landraces from the same geographic origin (locality and landscape), a name of landrace and kernel shape (no difference for ACC38 which is oval), respectively. Structure plot also shows that individual plants from landraces ACC14 (87.3 % red, 12.7 % green), ACC50 (20.1 % red; 79.9 % green), ACC26 (28.0 % red, 72.0 % green), and ACC28 (75.0 % red, 25.0 % green) are sharing on average higher proportion of germplasm belonging to both genetic clusters (Figure 2). According to the data in Table 1, four landraces named Kolomboq (vernacular name or local name), originating from flat landscape have the longest flowering period of more than 72 days. There is one exception in this cluster, landrace ACC28 Miser (local name), originating from the valley (Suharekë) with the longest flowering period (77 days). A difference in landrace distributions for genetic clustering, when comparing the structure plot (Figure 2) and dendrogram (Figure 3) could be a logical consequence of different algorithms/approaches applied for specific purpose, required for genetic diversity assessment.

According to the landrace-specific parameters of genetic diversity (Figure 4), landrace ACCmk represents a potentially interesting source for further genetic studies and germplasm improvement. A high number of private bands actually represents unique copies of *Hbr* transposons that ACCmk landrace harbors compared to private alleles observed in other studies using SSR markers. Private alleles in those cases could reflect accumulation and conservation of introduced genes via out-crossing along generations to the plant genome (Pipan et al., 2013). Calculated number of common bands with a frequency > 5 % (Figure 4) for landraces ACC40, ACC32 and even ACCmk, are sharing the highest number of scored bands with other landraces, even though that the three landraces belong to the second (green) genetic cluster/group (Figures 2 and 3). Evaluation of maize landraces from Kosovo was successfully assessed by a rare type of marker system, miniature inverted repeat transposable element - Hbr marker, using three selective primer combinations. To distinguish genotypes between and within different landraces, application of only one selective marker could be sufficient, as reported Kavar et al. (2007). Application of SSR markers as a codominant marker system is also used in genetic diversity studies of maize landraces (Ignjatović-Micić et al., 2015). According to the results presented, there are strong genetic relations between different landraces, comprising two genetic groups, which could be assigned to the two general micro centers of diversity in Kosovo. As a measure of a population structure influenced by genetic drift and migration, Fst values for each genetic cluster generated using Bayesian cluster analysis, were obtained. Higher Fst (0.4027) was calculated within the first (red) genetic group compared to the second (green) one (0.2001), reflecting higher levels of out-crossing and conservation between landraces from the first genetic cluster. This result could be also a consequence of simultaneous cultivation and uncontrolled gene flow between observed maize landraces listed in Table 1 within the same production area of Kosovo in the past.

5 CONCLUSIONS

Related to mean r value, there is 29.7 % of a variable genetic part, which is dispersed along included landraces from Kosovo. It is important to point out that landraces evaluated, originated from different localities with a diverse landscape (flat, mountain, and valley) and from various production areas. According to the results, there are strong genetic relations between different landraces, comprising from two genetic groups, which could indicate on two micro diversification locations of flint type in Kosovo. The results provided, we can conclude that MITE-Hbr markers are highly applicable and cost-effective tool for maize genetic diversity studies and as in this case for a genetic distinction between and within landraces collected in Kosovo.

6 ACKNOWLEDGEMENT

This work was in part financially supported by grant P4-0072 (Agrobiodiversity programme) from the Slovenian Research Agency. We are thankful to the mag. Romana Rutar providing seedlings, dr. Katarina Rudolf-Pilih for an extra hand with DNA extraction and to the dr. Tatjana Kavar for useful tips optimizing the Hbr protocol.

7 REFERENCES

Aliu, S., Fetahu, S. and Rozman, L. (2010). Variation of physiological traits and yield components of some maize hybrid (*Zea mays* L.) in agroecological conditions of Kosovo. *Acta Agriculturae Slovenica., 95*(1), 35-41.

Aliu, S., Fetahu, S., Rozman, L. and Salillari, A. (2008). General and specific combining ability studies for leaf area in some maize inbreds in agroecological conditions of Kosovo. *Acta Agriculturae Slovenica.*, *91*(1), 67-73. https://doi.org/10.2478/v10014-008-0007-4

Casa, A. M., Brouwer, C., Nagel, A., Wang, L., Zhang, Q., Kresovich, S. and Wessler, S. R. (2000). The MITE family heartbreaker (Hbr): Molecular markers in maize. *Proceeding of the National Academy of Sciences of the. USA*, *97*, 10083–10089. https://doi.org/10.1073/pnas.97.18.10083

Casa, A. M., Mitchell, S. E., Smith, O. S., Register, III J. C., Wessler, S. R. and Kresovich, S. 2002. Evaluation of Hbr (MITE) markers for assessment of genetic relationships among maize (*Zea mays* L.) inbred lines. *Theoretical and Applied Genetics*, *104*, 104–110. https://doi.org/10.1007/s001220200012

Đalović, I., Jocković, Đ., Chen, Y., Bekavac, G., Šeremešić, S., Jaćimović, G. and Brdar-Jokanović, M. 2015. Maize nutrient uptake affected by genotype and fertilization. *Genetika*, *47*(3), 941-950. https://doi.org/10.2298/GENSR1503941D

Dice, L. R. (1945). Measures of the amount of ecologic association between species. *Ecology*, *26*(3), 297–302. https://doi.org/10.2307/1932409

Earl, D. A. and Von Holdt, B. M. (2011). Structure harvester: A website and program for visualizing structure output and implementing the Evanno method. *Conservation Genetics Resource, 3*, 429–431.

Evanno, G., Regnaut, S. and Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: Asimulation study. *Molecular Ecology*, *14,* 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Ignajtović-Micić, D., Ristić, D., Babić, V., Adjelković, V. and Vancetović, J. (2015). A simple SSR analysis for genetic giversity estimation of maize landraces. *Genetika*, *47*(1), 53-62. https://doi.org/10.2298/GENSR1501053I

Kavar, T., Meglič, V. and Rozman, L. (2007). Diversity of Slovenian maize (*Zea mays* L.) populations by Hbr (MITE) markers and morphological traits. *Russian Journal of Genetics, 43*(9), 989-995. https://doi.org/10.1134/S1022795407090049

Maras, M., Pipan, B., Šuštar-Vozlič, J., Todorović, V., Đurić, G., Vasić, M., Kratovalieva, S., Ibusoska, A., Agić, R., Matotan, Z., Čupić, T. and Meglič, V. (2015). Examination of genetic diversity of common bean from the Western Balkans. *Journal of American Society for the Horticultural Science, 140*(4), 208-316. https://doi.org/10.21273/JASHS.140.4.308

Page, R. D. M. (1996). TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Bioscience, 12*, 357–358. https://doi.org/10.1093/bioinformatics/12.4.357

Pavliček, A., Hrda, S. and Flegr, J. (1999). Freetree - freeware program for construction of phylogenetic trees on the basis of distance data and bootstrap/jackknife analysis of the tree robustness. Application in the RAPD analysis of the genus *Frenkelia. Folia Biologica (Praha), 45*, 97–99.

Peakall, R. and Smouse, P. E. (2006). GenAlEx 6: Genetic analysis in excel. Population genetic software for teaching and research. *Molecular Ecology Notes, 6*, 288–295. https://doi.org/10.1111/j.1471-8286.2005.01155.x

Pipan, B., Šuštar-Vozlič, J. and Meglič, V. (2011). Cultivation, varietal structure and possibilities for cross-pollination of *Brassica napus* L. in Slovenia. *Acta Agriculturae Slovenic.*, *97*(3), 247-258. https://doi.org/10.2478/v10014-011-0019-3

Pipan, B., Šuštar-Vozlič, J. and Meglič, V. (2013). Genetic differentiation among sexually compatible relatives of *Brassica napus* L. *Genetika*, *45*(2), 309–327. https://doi.org/10.2298/GENSR1302309P

Pipan, B., Žnidarčič, D. and Meglič, V. (2016). Evaluation of genetic diversity of sweet potato [*Ipomoea batatas* (L.) Lam.] on different ploidy levels applying two capillary platforms. *Journal of Horticulture Science and Biotechnology*, doi.org/10.1080/14620316.2016.1249963. https://doi.org/10.1080/14620316.2016.1249963

Pritchard, J. K., Wen, X. and Falush, D. (2009). *STRUCTURE ver. 2.3*. Univ. Chicago, Chicago, IL. Available at http://pritch.bsd.uchicago.edu/ (April 2016).

Rusjan, D., Pipan, B., Pelengić, R. and Meglič, V. (2012). Genotypic and phenotypic discrimination of grapevine (*Vitis vinifera* L.) varieties of the 'Vitovska' and 'Garganja' denominations. *European Journal of Horticultural Science*, *77*(2), 84–94.

Rusjan, D., Pelengić, R., Pipan, B., Or, E., Javornik, B., and Štajner, N. (2015). Israeli germplasm: Phenotyping and genotyping of native grapevines (*Vitis vinifera* L.). *Vitis*, *54*, 87-89.

Sharifi-Rad, J., Sharifi-Rad, M. and Teixeira da Silva, J. A. (2016). Morphological, physiological and biochemical responses of crops (*Zea mays* L., *Phaseolus vulgaris* L.), medicinal plants (*Hyssopus officinalis* L., *Nigella sativa* L.), and weeds (*Amaranthus retroflexus* L., *Taraxacum officinale* F. H. Wigg) exposed to SiO2 nanoparticles. *Journal of Agricultural Science and Technology*, *18,* 1027-1040.

Singh Brar, H., Kumar Vashist, K. and Bedi, S. (2016). Phenology and yield of spring maize (*Zea mays* L.) under different drip irrigation regimes and planting methods. *Journal of Agricultural Science and Technology*, *18*, 831-843.

Wang, X. J., Jia, Zh. K., Liang, L. Y. and Kang, Sh. Zh. (2013). Effect of manure management on the temporal variations of dryland soil moisture and water use efficiency of maize. *Journal of Agricultural Sciencem and Technology*, *15*, 1293-1304.

doi:10.14720/aas.2022.118.3.2643 Original research article / izvirni znanstveni članek

Efficacy of *Bacillus subtilis* (Ehrenberg1835) Cohn1872, in suppressing *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen, the causal agent of root rot of date palm offshoots (*Phoenix dactylifera* L.) in Iraq

Naji Salim JASSIM [1, 2], Muntaha Abd. ATI [1]

Received March 31, 2022; accepted July 03, 2022.
Delo je prispelo 31. marca 2022, sprejeto 3. julija 2022

Efficacy of *Bacillus subtilis* (Ehrenberg1835) Cohn1872, in suppressing *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen, the causal agent of root rot of date palm offshoots (*Phoenix dactylifera* L.) in Iraq1

Abstract: Date palm root rot disease is one of the most important diseases of date palms and offshoots. It is caused by many soil-borne pathogenic fungi. Pathogenicity assays of the isolated fungi showed that the major causative agents of root rot disease in date palm plantlets were *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen, *F. proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg S1, *F. proliferatum* S2, *Gibberella fujikuroi* (Sawada) Wollenw., and *Rhizoctonia solani* J.G. Kühn. The most virulent fungus was *F. oxysporum* with a severity index of 82.16 % of root rot, while *R. solani* was the least harmful with a disease severity rate of 12.42 %. In laboratory tests, *Bacillus subtilis* reduced the radial mycelial growth of *F. oxysporum* on PDA medium by 86.6 %. The application of *B. subtilis* in combination with *F. oxysporum* substantially inhibited the severity of root rot disease relative to plantlets treated with only *F. oxysporum*. In addition, *B. subtilis* application in the presence or absence of *F. oxysporum* improved the plant physiology of plantlets, including total chlorophyll, total carotenoid, antioxidant enzyme levels (catalase and peroxidase), and total proline content.

Key words: *B. subtilis*; date palm; *F. oxysporum*; plant physiology

Učinkovitost bakterije *Bacillus subtilis* Ehrenberg 1835) Cohn 1872 pri zatiranju glive *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen, kot povzročiteljice koreninske gnilobe pri dateljevi palmi (*Phoenix dactylifera* L.) v Iraku

Izvleček: Koreninska gniloba je najpomembnejša bolezen dateljeve palme. Povzročajo jo številne talne patogene glive. Preiskus patogenosti z izolati gliv na sadikah dateljeve palme je pokazal, da so bili glavni povzročitelji njene koreninske gnilobe naslednje glive: *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen, *F. proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg S1, *F. proliferatum* S2, *Gibberella fujikuroi* (Sawada) Wollenw., and *Rhizoctonia solani* J.G. Kühn. Najbolj virulentna je bila gliva *F. oxysporum*, z indeksom virulentnosti 82,16 % med tem, ko je bila gliva *R. solani* najmanj škodljiva z indeksom povzročitve koreninske gnilobe 12,42 %. V laboratorijskem poskusu je bakterija *B. subtilis* na PDA gojišču zmanjšala radialno rast micelija glive *F. oxysporum* za 86,6 %. Uporaba bakterije *B. subtilis* je v kombinaciji z glivo *F. oxysporum* znatno zavrla razvoj koreninske gnilobe na sadikah dateljeve palme v primerjavi s sadikami, ki so bile tretirane samo z glivo. Dodatno je uporaba bakterije *B. subtilis* v prisotnosti ali odsotnosti glive *F. oxysporum* izboljšala fiziološke parametre sadik kot so vsebnost celokupnega klorofila in karotenoidov, aktivnost antioksidacijskih encimov katalaze in peroksidaze ter vsebnost celokupnega prolina

Ključne besede: *B. subtilis*; dateljeva palma; *F. oxysporum*; fiziološki parametri rastline

1 Plant pathology, Date Palm Research Center, University of Basrah, Basrah, Iraq
2 Corresponding author, e-mail: ahmidnaji916@gmail.com

1 INTRODUCTION

Date palm (*Phoenix dactylifera* L.), Palmaceae (Arecacea), is a tropical and subtropical plant native to southern Asia and Africa. Selective breeding over thousands of years has resulted in the 3,000 variants presently farmed across the world in areas where the date palm thrives in hot, dry climates (Zaid, 2002). Dates are high in nutrients and provide a wonderful source of energy. Date fruits are composed of 70 % carbohydrates, primarily sugars, and 15 %-30 % water. Dates are also a good source of minerals, including iron, potassium, and calcium, and are low in salt and fats (Thabet et al., 2010; Dayani et al., 2012).

Date palms are infected by many soil-borne pathogenic fungi that threaten mature trees and offshoots, resulting in substantial tree damage and yield losses across the world (El-Morsi et al., 2009; Maitlo et al., 2013). In several locations, pathogenic fungi of root rot and wilt disease caused by *Fusarium oxysporum*, *Fusarium solani*, *Fusarium moniliform*, and *Rhizoctonia solani* have been isolated from young offshoots and adults of the date palm (*Phoenix dactylefera* L.) (Alwahshi et al., 2019; Arafat et al., 2012; Baraka et al., 2011).

Chemical pesticides used to manage soil-borne diseases can result in pathogen resistance, negative effects on people and beneficial soil organisms, and pollution. Several soil fumigants and fungicide compounds are expected to be phased out soon. However, in order to achieve optimal plant development and production, soil pathogens will still need to be managed (Gerhardson, 2002). As a result, efficient beneficial microbes that can be used as an alternate method for controlling soil pathogens need to be discovered. For managing many soil-borne diseases, microbial antagonists such as bacteria and fungi have the potential to be a low-cost, healthy, and ecologically friendly solution (Caron et al., 2002; Gravel et al., 2004).

Bacillus species proliferate quickly, are resistant to harsh environmental conditions, and have been identified as beneficial microorganisms. Antibiotics; competition with pathogens for space or resources; destruction of pathogen hyphae; synthesis of siderophores and phytohormones that stimulate plant development; and induced systemic resistance (ISR) in the host plant are all modes of action by which *Bacilus subtilis* suppresses plant pathogens (Cao et al., 2012; Chen et al., 2020; Li et al., 2013). By induction of systemic resistance in plants, *B. subtilis* generates volatile chemicals that influence plant development and activate the plant defense mechanism (Hashem et al., 2019; Wang et al., 2018). *Bacillus* spp. also produce endospores, which allow the bacteria to live in harsh environments, allow for germination in response to varied environmental circumstances, allow for long-period storage of biopesticide, and make the formulation process easier (Collins & Jacobsen, 2003). The US Food and Drug Administration (USFDA) classifies *B. subtilis* as "generally recognized as healthy" (GRAS) for use in the food processing industry.

This work aimed to measure the efficacy of *B. subtilis* strains against *F. oxysporum* under greenhouse conditions. Second, levels and activity of some physiological and biochemical components were measured in date palm during infection by *F. oxysporum* in the presence and absence of *B. subtilis*, and compared to the control treatments.

2 MATERRIALS AND METHODS

2.1 PATHOGENICITY ASSAY OF ISOLATED FUNGI

Representative fungal isolate strains used throughout this study were obtained from a previous study carried out in the Biology Department, College of Science, University of Basrah, Basrah by Kazaal (2019). These isolates are *Fusarium oxysporum*, *Fusarium proliferatum* S1, *Fusarium proliferatum* S2, and *Fusarium fujikuroi*. *Rhizoctonia solani* isolate obtained from the lab of date palm diseases/Date Palm Research Center/University of Basrah. Infection trials with the recovered isolates run in a greenhouse trial at the Date Palm Research Center. Six-month-old plantlets (grown from seeds of the Halawii cultivar) were planted into plastic pots (2 kg pots) filled with sterilized soil (1 : 1 peat moss + sand). To prepare the fungi inoculant, each isolate was cultivated on PDA for 5–15 days at 27 °C. The spore suspension of each isolate was made by flooding plates of 15-day-old cultures with sterile distilled water, scraping with a sterilized glass rod, filtering, and adjusting to a 10^6 spore ml^{-1} concentration using a Neubauer haemocytometer before adding to the potted soil. Potted soil was injected with each fungus inoculum at a concentration of 10^6 spores ml^{-1} with irrigation water (Al-Ani et al., 2012). Each fungus (treatment) has five pots (replicates) with three plantlets, and a control treatment (uninfected soil). The pots were in the greenhouse under favorable conditions. For three months, pots were kept at 90 % soil humidity. The pots were carefully watered every time at the level of the field capacity. The percentage of disease severity is calculated after 60 days from inoculation using the following scale (with little modulation) of 0–5: where 0 = healthy; 1 = 1-25 % of the plant has a few spots on the roots; 2 = 26-50 % of the roots have spots and one leaf is wilting; 3 = 51-75 % of the roots have big black spots and all leaves are

wilting; 4 = up to 76 % of roots are rotted and all leaves are wilting, and5 = dead plants (Abdou et al., 2003).

The disease severity index (*DSI*) of each replicate was calculated according to the method described by Liu et al. (1995) as follows: $DSI = \Sigma d/(d_{max} \times n) \times 100$, where d = the disease rating of each plantlet, d_{max} = the maximum rate of disease, and n = the total number of plantlets in each replication assessed.

2.2 EFFECT OF *B. SUBTILIS* AGAINST THE GROWTH OF *F. OXYSPORUM IN VITRO*

The purpose of the experiment was to determine the antagonistic connection between the most virulent fungus, *F. oxysporum* and *B. subtilis* (*Bacillus subtilis* was isolated by serial dilution technique on nutrient agar medium (NAM). A 0.5 g of BioHealth biopesticide was separated and vortexed for 15 minutes in 10 ml of distilled water. From 10^{-1} to 10^{-6}, the suspension was serially diluted. 1 ml of suspension was pipette out and distributed with a glass rod in an L shape onto nutrient agar plates and incubated at 37 °C for 24 hours. For subsequent research, the most conspicuous colonies were separated and kept at 4 °C. The *in vitro* effect of *B. subtilis* on colony growth of *F. oxysporum* was assessed by the dual culture method. A 0.7 cm dia. disc from the *F. oxysporum* culture was chosen from the colony's edge (5 days) and was placed in the center of the PDA plate. After that, four-discs (0.7 cm dia. each) were taken from a three-day old *B. subtilis* colony on nutrient ager (NA) medium and placed at the periphery of the petri dish with equal dimension to each other and 1.5 cm from the edge of the petri dish. For the control treatment, a 0.7 cm dia. disc from the same pathogen colony was added to the sterilized PDA plate (without adding *B. subtilis*). For both the antagonism treatment and control, there were five plates (= replicates). All the plates were incubated at 28 °C. After incubation, in the antagonism treatment, the radial growth mycelium of the fungus was measured when the radial growth mycelium in the control reached the edge of the growth plates. The percentage of fungal growth inhibition (*FGI*) was calculated as the ratio of growth between fungal growth in the treatment opposite to the control: *FGI* % = [1 – (*FG* in antagonism/*FG* in control)] 100.

2.3 THE NATURE OF EXPERIMENT

This experiment was conducted in the greenhouse of the Date Palm Research Center, University of Basrah, during the 2019-2020 growing season. The experiment was repeated twice. Plantlets were 12 months old (they were grown from seeds of the Halawii cultivar) and were planted in plastic pots (4 kg each) filled with sterilized soil (1 : 1 peat moss + sand). The pots (=replicates) experiments were arranged and conducted in a completely randomized design. The pots in the experiment were divided into four treatments: (1) controls (no added any bacterial or fungal inoculation); (2) plantlets inoculated with *B. subtilis* only; (3) plantlets inoculated with *F. oxysporum* only; and (4) plantlets inoculated first with *B. subtilis* at a concentration of 10^8 spores ml^{-1} (*B. subtilis* inoculum concentration used according to the manufacture recommendation, 0.5 g of BioHealth biopesticide 10 mL of water, the concentration of *B. subtilis* was 10^8 spores ml^{-1}) and, after 48 hours, also inoculated with *F. oxysporum* at a concentration of 10^6 spores ml^{-1} (for both organisms, the inoculum was added at a rate of 150 ml/pot with irrigation water) (Al-Ani et al., 2012). All pots were placed in the greenhouse under favorable conditions (28–30 ˚C, watering and fertilization). Treatments were applied to the plantlets, which were then held for 28 days. Each treatment was replicated five times (each replicate having one pot with three plantlets). At the end of the experiment (day 28), we measured four response parameters: (1) photosynthetic pigment content, (2) antioxidant enzyme levels, and (3) total soluble proline, as described in detail below:

Photosynthetic pigment content was measured according to the protocol of Metzner et al. (1965).

The total chlorophyll and carotenoids were determined spectrophotometrically (CECL, 2021 spectrophotometer, UK).The absorbance was calculated against a blank of pure 85 % aqueous acetone at 452 and 663 nm, represented as mg. g fresh mass (FM). Using the following equations: total chlorophyll and total carotenoids: photosynthetic pigments represented as mg. g FM^{-1}.

For antioxidants, the activity of catalase (CAT) was determined according to Luck (1974), and the activity was expressed as a unit/mg of protein. Peroxidase (POD) activity was estimated according to Kara & Mishra (1976). The amount was evaluated by the absorbance at 420 nm, and the enzyme activity was expressed as a unit/mg of protein.

The protocol of Bates et al. (1973) was used to determine the total soluble proline leaf content. The toluene reagent was aspirated from the aqueous phase, and the solution absorbance was measured at 520 nm. Proline content was determined by measuring it from a standard curve and was calculated as mg. g dry $mass^{-1}$ (DM).

2.4 DATA ANALYSIS

The experimental design was completely randomized. The statistical analysis data was carried out by analysis of variance ANOVA using SPSS-21 software, the differences in the means were determined by the least significant difference test (LSD) ($p < 0.05$).

3 RESULTS

3.1 PATHOGENICITY TESTS OF ISOLATED FUNGI

Fusarium oxysporum, *F. proliferatum* S1, *F. proliferatum* S2, *F. fujikuroi*, and *R. solani*, were responsible for root rot infections in date palm plantlets (Table1). *F. oxysporum* was the most pathogenic fungus, causing 82.16 % of root rot severity, with highly significant differences compared with other fungi, followed by *F. proliferatum* S1, *F. proliferatum* S2, and *F. fujikuroi*, which caused 30.12 %, 24.26 %, and 18.56 % severity, respectively. *R. solani* was the least harmful species as it showed a disease severity 12.42 %.

Table 1: Pathogenicity of fungal isolates recovered from date palm plantlets after greenhouse inoculations, 60 days post-inoculation

	Disease severity of root rot disease		
Fungi tested	D	DSI	% Plant survival
F. oxysporum	4	82.16 a	12.82 a
F. proliferatum S1	3	30.12 b	68.64 b
F. proliferatum S2	3	24.26 c	76.10 c
F. fujikuroi	2	18.56 c	82.20 d
R. solani	2	12.42 d	90.00 d
Control(untreated)	0	_	100 e
LSD at p = 0.05	NA	2.86	4.93

Average scores for 15 plantlets for each treatment, where; D: disease rating scale and, DSI: disease severity index

3.2 EFFECT OF *B. SUBTILIS* AGAINST THE GROWTH OF *F. OXYSPORUM IN VITRO*

Bacillus subtilis reduced colony spread of *F. oxysporum* on PDA by 86.6 % (Fig.1) (1.2 cm mean radial colony growth with *B. subtilis* versus 9.0 cm mean colony growth without *B. subtilis*) after 6 days of incubation at 27 °C.

3.3 EFFECTS ON TOTAL CHLOROPHYLL AND TOTAL CAROTENOIDS

The results showed that date palm plantlets treated with *B. subtilis* in the presence and absence of *F. oxysporum* resulted in a highly significant increase in total chlorophyll and total carotenoid, in comparison to the pathogen alone (*F. oxysporum*). Plantlets treated with *B. subtilis* had the highest scores, while the pathogen treatment resulted in low values (Fig.2). *B. subtilis* significantly raised

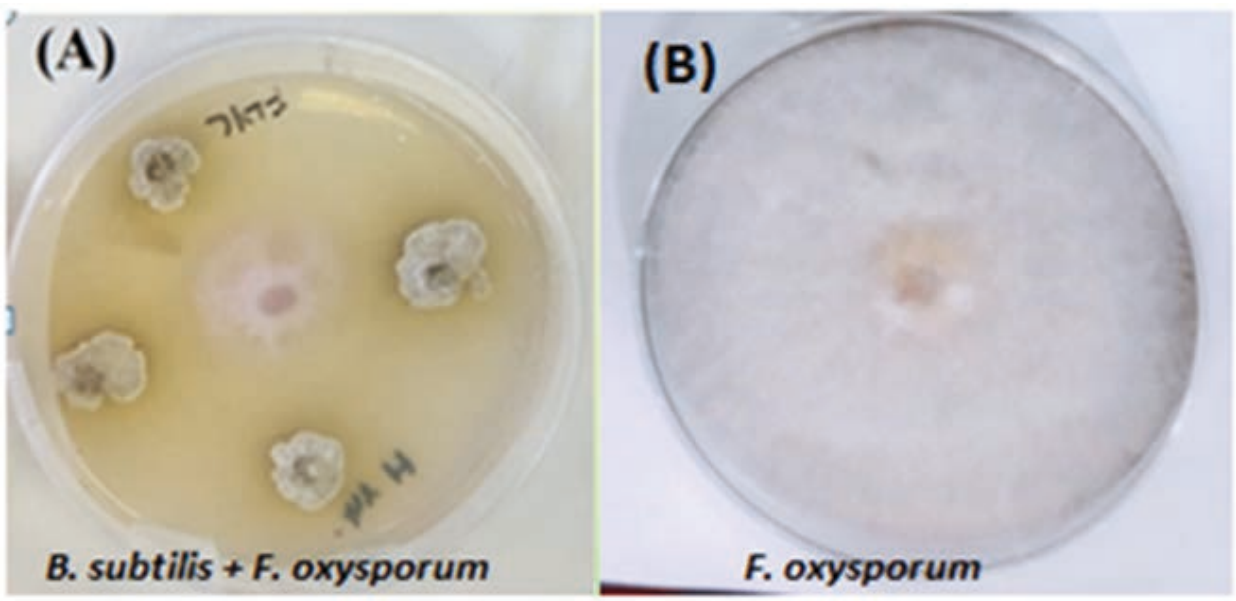


Fig. 1: *In vitro* inhibition of mycelial growth of *F. oxysporum* by *B. subtilis* on PDA (A: antagonism, B: *F. oxysporum*)

the total chlorophyll and total carotenoids in comparison with the *F. oxysporum* treatment. When plantlets infected with *F. oxysporum* were treated with the bacterium *B. subtilis*, chlorophyll levels were restored to roughly 90 % of control levels, and carotenoid levels were restored to control levels.

3.4 LEVELS OF CATALASE (CAT) AND PEROXIDASE (POD) ENZYMES

The obtained data showed that *B. subtilis* augmented the levels of the antioxidant enzymes CAT and POD significantly in *F. oxysporum*-treated plantlets, about 31.2 % and 5.2 %, respectively, compared to inoculated plantlets with *F. oxysporum* alone (Fig.3). Plantlets inoculated with *F. oxysporum* had lower levels of catalase (CAT) and peroxidase (POD) enzyme activity than those in the control. The data analyses showed highly significant enhancement of the antioxidant enzyme activities (CAT and POD) as a result of treating the plantlets with the *B. subtilis* strain in the presence and absence of the pathogenic fungus *F. oxysporum*.

3.5 EFFECT ON TOTAL PROLINE CONTENT

Data from our study revealed that total proline was reduced in response to inoculation with *F. oxysporum* compared with un-inoculated plantlets (control). Infection by *F. oxysporum* reduced proline levels, but co-application of *B. subtilis* restored proline to normal levels seen in the control (Fig.4). Data from the ANOVA table showed that the value of total proline content was increased in plantlets inoculated with *B. subtilis* in the presence of *F. oxysporum* by about 47.6 % compared with plantlets inoculated with *F. oxysporum* alone. *F. oxysporum* reduced total proline content by approximately 48.2 % (compared to the control (healthy).

4 DISCUSSION

4.1 PATHOGENICITY TESTS

The results of the pathogenicity test showed that *Fusarium* isolates were highly pathogenic to date palm plantlets. Species of *Fusarium* are known to produce toxins such as fumosisin, fusaric acid, fusaproliferin, fusarin, zearalenone, and others, which aid in the attack and parasitism of plant hosts (Hernandez et al., 2010). According to El Modafar & El Bostani (2000), *F. oxysporum* releases cell wall hydrolytic enzymes that hydrolyze host ingredients, allowing the pathogen to move into root tissues, and these enzymes are linked with disease progression. Our results agree with those of other researchers, showing that date palm trees and offshoots are attacked by many soil-borne pathogenic fungi capable of causing severe losses and degradation (Arafat et al., 2012; Baraka et al., 2011), including *F. oxysporum, F. solani, F. moniliforme, F. smitectum, R. solani,* and *Thielaviopsis paradoxa* (De Seynes) Höhn.(Ahmed, 2018; El-Morsi et al., 2012; Maitlo et al., 2013).

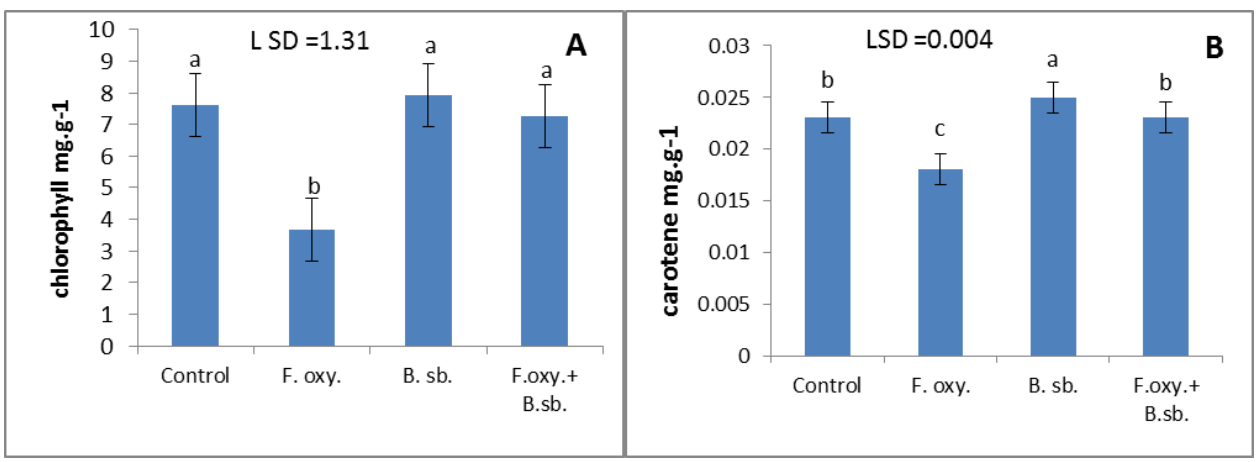


Fig. 2: Effectiveness of *B. subtilis* in presence and absence of *F. oxysporum* on: A: total chlorophyll content and, B: Total carotenoid content.(Each value is the mean of five replicates, means in the columns followed by the different letters are significantly different at $p < 0.05$ test)

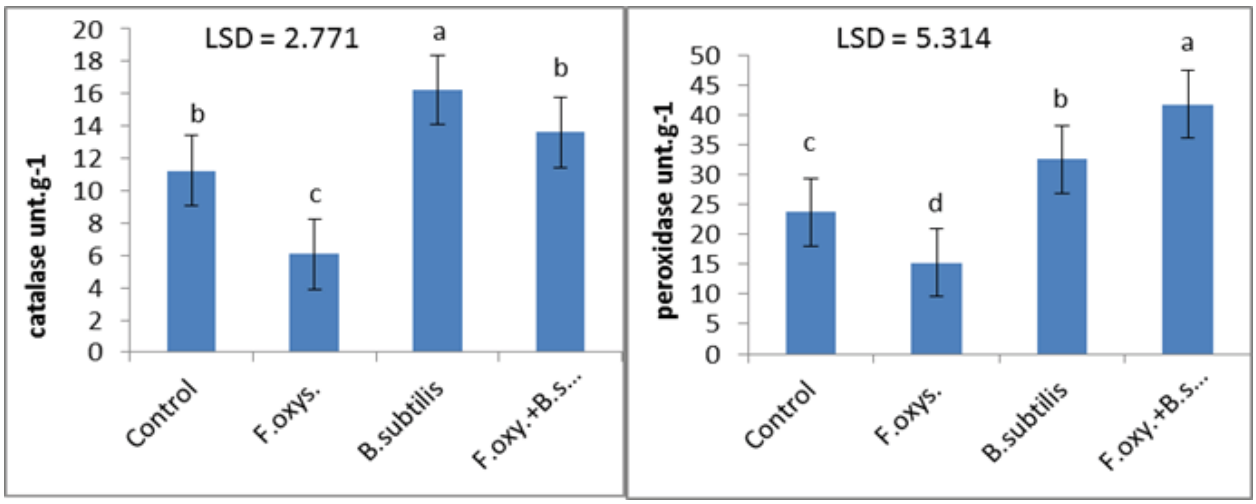


Fig. 3: Effectiveness of *Bacillus subtilis* in presence and absence of *Fusarium oxysporum* on catalase enzyme, and peroxidase enzyme (Each value is mean of five replicates, means in the columns followed by the different letters are significantly different at $p < 0.05$ test)

4.2 EFFECT OF *B. SUBTILIS* AGAINST THE GROWTH OF *F. OXYSPORUM IN VITRO*

The results of a double culture in Petri dishes containing PDA medium revealed that *Bacillus subtilis* has the ability to suppress radial mycelial growth to a large extent. *Bacillus subtilis* suppresses pathogen growth directly by the synthesis of many secondary metabolites, such as hormones, cell wall degrading enzymes, and antioxidants. Our results were in agreement with studies by Cao et al. (2012), who observed that *B. subtilis* produces many antibiotic compounds, including fengycin, iturin, and bacillomycin, and these compounds inhibit mycelial growth and spore germination of the fungal pathogen *F. oxysporum*. Jassim (2015) showed that *B. subtilis* completely inhibited the mycelial growth of the pathogen *F. moniliforme* in the PDA medium. Siala et al. (2016) found that an endophytic strain of *B. subtilis* slowed the growth of *Fusarium* species on PDA and that this strain produced proteases, contributing to the degradation of the cell walls of fungal pathogens. Isolate *B. subtilus* 174 has been shown to have strong biocontrol activity and to cause significant suppression of disease severity in Fusarium wilt disease in tomato plants caused by *F. oxysporum*, likely due to induced resistance (Akarm and Anjum, 2011). Several important plant pathogens, including *Fusarium* sp. (Zhao et al., 2013), *Rhizoctonia solani* (Kumar et al., 2012), and *Verticillium dahliae* Kleb (Li et al., 2013), can be suppressed by *B. subtilis*. Bhusal & Mmbaga (2020) examined three *Bacillus* spp. isolates as biological control agents against the pathogen *Phytophthora capsici* Leonian. These isolates suppressed the mycelial growth of *P. capsici in vitro* and reduced the incidence of disease in plants grown in soil infested with *Phytophthora* inoculum under greenhouse conditions.

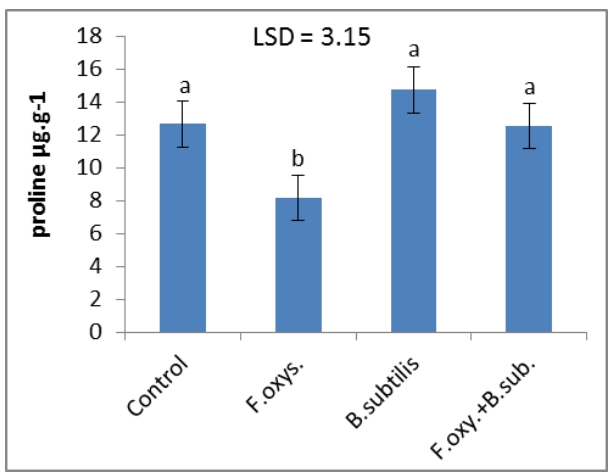


Fig. 4: Effectiveness of *Bacillus subtilis* in the presence or and absence of *Fusarium oxysporum* on proline content (Each value is mean of five replicates: means in the columns followed by the different letters are significantly different at $p < 0.05$ test)

4.3 EFFECTS ON TOTAL CHLOROPHYLL AND TOTAL CAROTENOIDS

Our results show that plantlets inoculated with *F. oxysporum* reduced photosynthetic activity (mostly photosynthesis), perhaps due to reduced levels of key proteins in the thylakoid membranes and/or the reduction of RuBPC-specific leaf soluble proteins (Weintraub & Jones,

2010). Huang et al. (2012) found that stress and infection by fungi led to a decline in leaf chlorophyll that was due to increased chlorophyllase activity, increased active oxygen products, and destabilization of ionic equilibrium. Reduction in chlorophyll may be due to the toxic action of compounds released by pathogenic fungus; such compounds lead to necrosis and chlorosis due to their toxic effects on chloroplasts in the host cells (Bashan et al., 1995). The reduction in the absorption of minerals (e.g., magnesium) required for chlorophyll synthesis can also indirectly reduce the chlorophyll content in plants infected by pathogens (Murkute et al., 2006; Sheng et al., 2008).

Nevertheless, in response to *F. oxysporum* infection, *B. subtilis* greatly increased the production of antioxidant enzymes, several secondary metabolites, growth regulator hormones, and enzymes to degrade cell walls (Hashem et al., 2019). According to Cazorla et al. (2007), because *B. subtilis* can emit antibiotics and hydrolytic enzymes, it may change its environment for the better and develop resistant endospores to survive in harsh environments. In mung beans, *B. subtilis* alleviated symptoms of infection by the fungal pathogen *Macrophomina phaseolina* (Tassi) Goid., reducing charcoal rot disease and enhancing total chlorophyll (Hashem et al., 2017). Shi et al. (2010) showed that *B. subtilis* elevated the photosynthetic capacity and total content of chlorophyll of sugar beet, resulting in a consequently enhanced synthesis of carbohydrates. In date palms, *B. subtilis* increased the total chlorophyll and total carotenoid in plants under abiotic stress and prevented the harmful effects of stress (Jassim et al., 2020). *F. oxysporum* infection in date palm plantlets generates reactive oxygen species (ROS), such as radicals of superoxide(O^{-2}), hydroxyl (OH^{-}), and molecules of hydrogen peroxide (H_2O_2). The accumulation of ROS in infected plant cells causes major and important injuries in all plant functions (Manhas & Kaur, 2016).

4.4 LEVELS OF CATALASE (CAT) AND PEROXIDASE (POD) ENZYMES

Antioxidant enzymes mitigate the damage level from reactive oxygen species (ROS) in plants, which is a source of oxidative stress from pathogen infection (Asada, 1999). Catalase (CAT) is one of the most prevalent detoxifying enzymes in plants, and it plays an important role in regulating ROS generation and buildup. In contrast, the catalase enzyme converts H_2O_2 into H_2O and O_2, so any increase in CAT activity is more likely to result in a decrease in H_2O_2 generation, as shown at low concentration in the plantlet treatments inoculated with both *B. subtilis* in the presence or absence of *F. oxysporum*. At high concentrations, plantlets inoculated with *F. oxysporum* alone showed the opposite effect, with an increase in H_2O_2 production accompanying a decrease in CAT activity. The increase in antioxidant enzyme activity levels enhances disease resistance in the host plants (Shi et al., 2010). Selvaraj & Chellappan (2006) explain that POD enzymes play an important role in producing ethylene, resisting disease, promoting wound healing, and lignin formation, as well as in building cell walls by changing polymerizing hydroxyl and methoxy cinnamic alcohols into lignin.

Xie et al. (2021) found that *B.subtilis* strain LZ88 induced plant resistance with an enhanced expression in tobacco leaves of the antioxidant enzymes peroxidase (POD) and polyphenol oxidase (PPO). *B. subtilis* induced systemic resistance and alleviated the harmful effects of pathogens by increasing the activity of antioxidant enzymes on plants (Hashem et al., 2019).In a recent study on date palms, Jassim et al. (2020) showed that *B. subtilis* protected date palms from the harmful effects of salt stress and increased the activity of antioxidant enzymes (CAT and POD). In our study we found that *F. oxysporum*, in the absence of *B. subtilis,* increased the levels of CAT and POD activity, which confirms that oxidative damage is associated with ROS scavenging, while the *B. subtilis* bacteria inhibited the activity of the pathogen fungus *F. oxysporum* and significantly reduced the negative effects on the vital processes in the plant, which reflected its effect on the plant, and that led to mitigation in the antioxidant enzymes compared to the pathogen treatment alone.

4.5 EFFECT ON TOTAL PROLINE CONTENT

There were significant differences in the total proline content among plants treated with *B. subtilis* and *F. oxysporum* compared with *F. oxysporum.* This can be attributed to the ability of *B. subtilis* to limit the activity of the fungus pathogen through the ability to excrete antibiotics such as subtilin, bacteriocins, iturins, and bacilomycin, which act to inhibit the growth of fungus (Meena & Kanwar, 2015; Wang et al., 2015). Proline is essential in plants and accumulates during pathogen attacks in a variety of species (Rehman et al., 2014). According to the study by Wang et al. (2012), the inoculation of cucumber plants (*Cucumis sativa* L.) with a mixture of three plant growth promoting rhizobacteria (PGPR) strains (*B. cereus* AR156, *B. subtilis* SM21, and *Serratia* sp. XY21) elevated leaf proline content by 3–4 fold compared to uninoculated plants. Proline catabolism plays an essential role in controlling the cellular ROS balance and can also control various other regulatory pathways. It has also

been demonstrated that proline accumulation activates the pathways of alternative detoxification by the maintenance and duration of ROS-eliminating enzymes (Hayat et al., 2012). Plants inoculated with *B. subtilis* showed less damage from the harmful effects of *M. phaseoline* and an increase in the accumulation of sugars, proline, and free amino acids, which are considered to be the key osmolytes for sustaining the content of cellular water to protect the structures and functions of cell organelles (Hashem et al., 2017).

5 CONCLUSION

Pathogenicity studies showed that *Fusarium oxysporum* is the most causative agent of root rot disease in date palm plantlets, with a severity index of 82.16 % for root rot. The *Bacillus subtilis* strain reduced the mycelial growth of *F. oxysporum in vitro* as well as *in vivo*. Our results show that *B. subtilis* is a beneficial microorganism for controlling the root rot disease of date palm plantlets caused by *F. oxysporum*. *B. subtilis* inhibited oxidative damage caused by the pathogenic fungus and significantly improved all measured physiological characteristics that were adversely affected by the fungus pathogen. More work is needed to determine the potential of *B. subtilis* for biological control of this pathogenic fungus and regulation of plant growth.

6 CONFLICT OF INTEREST

Authors declare no conflict of interest.

7 REFERENCES

Abdou, E., Abd-Alla, H.M.& Galal, A.A. (2003). Survey of sesame root rot and wilt disease in *Minia* and their possible control by ascorbic and salicylic acids. *Assuit Journal of Agricultural Sciences*, *32*,135–152.

Ahmed, F.A. (2018). Management of date palm root rot diseases by using some biological control agents under organic farming system. *Novel Research in Microbiology Journal*, *2*(2), 37–47. https://doi.org/10.21608/NRMJ.2018.6619

Akarm, W. & Anjum, T. (2011). Quantitative changes in defense system of tomato induced by two strains of *Bacillus* against *Fusarium* wilt. *Indian Journal of Fundamental and Applied Life Sciences*, *1*(3), 7–13. https://www.researchgate.net/publication/266200032

Al-Ani, R.A., Adhab, M.A., Mahdi, M.H. & Abood, H.M. (2012). *Rhizobium japonicum* as a biocontrol agent of soybean root rot disease caused by *Fusarium solani* and *Macrophomina phaseolina*. *Plant Protection Sciences*, *48*(4), 149–155. https://doi.org/10.17221/16/2012-PPS

Alwahshi. K.J., Saeed, E. I Sham, A., lblooshi, A.A., Alblooshi, M.M., El-Tarabily, K.A., & AbuQamar, S.F. (2019). Molecular identification and disease management of date palm sudden decline syndrome in the United Arab Emirates. *International Journal of Molecular Sciences*, *20*, 923. https://doi.org/10.3390/ijms20040923

Arafat, K.H., Mohamad, A.M. & Elsharabasy, S. (2012). Biological control of date palm root-rots diseases using Egyptian isolates of *Streptomyces*. *Research Journal of Agriculture and Biological Sciences*, *8*(2), 224–230.

Asada, K. (1999).The water-water cycle in chloroplast scavenging of active oxygen and dissipation of excess photons. *Annual Review of Plant Physiology and Plant Molecular Biology*, *50*, 601–639. https://doi.org/10.1146/annurev.arplant.50.1.601

Baraka, M.A., Radwan, F.A. & Arafat, K.H. (2011). Survey and identification of major fungi causing root rot on date palm and their relative importance in Egypt. *Journal of Biological Chemistry and Environmental Science*, *6*(2), 319–337. www.acepsag.org

Bashan, B., Levy, R., Cojocaru, M. & Levy, Y. (1995). Purification and structural determination of a phytotoxic substance rom *Exserohilum turcicum*. *Physiological and Molecular of Plant Pathology*, *47*(4), 225–235. https://doi.org/10.1006/pmpp.1995.1054

Bates, L.S., Waldern, R.P. & Teara, L.D. (1973). Rapid determination of free proline for water-stress studies. *Plant Soil*, *39*, 205–207. https://doi.org/10.12691/wjar-2-2-2

Bhusal, B. & Mmbaga, M.T. (2020). Biological control of Phytophthora blight and growth promotion in *Bacillus* species. *Biological Control*, *150*. https://doi.org/10.1016/j.biocontrol.2020.104373

Cao, Y., Zhang, Z.H., Ling, N., Yuan, Y.J., Zheng, X.Y., Shen, B.A. & Shen, Q.R. (2012). *Bacillus subtilis* SQR 9 can control *Fusarium* wilt in cucumber by colonizing plant roots. *Biological and Fertility of Soils*, *47*, 495–506. https://doi.org/10.1007/s00374-011-0556-2

Caron, J., Laverdière, L., Thibodeauand, P.O. & Bélanger, R.R. (2002). Use of an indigenous strain of *Trichoderma harzianum* against five plant pathogens on greenhouse cucumber and tomato in Québec. *Phytoprotection*, *83*(2), 73–87. https://doi.org/10.7202/706230ar

Cazorla, F., Romero, D., Perez- Garcia, A., Lugtenberg, B., Vicente, A.D., Bkoennberg, G. (2007). Isolation and characterization of antagonistic *Bacillus subtilis* strains from the vocado rhizplane displaying biocontrol activity. *Journal Applied of Microbiology*, *103*(5), 1950–1959. https://doi.org/10.1111/j.1365-2672.2007.03433.x

Chen, K., Tian, Z., He, H., Long, C. & Jiang, F. (2020). *Bacillus* species as potential biocontrol agents against citrus diseases. *Biological control*, *151*. https://doi.org/10.1016/j.biocontrol.2020.104419

Collin, D.P. & Jacobsen, B.J. (2003). Optimizing a *Bacillus subtilis* isolate for biological control of sugar beet *Cercospora* leaf spot. *Biological Control*, *26*(2), 153–161. https://doi.org/10.1016/S1049-9644(02)00132-9

El Modafar, C. & El Boustani, E. (2000). Relationship between cell wall susceptibility to cellulose and pectinases of *Fusarium oxysporum* and susceptibility of date palm

cultivars. *Biologia Plantarum*, *43*(4), 571–576. https://doi.org/10.1023/A:1002886104333

El-Morsi, M.E.A., Abo Rehab, M.E.A., El-Morsy, S.A. (2012). Surviey and control trials of root rot/wilt of date palm offshoots in New Valley Governorate. *Egyptian Journal of Agricultural Researches*, *90*, 1403–1414. https://doi.org/10.21608/ejar.2012.163415

El-Morsi, M.E.A., Kamhawy, M.A.M. & Sallam, M.A.A. (2009). Effectiveness of some organic compounds in controlling pathogenic fungi associated with roots of date palm offshoots in New Valley Governorate, Egypt. *Assiut Journal of Agriculture Sciences*, *40*, 137–150.

Gerhardson, B. (2002). Biological substitutes for pesticides. *Trends Biotechnology*, *20*, 338–343. https://doi.org/10.1016/S0167-7799(02)02021-8

Gravel, V., Martinez, C., Antoun, H. & Tweddell, R.J. (2004). Evaluation of antagonistic microorganism as biological control agents (BCAs) of root rot (*Pythium ultimum*) of greenhouse tomatoes in rock wool. *Canadian Journal of Plant Pathology*, *26*, 152–159.

Hashem A., Elsayed F.A.A., Alqarawi A.A., Radhakrishnan R. & Kumar A. (2017). Plant defense approach of *Bacillus subtilis* (BERA71) against *Macrophomina phaseolina* (Tassi) Goidin mung bean. *Journal of Plant Interactions*, *12*(1), 390–401. https://doi.org/10.1080/17429145.2017.1373871

Hashem, A.T., Tabassum, B. & Abd-Allsh, E.F. (2019). *Bacillus subtilis*: A plant-growth promoting rhizobacterium that also impacts biotic stress. *Saudi Journal of Biological Sciences*, *26*, 1291–1297. https://doi.org/10.1016/j.sjbs.2019.05.004

Hayat, S., Hayat, Q., Alyemeni, M.N., Wani, A.S., Pichtel, J. & Ahmad, A. (2012). Role of proline under changing environments: a review. *Plant Signal Behaves*, *7*, 1456–1466. doi: 10.4161/psb.21949. https://doi.org/10.4161/psb.21949

Hernandez, H., Espino, J., Rodriguez, R.A., Manuel, J., Perez, S., Ana, P.S. & et al. (2010). Survey of diseases caused by *Fuasarium* spp. on palm trees in the Canary Islands. *Phytopathology Mediterranea*, *49*, 84–88.

Huang, C.H., Roberts, P.D. & Datnoff, L.E. (2012). Fusarium diseases of tomato. In: Gullin Katan J and Garibaldi. A. (eds.). *Fusarium wilts of greenhouse vegetable and ornamental crops*. American Phytopathology and Society St. Paul, 145–158. https://doi.org/10.1094/9780890544822.018

Jassim, N.S. (2015).The efficiency of *Bacillus subtilis* and salicylic acid on the growth and pathogenecity of *Fusarium moniliforme* Sheldon the causal agent of date palm offshoot decline. *Basra Journal of Date Palm Research*, *14*(2), 55–75 (Abstract).

Jassim, N.S., Ati, M.A. & Alhamd, A.D. (2020). Effect of biological agent *Bacillus subtilis* on chemical composition and antioxidant enzymes of date palm (*Phoenix dactylifera* L) under salts tress. *Biochemical and Cellular Archive*, *20*(1), 1371–1380.

Kara, M. & Mishra, D. (1976). Catalase, peroxidase, polyphenyl oxidase activities during rice leaf senescence. *Plant Physiology*, *57*, 315–319. https://doi.org/10.1104/pp.57.2.315

Khazaal, F.A-K. (2019). *Isolation and identification of fungi associated with sudden decline disease on date palm trees (Phoenix dactylifera L). in Basrah/Iraq*. M. Sc. Thesis, Biology Department, University of Basrah, Iraq.

Kumar, K.V.K., Yellareddygari, S.K., Reddy, M.S., Kloepper, J.W., Lawrence, K.S., Zhou, X.G., et.al. (2012). Efficacy of *Bacillus subtilis* MBI 600 against sheath blight caused by *Rhizoctonia solani* and on growth and yield of rice. *Rice Sciences*, *19*(1), 55–63. https://doi.org/10.1016/S1672-6308(12)60021-3

Li, S., Zhang, N., Zhang, Z., Luo, J., Shen, B., Zhang, R. & Shen, Q. (2013). Antagonist *Bacillus subtilis* HJ5 controls *Verticillium* wilt of cotton by root colonization and biofilm formation. *Biology and Fertility of Soils*, *49*, 295–303. https://doi.org/10.1007/s00374-012-0718-x

Liu, L., Kloepper, J.W. & Tuzun, S. (1995). Induction of systemic resistance in cucumber against *Fusarium* wilt by plant growth-promoting rhizobacteria. *Phytopathology*, *85*, 695–698. https://doi.org/10.1094/Phyto-85-695

Luck, H. (1974). Catalase, In: Bregmeyer HU, editor, *Methods of enzymatic analysis*, New York, NY: Academic Press, p 885–893. https://doi.org/10.1016/B978-0-12-395630-9.50158-4

Maitlo, W.A., Markhand, G.S., Abul-Soad, A., Lodhi, A.M. & Jatoi M.A. (2013). Chemical control of sudden decline disease of date palm (*Phoenix dactylifera* L.) in Sindh, Pakistan. *Pakistan Journal of Botany*, *45*, 7–11.

Manhas, R.K. & Kaur, T. (2016). Biocontrol Potential of *Streptomyces hydrogenans* Strain DH16 toward *Alternaria brassicicola* to control damping off and black leaf spot of *Raphanus sativus*. *Frontiers in Plant Science*, 7, 1869. https://doi.org/10.3389/fpls.2016.01869

Meena, K.R. & Kanwar, S.S. (2015). Lipopeptides as the antifungal and antibacterial agents: Applications in food safety and therapeutics. *Hindawi Publishing Corporation BioMed Research International Article ID 473050*, 9 pages. https://doi.org/10.1155/2015/473050

Metzner, H., Rau, H. & Senger, H. (1965). Untersuchungen zur Synchronisierbakeit einzelner Pigmentmangel- Mutanten von *Chlorella*. *Planta*, *65*,186–194. https://doi.org/10.1007/BF00384998

Murkute, A.A., Sharma, S. & Singh, S. K. (2006). Studies on salt stress tolerance of citrus rootstock genotypes with arbascular mycorrhizal fungi. *Horticulure Science*, *33*, 70–76. https://doi.org/10.17221/3742-HORTSCI

Muthomi, J.W., Otieno, P.E., Chemining'wa, G.N., Nderitu, J.H. & Wagacha, J.M. (2007). Effect of legume root rot pathogens and fungicide seed treatment on nodulation and biomass accumulation. *Journal of Biological Sciences*, *7*, 1163–1170. https://doi.org/10.3923/jbs.2007.1163.1170

Rehman, F., Khan, F.A. & Anis, S.B. (2014). Assessment of aphid infestation levels in some cultivars of mustard with varying defensive traits. *Achieves Phytopathology and Plant Protection*, *47*, 1866–1874. https://doi.org/10.1080/03235408.2013.860724

Selvaraj, T. & Chellappan, P. (2006). Arbuscular mycorrhizae: adiverse personality. *Journal of Central European Agriculture*, *7*(2), 349–358.

Sheng, M., Tang, M., Chen, H., Yang, B., Zhang, F. & Huang, Y. (2008). Influence of arbuscular mycorrhizae on photosynthesis and water status of maize plants under salt stress. *Mycorrhiza*, *18*(6–7), 287–296. https://doi.org/10.1007/s00572-008-0180-7

Shi, Y., Lou, K. & Li, C. (2010). Growth and photosynthetic ef-

ficiency promotion of sugar beet (*Beta vulgaris* L.) by endophytic bacteria. *Photosynthesis Research*, *105*, 5–13. https://doi.org/10.1007/s11120-010-9547-7

Siala, R., Ben Chobba, I., Vallaeys, T., Triki, MA., Jrad, M., et al. (2016). Analysis of the cultivable endophytic bacterial diversity in the Date palm (*Phoenix dactylifera* L.) and evaluation of its antagonistic potential against pathogenic *Fusarium* species that cause date palm bayound disease. *Journal of Applied and Environmental Microbiology*, *4*(5), 93–104. doi: 10.12691/jaem-4-5-2.

Thabet, I., Francis, F., de Paw, E., Besbes S., Attia, H., Devanne, C. & Blecker, C. (2010). Charcterisation of proteins from date palm sap (*Phoenix dactylifera* L.) by a protein approach. *Food Chemistry*, *123*, 765–770. https://doi.org/10.1016/j.foodchem.2010.05.024

Wang, C., Yang, W., Wang, C., Gu, C., Niu, D. & Liu, H. (2012). Induction of drought tolerance in cucumber plants by a consortium of three plant growth promoting rhizobacterium strains. *PLoS One*, *7*, e52565. https://doi.org/10.1371/journal.pone.0052565

Wang, X., Zhao, D., Shen, L., Jing, C. & Zhang, C. (2018). Application and mechanisms of *Bacillus subtilis* in biological control of plant disease. *Role of Rhizospheric. Microbes in Soil*, Springer, pp. 225–250. https://doi.org/10.1007/978-981-10-8402-7_9

Wang, T., Liang, Y., Wu, M., Chen, Z., Lin, J. & Yang, I. (2015). Natural products from *Bacillus subtilis* with antimicrobial properties. *Chinese Journal of Chemical Engineering*, *23*(4), 744–754. https://doi.org/10.1016/j.cjche.2014.05.020

Weintraub, P.G. & Jones, P. (2010). *Phytoplasmas: genomes, plant hosts and vectors*. Walling ford, UK: CABI. https://doi.org/10.1079/9781845935306.0000

Xie, Z., Li, M., Wang, D., Wang, F., Shen, H., Sun, G.J. et al. (2021). Biocontrol efficacy of *Bacillus siamensis* LZ88 against brown spot disease of tobacco caused by *Alternaria alternata*. *Biological Control*, *154*,104508. https://doi.org/10.1016/j.biocontrol.2020.104508

Zaid A. (2002). Date Palm Cultivation. *FAO Agricultural Services Bulletin No. 156*, Food and Agriculture Organization of the United Nations, Rome.

Zhao, Q., Ran, W., Wang, H., Li, X., Shen, Q., Shen, S. & Xu, Y. (2013). Biocontrol of *Fusarium* wilt disease in muskmelon with *Bacillus subtilis* Y-IVI. *BioControl*, *58*, 283–292. https://doi.org/10.1007/s10526-012-9496-5

doi:10.14720/aas.2022.118.3.1208 Original research article / izvirni znanstveni članek

Clonal propagation of *Tetragonolobus palaestinus* Bioss: A Jordanian medical plant

Mawadda MEHERAT [1], Mohammad SHATNAWI [1], Rida SHIBLI [2], Tamara QUDAH [3, 4], Saida ABU MALLOH [3], Tamadour AL-QUDAH [5]

Received July 03, 2019; accepted July 05, 2022.
Delo je prispelo 20. julija 2019, sprejeto 5. julija 2022

Clonal propagation of *Tetragonolobus palaestinus* Bioss: A Jordanian medical plant

Abstract: *Tetragonolobus palaestinus* Bioss (Aljalaton) is one of the Jordanian medicinal plants that can be used to treat stomach pain and some infections. This study was done in order to establish optimal *in vitro* propagation method for *T. palaestinus*. Factors of *in vitro* shooting, rooting, and acclimatization of the *in vitro Tetragonolobus palaestinus* seedlings were studied using different growth regulators. For *in vitro* shooting, different cytokinins including benzylamino purine (BAP), kinetin, TDZ, and zeatin were used in increasing concentrations (0.0, 0.3, 0.6, 0.9, 1.2, 1.5, and 2.0 mg l^{-1}). Using benzylamino purine (BAP produced a maximum of 2.0 shoots/explants on Murashige and Skoog (MS) medium supplemented with 0.3 mg l^{-1}. Moreover, the effect of different concentrations of IBA (indole-3-butyric acid), IAA (indole-3-acetic acid), andnaphthalene acetic acid (NAA) was evaluated for *in vitro* rooting. The highest number of roots (4.06 roots/explant) was obtained on MS medium supplemented with 0.3 mg l^{-1} IBA. All of the plants (100 %) were grown normally after the acclimatization process. Based on these results simple protocol of *T. palaestinus in vitro* culture was optimized for the first time which can be utilized to do more studies on cell culture and production of active secondary metabolites.

Key words: acclimatization;, *in vitro*; shoot multiplication;, rooting

Klonsko razmnoževanje vrste *Tetragonolobus palaestinus* Bioss: jordanske zdravilne rastline

Izvleček: Vrsta *Tetragonolobus palaestinus* Bioss (Aljalaton) je jordanska zdravilna rastlina, ki se lahko uporablja za blaženje bolečin v želodcu in zdravljenje nekaterih okužb. Namen raziskave je bil vzpostaviti optimalen način *in vitro* razmnoževanja te rastline. Preučevani so bili dejavniki *in vitro* gojenja (vkoreninjenja, tvorbe poganjkov) in aklimatizacije sadik te rastline z uporabo različni rastnih regulatorjev. Za *in vitro* tvorbo poganjkov so bili uporabljeni različni citokinini in sicer benzilamino purin (BAP), kinetin (TDZ) in zeatin v naraščajoči koncentraciji (0,0; 0,3; 0,6; 0,9; 1,2; 1,5 in 2,0 mg.l^{-1}). Uporaba benzilamino purina (0,3 mg l^{-1}) je dala pri gojenju na Murashige in Skoog (MS) gojišču največ poganjkov, dva na izseček. Učinek različnih koncentracij rastnih regulatorjev (IBA-indol-3-maslene kisline, IAA -indol-3-ocetne kisline in naftalen ocetne kisline NAA) je bil ovrednoten pri *in vitro* vkoreninjenju. Največje število korenin (4,06 korenin/izseček) je bilo dobljeno na MS gojišču, z dodatkom 0,3 mg l^{-1} IBA. Vse rastline (100 %) so po obdobju aklimatizacije rastle normalno. Na osnovi teh rezultatov je bil prvič optimiziran enostaven protokol za *in vitro* gojenje te vrste, ki bi se lahko uporabil v nadaljnih raziskavah na celičnih kulturah in produkciji aktivnih sekundarnih metabolitov.

Ključne besede: aklimatizacija; *in vitro*; namnoževanje poganjkov; vkoreninjanje

1 Faculty of Agricultural technology, Al Balqa Applied University, Salt, Jordan
2 Al Ahliyya Amman University (and The University of Jordan), Faculty of Agricultural technology, Amman, Jordan
3 Hamdi Mango Centre for Scientific Research, University of Jordan, Amman
4 Corresponding author,e-mail: t.alqudah@ju.edu.jo
5 Department of Nutrition and Food Technology, Faculty of Agriculture, Mutah University, Karak, Jordan

1 INTRODUCTION

Tetragonolobus palaestinus is a wild plant from the Fabaceae family. Its natural habitat can be found in the northern parts of Jordan on the rocks and meadow areas (Afifi & Abu-Irmaileh, 2000). *T. palaestinus* is a herbaceous plant, it starts to grow after seasonal rainfall in the winter, and has a red flower and a small fruit (pod) which can be served as fresh or boiled to eat, it is well-known among the people as Aljlatoun (Al-Karaki, 2000). Most Jordanian wild plants are becoming endangered due to the expansion of urban and rural settlements, uncontrolled deforestation, illegal collection, industrial pollution, and low level of environmental awareness (Al-Bakri et al., 2011). Therefore, to solve such problem, alternative methods for massive plant propagation like plant tissue culture techniques and other biotechnological approaches are used for producing medicinal plants, isolating medicinal secondary products, conserving and rapid propagating valuable, rare, and endangered plant species (Arafeh et al., 2006; Al-Mahmood et al., 2012; Qrunfleh et al., 2013; Shatnawi , 2013). Propagation methods of *T. palaestinus* using seeds are not preferred due to the low germination percentage (Al-Karaki, 2000).

In vitro culture of *T. palaestinus* can solve propagation problems as it guarantees mass production of plant material without compromising the natural resources and it also improves and conserves this plant (Alenizi et al., 2020; Ebrahim et al., 2007; Shatnawi 2006, Shibli et al., 2018). The use of *in vitro* culture technique is the best solution to overcome *T. palaestinus* propagation problems and can also enhance mass production without threatening the natural resources (Shibli et al., 2003; Makhadmeh & Shatnawi, 2008; Shatnawi et al., 2011, Al Qudah et al., 2011). Also, it is an important tool in both basic and applied studies and for commercial applications (Arafeh et al., 2006; Ahmad et al., 2010). Using micropropagation; the plant developed from this technique is true to type or genetically uniform with the mother plant (Shibli et al., 2003). Production of a large number of genetically uniform disease-free plants is known to be a reliable technique system. The conventional method of propagation is done by vegetative methods through root suckers or terminal cutting which is classified as very slow (George et al., 2008). *In vitro* propagation plays a major role in the rapid production of disease-free planting material of newly improved varieties year rounded basis (Ebrahim et al., 2007; Shatnawi 2006, Shatnawi et al., 2011). Shoot tip culture is a relatively simple *in vitro* technique for the rapid propagation of selected pathogen-free plant materials. Therefore, many simple protocols have been developed for the rapid multiplication of newly released commercially important genotypes through apical meristem cultures. Successful commercial micropropagation protocol depends on successful rooting and acclimatization of *in vitro* derived plantlets (Ebrahim et al., 2007; Shatnawi 2006, Shatnawi et al., 2011). Till now; there are no available data on the *in vitro* propagation of *T. palaestinus*. So, this study was initiated to develop an applicable and simple protocol for in vitro establishment, multiplication, rooting, and acclimatization of *T. palaestinus*.

2 MATERIAL AND METHODS

2.1 ESTABLISHMENT OF *IN VITRO* CULTURE

Plant seeds of wild *T. palaestinus* were collected in mid of April in north Jordan – "Al-Sareeh, Irbid" (32.3306° N latitude and 35.8951° E Longitude). Firstly, surface sterilization of seeds was done by washing seeds with tap water for 5 min. After that, seeds were immersed in (4 %) sodium hypochlorite for 15 min. The following sterilization steps were performed under sterile conditions in a laminar air flow chamber; the seeds were washed 3 times in sterile distilled water, then soaked in 70 % ethanol solution for 30 s and washed several times with sterile distilled water. Seeds were cultured on the surface of hormone free Murashige and Skoog (MS) medium (1962) inside Petri dishes (five seeds/ Petri dish). Murashige and Skoog (MS) medium was supplemented with vitamins and 30 g $l^{-1}$of sucrose. After the final volume of the MS media was adjusted to 1 l and the pH to 5.75, 8 g of agar was added to the media mixture with constant stirring and heating until the agar was completely dissolved. After that, 100 ml of medium was poured into *Erlenmeyer flask*s. Then flasks were plugged and autoclaved at 121 °C for 15 min. After that cultures of seeds were kept in a growth room in the dark and moderate temperature 24 ± 2 °C for four weeks until full germination. The germinated seedlings were transferred to light conditions in the growth room under the light regime (16/8 h (light/dark) and a light intensity of 50 µmol $m^{-2}s^{-1}$. Afterward, cultures were transferred to the new medium and further grown. Then, cultures were transferred to MS medium provided with growth regulators, i.e. 0.3 mg l^{-1} benzyl amino purine (BAP) and 0.05 mg l^{-1} naphthalene acetic acid (NAA) with 30 g l^{-1} sucrose, to increase the growth of the cultures.

2.2 SHOOT PROLIFERATION

Microshoots of 10 mm in length, were treated with different concentrations of cytokinins for shoot proliferation experiments. MS media were supplemented with 0.0, 0.3, 0.6, 0.9, 1.2, 1.5 or 2.0 mg l^{-1} of BAP, Kinetin, Thidiazuron (TDZ) or Zeatin. Five replications with three microshoots were used for each treatment. Data were collected after five weeks for the microshoots growth parameters as shown in Table 1.

2.3 ROOT FORMATION OF *IN VITRO* CULTURES

Microshoots, 10 mm in length, were treated with different concentrations of auxins. For root formation MS media were supplemented with 0.0, 0.3, 0.6, 1.2, 1.5 or 2.0 mg l^{-1} of indole-3-butyric acid (IBA), indole acetic acid (IAA) or naphthalene acetic acid (NAA). Ten replications were used with one microshoot / replicate. Data were collected for the number of axillary shoots/ explant, shoot length, root length, and rooting (%) after five weeks.

2.4 ACCLIMATIZATION

The fully *in vitro* rooted microshoots were hardened gradually from *in vitro* tubes. Firstly, the tubes plugs were removed for three days and the cultures were left in the growth room. After that microshoots were gently transferred from test tubes and washed until all agar residues were removed and grown in plastic pots that have a suitable mixture of (1 peat : 3 perlite). Cultures were covered with perforated plastic bags for 3 days with continuous wetting with sterile distilled water. After that, plastic bags were removed and the pots were left for extra 2 weeks under growth room conditions with continuous wetting. At the end of the acclimatization experiment, the survival percentage of the acclimatized plants was registered.

2.5 EXPERIMENTAL DESIGN

The completely randomized design (CRD) was used with all the experiments. Data were analyzed in SPSS Software with Tukey HSD Multiple Range test at $p \leq 0.05$. Means and standards error of means were calculated.

3 RESULTS AND DISCUSSION

In this study, significant differences in the shoots growth parameters were obtained using BAP, Kinetin, TDZ, or zeatin at different concentrations. At 0.3 mg l^{-1} of BAP, maximum shoots numbers (2.0 shoot per explants) were obtained (Table 1 and Fig. 1). Additionally, the length of shoots and the number of leaves on average increase up to two- fold (30.0 mm and 12.2 leaves, respectively) in comparison with controls (18.0 mm, 7.0 leaves, respectively). At 0.9 mg l^{-1} BAP, a maximum fresh mass of 112.0 mg was obtained and it was 1.7-fold higher compared to controls (66.0 mg). Shoot length increased up to 30 mm and 26 mm at the lowest and the highest concentrations of BAP (0.3, 2.0 mg l^{-1}; respectively), comparing with the control (18.0 mm). But; at 1.2 and 1.5 mg l^{-1} BAP the length of the shoots was significantly smaller compared to the length of shoots in medium with 0.3 mg l^{-1} BAP. Furthermore; BAP at (0.3 and 0.6 mg l^{-1}) concentrations resulted in the highest number of leaves 12 (leaves). One of the best cytokinins that can be used to induce *in vitro* shoot formation is 6-benzylaminopurine (Singh et al., 2019). Thảo et al. (2013) reported the highest shoot induction in common bean (*Phaseolus vulgaris* L.) when BAP was used with NAA in media. Similarly, in *Securidaca longipedunculata* (Fresen) the combinations between BAP and IBA at (1.5 mg and 0.1 mg l^{-1}; respectively) produced a better short number and length per explant than other growth regulator combinations (Lijalem and Feyissa, 2020). Besides that, BAP has been reported in many previous studies for shoot multiplications. For example; *Trichosanthes dioica* Roxb. was established from nodal explants on MS medium containing 1.0 mg l^{-1} BAP (Tiwari et al., 2010). BAP also; gave the best results for *Prosopis cineraria* (L.) Druce in vitro establishment (Kumar and Singh, 2010). BAP gave the best outcome for shoot induction in the in vitro grain legume, *Phaseolus vulgaris* (Malik and Saxena, 1992)

Similarly, kinetin at 0.3 mg l^{-1} had increased the shoot length up to 32.0 mm which was 1.7-fold longer than control (18.0 mm) (Table 1, Fig 1). Moreover, maximum dry and fresh mass (140 and 114 mg; respectively) of *in vitro Tetragonolobus palaestinus* explants were obtained at 0.3 g l^{-1} of kinetin. Increasing concentrations of kinetin inhibited the growth of the shoots in length and their appearance was swelling and short. Kinetin induced expansion of growth by swelling rather than elongation, this was confirmed previously by Naeem (2004). Ahmadi et al. (2011) reported that using kinetin at 2.0 mg l^{-1} increased the *in vitro* shoot induction in

Matthiola incana (L.) W.T.Aiton. In *Moringa stenopetala* (Baker f.) Cufod.**;** maximum number of shoots per explant (3.43 ± 1.41) and 7.97 ± 4.18 leaves per explant were obtained on MS medium containing 0.5 mg l^{-1} kinetin with 0.01 mg l^{-1} NAA. **(**Adugna et al., 2020**).**

The addition of 0.3 mg l^{-1} TDZ to MS medium resulted in longer shoots (32.0 mm) compared to controls (18.0 mm), and the highest number of leaves per explant (14.8 leaves per explant) was obtained on MS medium supplemented with 1.2 mg l^{-1} TDZ. The Growth regulator TDZ had been used in previous studies in order to promote *in vitro* propagation of different plants species of the Fabaceae family; such as, *in vitro Psophocarpus tetragonolobus* (L.) D.C. (Singh et al., 2014); and common bean (Veltcheva et al., 2005). The results from the present work demonstrated that TDZ at low concentration was effective compared to other cytokinins (Table 1). However, it was found to be effective at low concentration. Low concentrations of TDZ (0.01 mg l^{-1}) were the most appropriate for shoot regeneration in *Abelmoschus moschatus Medik* (Sharma & Shahzad, 2008). The effect of TDZ on growth parameters is not entirely clear, and more studies are needed to understand its role in plant tissue cultures. (Ugandhar et al., 2012). TDZ in combination with NAA produced relatively shorter shoots when used with *Securidaca longipedunculata* (Fresen) (Lijalem and Feyissa, 2020). Furthermore; TDZ had been reported to have an adverse effects with *Vitex trifolia* L. (Ahmed and Anis, 2012).

When zeatin was used, a maximum number of shoots (1.4 shoots per explant) was obtained on MS medium supplemented with 0.3, 0.9, and 1.2 mg l^{-1} Zeatin (Table 1). While the maximum shoot length (28.0 mm) was produced on MS medium supplemented with 0.9 mg l^{-1} Zeatin. On the other hand, Vikram et al. (2012) reported that Zeatin at 1.2 mg l^{-1} produced a maximum number of multiple shoot formation in *Lycopersicum esculentum* L.. In addition, a highly efficient organogenesis protocol for in vitro regeneration of eggplant was developed using zeatin (García-Fortea et al., 2020). This may be due to that, zeatin suppress apical dominance which leads to increase numbers of multiple shoots and reduce the length of the shoot.

3.1 IN VITRO ROOTING

The in vitro rooting of T. palaestnius was significantly induced at a concentration of 0.3 mg l^{-1} of IBA with (4.06 roots/microshoot). The rooting percentage was 40 % with 3.33 mm/root long at 0.3 mg l^{-1} of IBA. Meanwhile; control and other concentrations of IBA showed lower in vitro rooting; as we can show in (Table 2 & Fig 2). Low concentrations of IBA (1.0 mg l^{-1}) also resulted in the highest in vitro rooting in Cicer microphyllum Benth. (Singh et al., 2019) and in common bean (Phaseolus vulgaris L.) (Thảo et al., 2013).

For rooting with IAA growth regulator; the maximum number of roots per microshoots was (2.14 roots/explant) obtained at 1.2 mg l^{-1} IAA with a maximum root length of 2.94 mm. The maximum root percentage (30 %) was also recorded on media supplemented with 1.2 mg l^{1} IAA. Using 0.3 mg l^{-1} NAA resulted in 0.5 developed root length of 1.94 mm (Table 2). No callus occurred at microshoots bases.

The current study showed that auxin is essential for the induction of root formation of in vitro T. palaestnius cultures. This is because auxin exerts a primary role in root formation by its involvement in successive and interdependent phases (Mineo, 1990). The rooting of leguminous species is dependent on the auxin type (Dewir et al.,

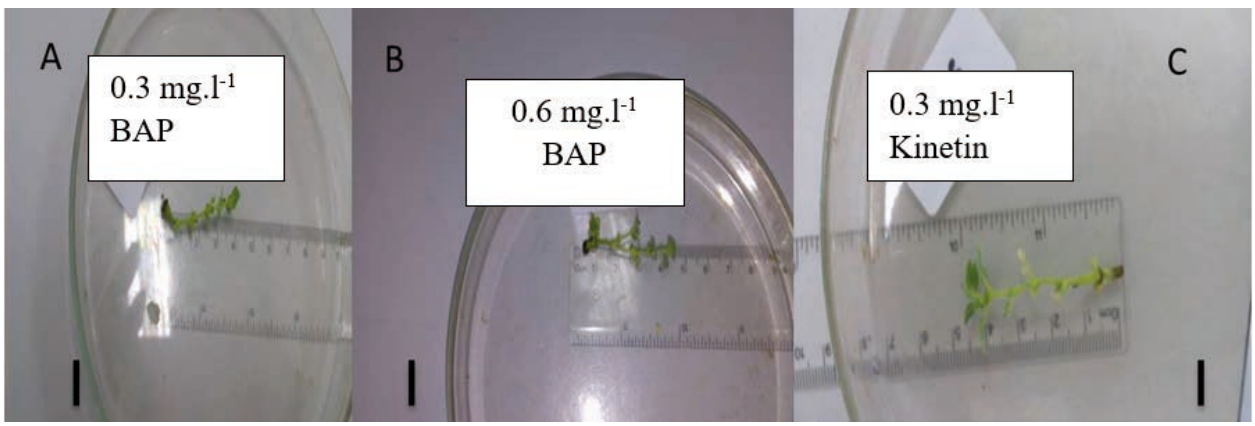


Figure 1: In vitro shoot formation of *Tetragonolobus palaestinus* after five weeks. A) MS with 0.3 mg l^{-1} (BAP). B) MS with 0.6 mg l^{-1} (BAP). C) MS medium with 0.3 mg l^{-1} Kinetin. Bars represent 1.0 cm

2016). Using IBA or NAA at a low concentrations such as 0.3 mg l^{-1} increased cell division and root primordial formation and proved to enhanced rooting percentage, number of roots per rooted explants and, root length as compared to IAA or IBA. Some studies found that NAA was the best rooting auxin in Vigna mungo (L.) Hepper (Mony et al., 2010). Furthermore; different legumes of the Fabaceae family have been in vitro rooted using different auxins types. For example; the in vitro rooting in Clitoria ternatea L. which is known as the butterfly pea plant was obtained by the addition of 1.50 mg l^{-1} NAA with the highest number of adventitious roots (12.86 ± 2.14) (Lee et al., 2021). While IBA had been used in the in vitro rooting of Lotononis bainesii Baker (Fabaceae) at the concentration of 0.049 µM IBA (Vidoz et al., 2012). Also, 83 % of in vitro rooting in Thermopsis turcica Kit Tan, Vural & Küçük. (Fabaceae) was attained on pulsed-IBA treated shoots (Cenkci et al., 2008). The growth regulator IAA, was used at 0.005–0.01 mg l^{-1} to induce in vitro rooting in Psoralea corylifolia L.(Fabaceae) which is

Table 1: The effect of different concentrations of the cytokinins on *in vitro* grown *Tetragonolobus palaestinus* after five weeks of incubations. *Values represent means ± standard error. *Means within the column for each growth regulator having different letters are significantly different according to Tukey HSD at $p \leq 0.05$

Concentrations mg L^{-1}	Number of axillary shoots/explant	Shoot length (mm)	Number of leaves/ explant	Fresh mass/five explants (mg)	Dry mass/five explants (mg)
Control 0.0	1.0 ± 0.0 b	18.0 ± 1.22 c	7.0 ± 0.32 c	66.0 ± 2.0 b	34.0 ± 2.4 c
			BAP		
0.3	2.0 ± 0.32 a	30.0 ± 3.1 a	12.2 ± 0.74 a	66.0 ± 2.0 b	34.0 ± 2.4 c
0.6	1.6 ± 0.20 ab	28.0 ± 2.0 ab	12.0 ± 0.44 a	90.0 ± 2.0 ab	56.0 ± 5.0 b
0.9	1.6 ± 0.40 ab	26.0 ± 2.4 ab	11.2 ± 0.37 ab	112.0 ± 22.0 a	106.9 ± 15.0 a
1.2	1.0 ± 0.24 b	22.0 ± 2.0 b	10.8 ± 0.37 b	92.0 ± 4.0 ab	52.0 ± 5.0 b
1.5	1.6 ± 0.00 ab	22.0 ± 2.0 b	11.4 ± 0.51 ab	52.0 ± 0.0 c	20.0 ± 5.0 d
2.0	1.8 ± 0.20 ab	26.0 ± 2.4 ab	11.8 ± 0.37 ab	88.0 ± 17.6 ab	62.0 ± 5.0 b
			Kinetin		
0.3	1.2 ± 0.20 a	32.0 ± 3.7 a	12.4 ± 1.12 a	140.0 ± 24.4 a	114.0 ± 25.0 a
0.6	1.2 ± 0.20 a	22.0 ± 0.0 b	11.8 ± 0.48 a	96.0 ± 14.7 ab	64.0 ± 9.21 ab
0.9	1.2 ± 0.2 a	24.0 ± 2.4 ab	11.8 ± 0.48 a	92.0 ± 3.7 ab	66.0 ± 5.12 ab
1.2	1.0 ± 0.0 b	15.0 ± 0.0 d	8.0 ± 0.20 c	44.0 ± 4.0 d	20.0 ± 3.1 c
1.5	1.4 ± 0.24 a	20.0 ± 0.0 b	10.2 ± 0.37 b	88.0 ± 2.0 b	66.0 ± 4.0 ab
2.0	1.0 ± 0.0 b	15.0 ± 0.0 d	4.0 ± 0.24 d	30.0 ± 0.0 d	14.0 ± 2.0 c
			TDZ		
0.3	1.6 ± 0.24 a	32.0 ± 2.0 a	10.8 ± 0.37 b	84.0 ± 4.4 b	58.0 ± 7.3 c
0.6	1.8 ± 0.37 a	20.0 ± 0.0 ab	12.8 ± 0.58 ab	90.0 ± 4.4 ab	64.0 ± 5.1 b
0.9	1.2 ± 0.20 b	22.0 ± 2.0 b	10.8 ± 0.37 b	84.0 ± 5.1 b	85.0 ± 5.8 ab
1.2	1.8 ± 0.37 a	30.0 ± 4.4 a	14.8 ± 0.24 a	136.0 ± 26.1 a	112.0 ± 24.1 a
1.5	1.6 ± 0.24 a	24.0 ± 2.4 ab	10.6 ± 0.40 b	92.0 ± 2.0 ab	60.0 ± 5.4 c
2.0	1.6 ± 0.24 a	22.0 ± 2.0 b	11.8 ± 0.41 b	90.0 ± 6.7 ab	66.0 ± 6.7 b
			Zeatin		
0.3	1.4 ± 0.25 a	26.0 ± 2.45 a	15.4 ± 0.81a	66.0 ± 1.8 b	106.9 ± 24.0 a
0.6	1.2 ± 0.20 ab	20.0 ± 0.0 ab	10.4 ± 0.24 ab	92.0 ± 2.0 ab	56.0 ± 4.8 b
0.9	1.4 ±0.40 a	28.0 ± 3.74 a	15.4 ± 1.02 a	112.0 ± 22.0 a	74.0 ± 5.1 ab
1.2	1.4 ± 0.24 a	24.0 ± 2.44 ab	10.0 ± 0.95 ab	92.0 ± 5.0 ab	52.0 ± 1.9 b
1.5	1.0 ± 0.00 b	11.0 ± 1.00 c	7.6 ± 0.51 c	52.0 ± 2.0 c	20.0 ± 6.0 d
2.0	1.2 ± 0.20 ab	20.0 ± 00 ab	11.60 ± 0.88 ab	88.0 ± 4.0 ab	62.0 ± 3.0 b

a rare and endangered herbaceous medicinal plant (Rout et al., 2010). We can see from this, that in vitro rooting depend on plant species and the type of auxin used.

3.2 ACCLIMATIZATION

The acclimatization process of *Tetragonolobus palaestinus* microshoots was proved its ability to the production of healthy acclimatized micro shoots. The mixture of (Peatmoss: Perlite) was suitable for roots growth and new leaves were formed after 2 weeks (Fig 3). All of the rooted plantlets were survived after acclimatization process after acclimatization process. No variations were observed visually among the acclimatized plantlets as shown in Fig (3). A lower survival rate of 65 % was obtained in *C. microphyllum* using different potting culture mixture (garden soil, vermiculite, and vermicompost (1:1:1) (Singh et al., 2019)

Plantlet needs an acclimatization period and this could be due to the effect of tissue differentiation, growth, and development (Quisen, 2013). The acclimatized plantlet may be affected by the change in environmental conditions during acclimatization, which may be due to the short acclimatization period evaluated in this study (Shatnawi., 2013). Different plants of the Fabaceae family have been successfully in vitro rooted and acclima-

Figure 2: The effect of 0.3 mg l^{-1} indole-3-butyric acid (IBA) on *in vitro* growth of *Tetragonolobus palaestinus* after five weeks growth. The bar represents 1.0 cm

Table 2: The effect of different auxin concentration on *in vitro* root formation of *Tetragonolobus palaestinus* after five weeks growth period. ˙Values represent means ± standard error. *Means within the column for each growth regulator having different letters are significantly different according to Tukey HSD at $p \leq 0.05$

Concentrations mg.l^{-1}	Number of axillary shoots/explant	Shoot length (mm)	Number of roots / explant	Root length (mm)	Rooting %
Control 0.0	1.01 ± 0.23* b	18.07 ± 7.46 ab	1.50 ± 0.48 b	2.00 ± 0.70 ab	20%
		IBA			
0.3	1.16 ± 0.32 a	19.0 ± 9.5 a	4.06 ± 0.67 a	3.33 ± 0.90 a	40%
0.6	1.09 ± 0.18 a	17.06 ± 7.06 ab	3.17 ± 0.52 b	1.44 ± 1.33b	20%
1.2	1.04 ± 0.15 a	12.28 ± 4.21 b	2.67 ± 0.78 ab	2.94 ± 1.86 a	30%
1.5	1.04 ± 0.16 a	13.33 ± 7.13 b	2.06 ± 0.78 ab	1.94 ± 2.83 b	30%
2.0	1.06 ± 0.17 a	12.04 ± 5.29 b	2.94 ± 0.81 ab	3.17 ± 0.86 a	20%
		IAA			
0.3	1.00 ± 0.22 b	13.78 ± 4.41 ab	0.56 ± 0.24 b	0.89 ± 0.40 b	20%
0.6	1.22 ± 0.18 a	12.61 ± 3.45 b	0.50 ± 0.20 b	2.61 ± 0.84 a	27%
1.2	1.06 ± 0.16 ab	19.33 ± 4.02 a	2.14 ± 0.17 a	2.94 ± 1.50 a	30%
1.5	1.09 ± 0.12 ab	12.28 ± 4.09 b	0.57 ± 0.60 b	1.83 ± 1.54 ab	26%
2.0	1.02 ± 0.13 b	12.61 ± 3.38 b	1.39 ± 0.41 ab	2.39 ± 1.11 a	20%
		NAA			
0.3	1.00 ± 0.22 b	17.00 ± 4.41 ab	0.56 ± 0.24 a	0.89 ± 0.40 c	30%
0.6	1.06 ± 0.18 ab	18.01 ± 3.45 a	0.50 ± 0.20 a	1.61 ± 0.84 ab	27%
1.2	1.01 ± 0.16 b	16.03 ± 4.02 b	0.44 ± 0.17 b	1.94 ± 1.50 a	20%
1.5	1.09 ± 0.12 ab	17.08 ± 4.09 ab	0.17 ± 0.60 b	1.83 ± 1.54 a	26%
2.0	1.02 ± 0.13 b	17.01 ± 3.38 ab	0.39 ± 0.41 ab	1.39 ± 1.11 b	30%

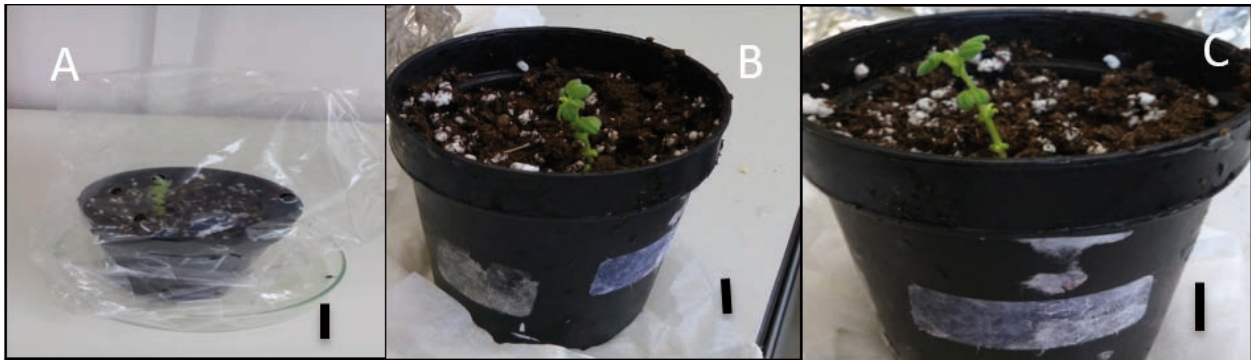


Figure 3: Acclimatization process of *Tetragonolobus palastinus* microshoots. Plantlets after: A) one week B) three weeks and C) five weeks of the Acclimatization process. Bars represent 1.0 cm

tized. For example, the regenerated plantlets of licorice (*Glycyrrhiza glabra* L.; Fabaceae) were acclimatized, with a survival rate of 77.7 %, when transferred to ex vitro conditions and showed no morphological abnormalities (Shaheen, 2020). While, the in vitro seedlings of *Caesalpinia ferrea* Mart. were acclimated without the presence of roots in different types of the substrate with 73.4 % surviving plantlets after 30 days of growth (Silva et al., 2018). On the other hand, in *A. leiocarpa* (L.A.S.Johnson ex G.J.Leach) K.R.Thiele & Ladiges plantlets the substrate composition did not affect the survival or growth of in vitro rooted plantlets during acclimatization (Haygert-Lencina it., 2017). We can notice from this, that acclimatization is a critical process and it depends on plant species and the successful adjustment of the environment of the acclimatized plants.

4 CONCLUSION

The current results indicat that *in vitro* propagation method of *Tetragonolobus palastinus* was successful, with full survival percentage for the first records of this plant species *in vitro*. The optimum *in vitro* propagation method of *Tetragonolobus palastinus* was obtained at 0.3 mg l^{-1} of Benzylamino purine (BAP) with 2.0 shoots/explants and 0.3 mg l^{-1} IBA with (4.06 roots/explants) and this method is recommended for *in vitro* clonal propagation in *T. palaestinus*.

5 REFERENCES

Adugna, A. Y., Feyissa, T., & Tasew, F. S. (2020). Optimization of growth regulators on in vitro propagation of *Moringa stenopetala* from shoot explants. *BMC Biotechnology*, *20*(1), 1-10. https://doi.org/10.1186/s12896-020-00651-w

Adwan, G., Abu-shanab, B., & Adwan, K. (2009). *In vitro* activity of certain drugs in combination with plant extracts against *Staphylococcus aureus* infections. *African Journal of Biotechnology*, *8*(17), 4239-4241.

Afifi, F. U., & Abu-Irmaileh, B. (2000). Herbal medicine in Jordan with special emphasis on less commonly used medicinal herbs. *Journal of Ethnopharmacology*, *72*(1-2), 101-110. https://doi.org/10.1016/S0378-8741(00)00215-4

Ahmad, N., Faisal, M., Anis, M., & Aref, I. M. (2010). *In vitro* callus induction and plant regeneration from leaf explants of *Ruta graveolens* L.. *South African Journal of Botany*, *76*(3), 597-600. https://doi.org/10.1016/j.sajb.2010.03.008

Ahmadi Hesar, A., Kaviani, B., Tarang, A. R., & Zanjani, S. B. (2011). Effect of different concentrations of kinetin on regeneration of ten weeks (*Matthiola incana*). *Plant Omics Journal*, *4*(5), 236-38.

Ahmed MR, Anis M (2012) Role of TDZ in the quick regeneration of multiple shoots from nodal explant of *Vitex trifolia* L. an important medicinal plant. *Applied Biochemistry and Biotecnology*, *168*, 957–966. https://doi.org/10.1007/s12010-012-9799-0

Al-Bakri, J. T., Al-Eisawi, D., Damhoureyeh, S., & Oran, S. (2011). GIS-based analysis of spatial distribution of medicinal and herbal plants in arid and semiarid zones in the Northwest of Jordan. *Annals of Arid Zone*, *50*(2), 99-115.

Alenizi, A., Shibli, R. A., Tahtamouni, R. W., Al-Qudah, T. S., & Abu-Iramaileh, B. (2020). *In vitro* propagation and enhancement of quercetins and isorhamnetin production in wild *Paronychia argentea* L. *Jordan Journal of Pharmaceutical Sciences*, *13*(1).

Al-Karaki, G. N. (2000). Morphological and yield traits of wild legume (*Tetragonolobus palaestinus* Boiss) populations. *Journal of Agronomy and Crop Science*, *184*(4), 267-270. https://doi.org/10.1046/j.1439-037x.2000.00393.x

Al-Mahmood, H. J., Shatnawi, M. A., Shibli, R. A., Makhadmeh, I. M., Abubaker, S. M., & Shadiadeh, A. N. (2012). Clonal propagation and medium-term conservation of *Capparis spinosa*: a medicinal plant. *Journal of Medicinal Plants Research*, *6*(22), 3826-3836. https://doi.org/10.5897/JMPR11.547

Al-Qudah, T. S., Shibli, R. A., & Alali, F. Q. (2011). *In vitro* propagation and secondary metabolites production in

wild germander (*Teucrium polium* L.). *In Vitro Cellular & Developmental Biology-Plant*, *47*(4), 496-505. https://doi.org/10.1007/s11627-011-9352-9

Arafeh, R. M., Shibli, R. A., Al-Mahmoud, M., & Shatnawi, M. A. (2006). Callusing, cell suspension culture and secondary metabolites production in Persian oregano (*Origanum vulgare* L.) and Arabian oregano (*O. syriacum* L.). *Jordan Journal of Agricultural Sciences*, *2*(3), 274-81.

Cenkci, S., Kargioglu, M., Dayan, S., & Konuk, M. (2008). In vitro propagation of an endangered plant species, *Thermopsis turcica* (Fabaceae). *Biologia*, *63*(5), 652-657. https://doi.org/10.2478/s11756-008-0125-9

Dewir, Y. H., Murthy, H. N., Ammar, M. H., Alghamdi, S. S., Al-Suhaibani, N. A., Alsadon, A. A., & Paek, K. Y. (2016). *In vitro* rooting of leguminous plants: Difficulties, alternatives, and strategies for improvement. *Horticulture, Environment, and Biotechnology*, *57*(4), 311-322. https://doi.org/10.1007/s13580-016-0060-6

Ebrahim, N., Shibli, R., Makhadmeh, I., Shatnawi, M., & Abu-Ein, A. (2007). *In vitro* propagation and in vivo acclimatization of three coffee cultivars (*Coffea arabica* L.) from Yemen. *World Applied Sciences Journal*, *2*(2), 142-150.

García-Fortea, E., Lluch-Ruiz, A., Pineda-Chaza, B. J., García-Pérez, A., Bracho-Gil, J. P., Plazas, M., ... & Prohens, J. (2020). A highly efficient organogenesis protocol based on zeatin riboside for in vitro regeneration of eggplant. *BMC plant Plant Biology*, *20*(1), 1-16. https://doi.org/10.1186/s12870-019-2215-y

George, E. F., Hall, M. A., & De Klerk, G. J. (2008). Micropropagation: uses and methods. In *Plant propagation by tissue culture,* (pp. 29-64). Springer, Dordrecht. https://doi.org/10.1007/978-1-4020-5005-3_2

Haygert-Lencina, K., Antônio-Bisognin, D., Kielse, P., & Pimentel, N. (2017). Rooting and acclimatization of *Apuleia leiocarpa* plantlets. *Agrociencia*, *51*(8), 909-920.

Kumar S, Singh N (2010). Micropropagation of *Prosopis cineraria* (L.) Druce.: a multipurpose desert tree. *Report and Opinion*, *2,* 44–47.

Lee, R. X., Hassan, Z., Subramaniam, S., & Chew, B. L. (2021). Adventitious root cultures of *Clitoria ternatea* L. and its potential as a memory enhancer alternative. *Plant Biotechnology Reports*, *15*(2), 163-176. https://doi.org/10.1007/s11816-021-00664-7

Lijalem, T., & Feyissa, T. (2020). In vitro propagation of *Securidaca longipedunculata* (Fresen) from shoot tip: an endangered medicinal plant. *Journal of Genetic Engineering and Biotechnology*, *18*(1), 1-10. https://doi.org/10.1186/s43141-019-0017-0

Makhadmeh, I. M., & Shatnawi, M. A. (2008). *In vitro* propagation of threatened *Pimelea spicata* from mature plant material. *Advances in Horticultural Science*, 212-217.

Malik KA, Saxena PK (1992) Regeneration in *Phaseolus vulgaris* L.: high frequency induction of direct shoot formation in intact seedlings by N6- benzylaminopurine and thidiazuron. *Planta*, *186,*384–389. https://doi.org/10.1007/BF00195319

Mineo, L. (1990). Plant tissue culture techniques. *Tested studies for laboratory teaching*, *11*, 151-174.

Mony, S. A., Haque, M. S., Alam, M. M., Hasanuzzaman, M., & Nahar, K. (2010). Regeneration of blackgram (*Vigna mungo* L.) on changes of hormonal condition. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, *38*(3), 140-145.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, *15*(3), 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Naeem, M., Bhatti, I. R. A. M., Ahmad, R. H., & Ashraf, M. Y. (2004). Effect of some growth hormones (GA3, IAA and Kinetin) on the morphology and early or delayed initiation of bud of lentil (*Lens culinaris* Medik). *Pakistan Journal of Botany*, *36*(4), 801-809.

Qrunfleh, I. M., Shatnawi, M. M., & Al-Ajlouni, Z. I. (2013). Effect of different concentrations of carbon source, salinity and gelling agent on *in vitro* growth of fig (*Ficus carica* L.). *African Journal of Biotechnology*, *12* (90), 936-940.

Quisen, R. C., Raizer, M. D. M., & Iriarte-Martel, J. H. (2013). Acclimatization of micropropagated plantlets of *Heliconia sexy* Pink. *Embrapa Amazônia Ocidental-Artigo em Periódico Indexado (ALICE), 56*, Supl., 1-5. https://doi.org/10.4322/rca.2013.073

Rout, G. R., Samantaray, S., & Das, P. (2000). In vitro rooting of *Psoralea corylifolia* Linn: Peroxidase activity as a marker. *Plant Growth Regulation*, *30*(3), 215-219. https://doi.org/10.1023/A:1006336819887

Shaheen, A., Ali, M., Ahmad, N., Hassan Dewir, Y., El-Hendawy, S., Gawad, A. E., & Ahmed, M. (2020). Micropropagation of licorice (*Glycyrrhiza glabra* L.) by using intermediate nodal explants. *Chilean Journal of Agricultural Research*, *80*(3), 326-333. https://doi.org/10.4067/S0718-58392020000300326

Sharma, R., & Shahzad, A. (2008). Thidiazuran (TDZ) induced regeneration from cotyledonary node explant of *Abelmoschus moschatus* Medik. L., (A valuable medicinal plant). *World Journal of Agricultural Sciences*, *4*(4), 449-452.

Shatnawi, M. A. (2006). Micropropagation and germplasm storage of *Prunus amygdalus* by the vitrification method. *Jordan Journal of Agricultural Sciences*, *2*(3), 222-233.

Shatnawi, M. A. (2013). Multiplication and cryopreservation of yarrow (*Achillea millefolium* L., Astraceae). *Journal of Agriculture Science and Technology*. *15*, 163-173.

Shatnawi, M. A., Shibli, R. A., Abu-Romman, S. M., Al-Mazra, M. S., Al Ajlouni, Z. I., Shatanawi, A., & Odeh, W. H. (2011). Clonal propagation and cryogenic storage of the medicinal plant *Stevia rebaudiana. Spanish Journal of Agricultural Research*, *9*(1), 213-220. https://doi.org/10.5424/sjar/20110901-021-10

Shibli, R. A., Sharaf, S. A., Kasrawi, M. A., & Al-Qudah, T. S. (2018). *In vitro* multiplication of the white wormwood, *Artemisia herba-alba* Asso. *Jordan Journal of Biological Sciences*, *11*(3).

Shibli, R. A., Shatnawi, M. A., & Swaidat, I. Q. (2003). Growth, osmotic adjustment, and nutrient acquisition of bitter almond under induced sodium chloride salinity in vitro. *Communications in Soil Science and Plant Analysis*, *34*(13-14), 1969-1979. https://doi.org/10.1081/CSS-120023231

Silva, D., Imakawa, A. M., Bruno, F. M. S., de Souza Costa, S., & Sampaio, P. D. T. B. (2018). In vitro propagation and seed-

ling acclimatization of *Caesalpinia ferrea* Mart., a valuable medicinal plant in the Amazon (Fabaceae). *Boletin do Museu Paraense Emílio Goeldi-Ciências Naturais*, *13*(1), 57-65. https://doi.org/10.46357/bcnaturais.v13i1.368

Singh, R. K., Anandhan, S., García-Pérez, L. M., Ruiz-May, E., Pérez, E. N., & Quiroz-Figueroa, F. R. (2019). An efficient protocol for *in vitro* propagation of the wild legume *Cicer microphyllum* Benth., a crop wild relative of chickpea (*Cicer arietinum* L.). *In Vitro Cellular & Developmental Biology-Plant*, *55*(1), 9-14. https://doi.org/10.1007/s11627-018-09958-y

Singh, V., Chauhan, N. S., Singh, M., Idris, A., Madanala, R., Pande, V., & Mohanty, C. S. (2014). Establishment of an efficient and rapid method of multiple shoot regeneration and a comparative phenolics profile in *in vitro* and greenhouse-grown plants of *Psophocarpus tetragonolobus* (L.) DC. *Plant Signaling & Behavior*, *9*(10), e970443. https://doi.org/10.4161/15592316.2014.970443

Thảo, N. T., Thảo, N. T. P., Hassan, F., & Jacobsen, H. J. (2013). *In vitro* propagation of common bean (*Phaseolus vulgaris* L.). *Journal of Science and Development*, *11*(6), 868-876.

Tiwari AK, Singh D, Tripathi S, Mishra N, Singh RB (2010) Effect of BAP and kinetin on shoot initiation of *Trichosanthes dioica* Roxb: An important medicinal plant. *Journal of Medicinal Plants, 2,* 59–61. https://doi.org/10.5958/j.0975-4261.2.1.008

Ugandhar, T., Venkateshwarlu, M., Sammailah, D., & Reddy, J. M. K. (2012). Rapid *in vitro* micro propagation of chick pea (*Cicer arietinum* L.) from shoot tip and cotyledonary node explants. *Journal Biotechnol Biomater*, *2*(6), 1-6.

Veltcheva, M. R., & Lilova Svetleva, D. (2005). *In vitro* regeneration of *Phaseolus vulgaris* L. via organogenesis from petiole explants. *Journal of Central European Agriculture*, *6*(1), 53-58.

Vidoz, M. L., Quesenberry, K. H., Real, D., & Gallo, M. (2012). Plant regeneration of *Lotononis bainesii* Baker (Fabaceae) through cotyledon and leaf culture. *African Journal of Biotechnology*, *11*(41), 9724-9731. https://doi.org/10.5897/AJB11.2680

Vikram, G., Madhusudhan, K., Srikanth, K., Laxminarasu, M., & Swamy, N. R. (2012). Zeatin induced direct multiple shoots development and plant regeneration from cotyledon explants of cultivated tomato (*Solanum lycopersicum* L.). *Australian Journal of Crop Science*, *6*(1), 31-35.

doi:10.14720/aas.2022.118.3.2626 Original research article / izvirni znanstveni članek

Isolation of salt-tolerant *Pseudomonas* strains with potential for alleviation of salt stress in peanut plant (*Arachis hypogaea* L.)

Quang Trung DO [1, 2], The Anh LUU [1, 3], Minh Truong DAO [1], Quoc Nam HOANG [3, 4], Trong Tri NGUYEN [5]

Received March 26, 2022; accepted July 07, 2022.
Delo je prispelo 26. marca 2022, sprejeto 7. julija 2022

Isolation of salt-tolerant *Pseudomonas* strains with potential for alleviation of salt stress in peanut plant (*Arachis hypogaea L.*)

Abstract: Plant growth-promoting rhizobacteria (PGPR) is a promising solution to improve plant growth under salt stress. Among PGPR, *Pseudomonas* is a genus of bacteria that possesses a variety of mechanisms in promoting plant growth and inducing resistance to biological as well as non-biological stress. This study aimed to isolate the genus *Pseudomonas* from the salty-contaminated rhizosphere of plant root collecting at Nam Dinh, and also investigate their functions in promoting the growth of peanut seedlings under salty conditions. Nine *Pseudomonas* bacteria were isolated, but only seven of them were identified by *Pseudomonas*-specific primers. Two of those seven isolates, ND06 and ND09, were chosen based on their characteristics in promoting plant growth such as the production of indole-3-acetic acid (IAA), phosphate solubilization, and nitrogen fixation. In addition, both two strains also carried the coding gene for 1-aminocyclopropane-1-carboxylate (ACC) deaminase which plays an important role in supporting plants to withstand various stress conditions. Especially, the ND09 strain improved the growth parameters of peanut seedlings under normal and salty stress conditions; while the ND06 only presented the plant growth enhancement under salty stress but not in normal conditions. These results suggest the ND09 strain may be used as a biological agent for eco-friendly agricultural practices in the future.

Key words: peanut plant; PGPR; *Pseudomonas*; salt stress resistance

Izolacija na sol tolerantnih sevov bakterij iz rodu *Pseudomonas* s potencialom zmanševanja solnega stresa pri arašidu (*Arachis hypogaea* L.)

Izvleček: Uporaba rast vzpodbujajočih rizobakterij (PGPR) je obetajoča rešitev za izboljšanje rasti rastlin v razmerah solnega stresa. Med PGPR imajo bakterije iz rodu *Pseudomonas* mehanizme, ki vzpodbujajo rast rastlin in povečujejo njihovo odpornost v razmerah biotičnega in abiotičnega stresa. Namen raziskave je bil izolirati bakterije iz rodu *Pseudomonas* iz rizosfere rastlin nabranih v zasoljenih tleh na območju Nam Dinh in preučiti njihove fukcije pri vzpodbujanju rasti sejank arašidov, rastočih v slanih tleh. Izoliranih je bilo devet vrst bakterij iz rodu *Pseudomonas*, vendar je bilo samo sedem od teh potrjenih s specifičnimi primerji za rod *Pseudomonas*. Dva od teh sedmih izolatov, ND06 in ND09, sta bila izbrana na osnovi njunih lastnosti vzpodbujanja rasti rastlin s tvorbo indol-3-ocetne kisline (IAA), raztaplanja fosfatov in fiksacije dušika. Dodatno sta oba seva vsebovala gen za kodiranje 1-aminociklopropan-1-karboksilaze (ACC), deaminaze, ki ima pomembnmo vlogo pri podpori rastlinam za prenašanje različnih stresnih razmer. Še posebej je sev ND09 izboljšal rastne parametre sejank arašidov v normalnih razmerah in ob solnem stresu. Med tem je sev ND06 izboljšal rast rastlin samo v razmerah solnega stresa, ne pa v normalnih razmerah. Razultati nakazujejo, da bi v prihodnosti sev ND09 lahko uporabili kot biotični aganes pri okolju prijaznem kmetovanju.

Ključne besede: arašid; PGPR; *Pseudomonas*; odpornost na solni stres

1 Central Institute for Natural Resources and Environmental Studies, Vietnam National University, Hanoi, Vietnam
2 Corresponding author: trungcnsinh@gmail.com
3 Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam
4 Institute of Geography, Vietnam Academy of Science and Technology, Hamoi, Vietnam
5 Vietnam National University of Forestry, Hanoi, Vietnam

1 INTRODUCTION

Peanuts (*Arachis hypogaea* L.), a plant of high economic value in agriculture, is considered the most widely produced and consumed oilseed plant all over the world and also in Vietnam. However, peanuts are quite sensitive to salt stress, which causes inhibitory effects to plant development and subsequently a decrease in peanuts production (Goswami et al., 2014; Sharma et al., 2016; Zörb et al., 2019). Recently, many methods have been applied to reduce soil salinity and acidity (Upadhyay and Singh, 2015; El-Nahrawy and Yassin, 2020). Among those, the phytoremediation and bioremediation methods are promising alternative approaches to retrieving salt-affected soils (Singh et al., 2015).

Plant growth-promoting rhizobacteria (PGPR) are bacteria that support growth and control pathogens in plants. Especially, the PGPR inoculation presented the alleviation of salt stress in the development of various plants such as tomato, pepper, canola, bean, *Arabidopsis*, and lettuce (Kang et al., 2009; Chu et al., 2019). The mechanism of plant growth stimulation of PGPR (such as *Rhizobium*, *Azospirillum*, *Pseudomonas*, *Flavobacterium*, *Arthrobacter*, and *Bacillus*) under saline conditions involves the biosynthesis of growth regulators such as indole-3-acetic acid (IAA); the enhancement of nutrients absorption for plants through the process of phosphate solubilization, nitrogen fixation; help plants maintain ionic balance; biosynthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase; inducing systemic tolerance (IST) (Goswami et al., 2014; Chu et al., 2019). Among those, fluorescent *Pseudomonas* sp. is the most studied and exploited bacteria because of some advanced abilities such as an excellent root-colonizing capability and plant growth-promoting activity (Egamberdieva, 2011); is also salt tolerant and able to alleviate salt stress in plants (Shafi et al., 2017). For example, Egamberdieva (2011) reported a significant increase in shoot length (up to 50 %) of beans in salt stress (at 5.0, 7.5, and 10.0 dS m^{-1}) when inoculated beans with *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU13. Hence, the selection of native PGPRs with characteristics related to bacterial suitability in the potential environment should be considered.

In this study, we aimed to isolate the genus *Pseudomonas* from the salt-contaminated rhizosphere of plants in Nam Dinh and to assess their abilities in stimulating peanuts' growth under conditions of salty stress. The results suggest a promising PGPR to further exploit as a bioinoculant in the future.

2 MATERIAL AND METHODS

2.1 ISOLATION AND SCREENING OF *PSEUDOMONAS* FOR SALT TOLERANCE FROM SALT-CONTAMINANT SOIL

Bacteria were isolated from 18 samples of the rhizosphere of corn (*Zea mays* L.), rice (*Oryza sativa L.),* and peanuts *(Arachis hypogaea*) obtained from salt-contaminated land in Quat Lam, Giao Thuy, Nam Dinh, Vietnam. The 10-fold serial dilutions of the samples were plated on sterile LB agar plates supplemented with 10 % NaCl. After 48 hours of incubation (30 ± 1) °C, the fluorescent colonies under a 366 nm wavelength UV lamp were selected, purified, sub-cultured, and preserved by deep freezing techniques at -80 °C.

Isolated strains were then reconfirmed by using PCR techniques to detect 16S rDNA sequences specifically for *Pseudomonas*. The bacteria proliferated on the Tryptone Soya Broth (TSB) agar medium around 18 hours in (30 ± 1) °C. Use the PureLink™ Genomic DNA Mini Kit (Thermo Fisher) to extract the bacterial gDNA according to the manufacturer's instructions. PCR reaction (25 µl) consists of 2.5 µl gDNA bacteria; 2.5 µl PCR reactive buffer solution (10X); 1 µl per primer (10 nM) and 1 U Phusion High-Fidelity DNA Polymerase (Thermo Fisher), and deionized water was added to get the desired volume. Forward primer (Psmn289: 5'–GGTCTGAGAGGATGATGATCAGT–3') and reverse primer (Psmn1258: 5'–TTAGCTCCACCTCGCGGC–3') were used (Widmer et al., 1998). The PCR program included 5 minutes at 95 °C; 25 cycles (15 seconds at 94 °C, 30 seconds at 55 °C, and 1 minute at 72 °C); 10 minutes at 72 °C. PCR product (about 960 bp) was then detected by electrophoresis on the 1 % agarose gel.

2.2 CHARACTERIZE THE SALT-TOLERANT BACTERIA STRAINS FOR PLANT-GROWTH PROMOTING PROPERTY

2.2.1 Salt tolerance

Strains of bacteria cultured on the TSB media supplemented with different NaCl concentrations ranging from 10 to 24 %. The culture was incubated on a shaker at 150 rpm at (30 ± 1) °C. The results were recorded after 1-4 days of incubation.

2.2.2 Phosphate solubilization

Strains of bacteria were grown on Pikovskaya

(PVK) media agar plates (Pikovskaya, 1948). The plates were incubated at 30 °C for 7 days. Each treatment was done in triplicates. The bacterial colonies with clear halos in the PVK agar plate indicated solubilizing activity of the phosphate. These were sub-cultured on PVK media (Biobasic, Canada).

The phosphate solubilization index (PSI) of bacteria grown on plates was measured as the following formula:

Phosphate solubilizing index (PSI) = [(colony diameter + clearing zone)/ colony diameter]

2.2.3 IAA production

The IAA content produced by isolates was determined by the color reaction with the improved Salkowski reagent (Glickmann and Dessaux, 1995). Bacteria were grown in TSB media containing 5 % NaCl, with an additional 0.1 g l^{-1} tryptophan. After 5 days of incubation at 150 rpm, (30 ± 1) °C, 1 ml of bacteria was collected and centrifuged to remove biomass. The bacterial supernatant was then added Salkowski reagent (1:2 ratio). The reaction was kept for 1 hour at room temperature. The positive reaction with the color from pink to red has measured the absorption at a wavelength of 530 nm to determine the IAA content based on the IAA standard line.

2.2.4 Nitrogen fixation

The bacterial isolates were cultured on a nitrogen-free mineral media containing 3 % NaCl (Wright and Weaver, 1981). Bacteria with the ability in forming colonies, and change the color of the media after 5 days of culture were identified as nitrogen-fixed bacteria.

2.2.5 Biofilm formation

The experiment was carried out in 96-well polystyrene microtiter plates (Biobasic, Canada), using the method described by O'Toole and Kolter (1998). The isolates were grown overnight in LB and LB + 0.3 M NaCl (Costa-Gutierrez et al., 2020a). Then overnight culture was diluted to an OD600 = 0.1 before placing in the wells. The plate incubation was done at 30 °C without agitation. After the indicated times, the biofilm formation was observed by staining with crystal violet (0.4 %) and then using 30 % glacial acetic acid solution to solubilize the dye before qualifying the biofilm formation by measuring absorbance at 540 nm.

2.2.6 Identification of the ACC deaminase encoding genes

PCR reaction (25 µl) consists of 2.5 µl bacterial g DNA; 2.5 µl PCR reactive buffer solution (10X), 1 µl per primer (10 nM), and 1 U Phusion High-Fidelity DNA Polymerase (Thermo Fisher) and deionized water was added to get the desired volume. Forward primer (5'–ATGAACCTGCTGCAACGATTC–3') and reverse primer (5'–TCAGCCGTCGGAAGAT–3') were applied (Saravanakumar and Samiyappan, 2007). The PCR program included 5 minutes at 95 °C; 25 cycles (15 seconds at 95 °C, 15 seconds at 58 °C, and 75 seconds at 72 °C); 5 minutes at 72 °C. PCR product (about 750 bp) was then detected by electrophoresis on the 1 % agarose gel.

2.3 EVALUATE THE PEANUT GROWTH PROMOTION OF BACTERIA UNDER SALTY STRESS AND IN VITRO CONDITIONS

Peanut seeds (*Arachis hypogaea* LDH12) were disinfected and germinated on cotton wool that was impregnated with ½ MS media. After 7-day incubation, the seedlings were transferred to ½ MS media with or without bacteria; and ½ MSmedia supplemented with 100 mM NaCl with or without bacteria (Sharma et al., 2016). The density of bacteria added to the media was 10^6 CFU ml^{-1}. The seedling was grown in long-day conditions (day/night ratio was 16 hours/8 hours); room temperature ranged from 23 to 27 °C. The fresh biomass of the seedling was measured after 4 weeks of growth.

2.4 EVALUATE THE PEANUT GROWTH PROMOTION OF BACTERIA UNDER SALTY STRESS AND GREENHOUSE CONDITION

Peanut seeds (*Arachis hypogaea* LDH12) are disinfected and germinated on cotton wool impregnated with ½ MS mineral media with or without bacterial supplements at a density of 10^6 CFU ml^{-1}. After the seeds germinate, peanut seedlings were transferred to pots containing soil not treated with salt or the soil is mixed with NaCl 75 mM (Goswami et al., 2014). The seedling was watered twice a week. In bacterial treatment experiments, bacterial suspension was added to the water reaching a density of 10^6 CFU ml^{-1}, and watered every 2 weeks. With the salt treatment test, 2 weeks will be additionally watered with a 50 mM NaCl solution (Goswami et al., 2014). After 40 days of sowing seeds, fresh biomass of seedlings was recorded. Temperature conditions (day: 34-38 °C; night: 29-32 °C) and relative humidity of 48-62 % in the nursery

were recorded during the experiment.

2.5 DATA ANALYSIS

All experiments were repeated three times the results were presented as mean values with ± SD. Tukey's honestly significant difference (HSD) method in SPSS (version 17) was applied to compare the means in all experiments.

3 RESULTS AND DISCUSSION

3.1 ISOLATION AND IDENTIFICATION OF BACTERIA

From 18 root rhizosphere soil samples, we isolated 10 bacteria strains including 6 strains (ND01, ND02, ND03, ND04, ND05, and ND06) from corn rhizosphere; 2 strains (ND07 and ND08) from rice rhizosphere, and 2 strains (ND09 and ND10) from peanut rhizosphere. In fact, *Pseudomonas* is a genus of bacteria that is very common in the soil and root rhizosphere of plants, but due to the diversity of specie composition along with the relatively low selective efficiency of the LB media limits the ability to isolate target bacteria, especially for those samples with mold growth on agar plates. To confirm that selected strains belong to the genus of *Pseudomonas*, the PCR reaction was used to amplify a 16S rDNA sequence specific for the *Pseudomonas* (Kim et al., 2013; Yadav et al., 2014). The experiment results showed that only 7 isolates (ND01, ND03, ND04, ND06, ND07, ND09, and ND10) gave a band on the electrophoresis. These bacteria strains were evaluated for growth-promoting characteristics.

3.2 SCREENING THE BACTERIAL ISOLATES FOR SALT TOLERANCE AND PGPR TRAITS

All 7 isolates were used to evaluate their possibilities of growing in high salt conditions. The isolates were cultured in the media containing a gradual increase in NaCl concentration from 10 % to 24 % (the gap between concentrations is 2 %). The results showed that isolated strains in Nam Dinh are likely to survive in media containing a quite high salt concentration, especially the two strains ND06 and ND09, which could grow in media supplemented with up to 18 % and 22 % NaCl, respectively (Table 1).

The experimental results also presented in Table 1 showed that all isolates were capable of producing IAA. However, the amount of IAA produced by bacterial strains after 7 days of culture was relatively low in the range of 2.021 – 3.549 μg ml^{-1} (Malik and Sindhu, 2011). According to Egamberdieva (2015), three main factors affecting the IAA production of rhizobacteria were the bacterial strains; culture time, bacterial growth stage; and precursor to IAA synthesis. Many studies have shown that different strains of bacteria have different IAA production. Several strains of bacteria that are prominent for IAA production, such as *Pseudomonas aureantiaca* TSAU22 (Sheehy et al., 1991), *Pseudomonas extremorientalis* TSAU6 and *Pseudomonas extremorientalis* TSAU20 (Egamberdieva, 2011), significantly increased root growth by up to 25 % under normal conditions and up to 52 % under 100 mM NaCl condition compared with control plants (Botelho and Mendonça-hagler, 2006; Egamberdieva, 2009).

One of the important traits of PGPR is nitrogen fixation. Hence, the isolates were also investigated the nitrogen fixation ability on media without nitrogen sources. The results were shown in Table 1, the majority of iso-

Table 1: Characterization of plant growth-promoting bacteria isolated under salty stress conditions

Bacterial strain	Highest NaCl concentration (%)	IAA concentration (μg ml^{-1})	Phosphate Solubility Index	Nitrogen fixation
ND01	12	3.521 ± 0.113^{a}	1.557 ± 0.211^{b}	-
ND03	12	3.327 ± 0.106^{ab}	2.013 ± 0.131^{a}	+
ND04	10	2.876 ± 0.132^{bc}	1.252 ± 0.124^{c}	-
ND06	18	3.021 ± 0.211^{b}	1.239 ± 0.102^{c}	+
ND07	10	2.417 ± 0.215^{c}	1.532 ± 0.079^{b}	+
ND09	22	3.549 ± 0.115^{a}	1.635 ± 0.063^{ab}	+
ND10	12	2.437 ± 0.102^{c}	1.781 ± 0.023^{ab}	-

Data are means ± SD (n = 3). Values in the same column with the same letter(s) are not significantly different as determined by Tukey's honestly significant difference test ($p < 0.005$). '-' mean no media color; '+' means media color changed

lated strains were capable of growth on the Nitrogen Free Mineral Medium (MNFM), excepted for ND01, ND04, and ND10. The MNFM is a media with no nitrogen sources, hence, the formation of colonies on this media demonstrates that bacterial strains were capable of using air nitrogen sources for cellular processes. In an MNFM, there was a supplement of blue bromophenol as a pH indicator, which is yellow when the pH < 7, green at pH = 7 and turns blue when the pH > 7. In this experiment, the environment changed from green to blue because nitrogen-fixed bacteria created NH_4^+ products that increased the pH of the media.

All 7 isolated strains of bacteria showed the phosphate solubilization capacity when produced a clear zone around colonies after 7 days (Table 1). The PSI ranged from 1.239 to 2.013. The mechanisms for dissolving phosphate by bacteria vary widely, but according to Sharma et al. (2013), there are three main mechanisms: organic acid production, inorganic acid release, and extracellular polymeric substances (EPSs) (Fatima and Arora, 2021).

3.3 EVALUATION OF ACC DEAMINASE PRODUCTION AND COLONIZATION OF BACTERIAL ISOLATES

In order to identify the presence of ACC deaminase in potential strains, the PCR to amplify the specific DNA sequence of this gene in *Pseudomonas* was performed as the method described by Sheehy et al. (1991). In addition, Saravanakumar and Samiyappan (2007) proved that this pair of primers is specific to characterize *Pseudomonas fluorescens* (Flügge 1886) Migula, 1895. Electrophoresis results showed that the target product of about 750 bp appeared in all two selected strains.

Moreover, the biofilm formation ability of isolates under salt stress conditions was also studied. The results were shown in Figure 1.

As can be seen from Figure 1A, under normal conditions, the biofilm formation dynamics of bacterial isolates were different. The ND09 presented the late production of biofilm formation compared to the ND06 strain, showing a lower OD_{540nm} value at the beginning and reaching a higher OD_{540nm} value after 24 hours while the ND06 presented a decline of OD_{540nm} value during the experiment. The results also indicated the delay effect of salt stress on the bacterial biofilm formation, which reached a maximum OD_{540nm} value after 6 hours and higher than the maximum OD_{540nm} value in normal conditions (Figure 1B). These results are consistent with some previous studies on the formation of bacterial biofilm under salt stress (Costa-Gutierrez et al., 2020b; Costa-Gutierrezet al., 2021).

3.4 EVALUATE THE ABILITY TO STIMULATE PEANUT GROWTH UNDER SALT STRESS

Based on the results of the growth-stimulating characteristics of the isolated strains, among the strains isolated from the soil rhizosphere, ND03, ND06, ND07, and

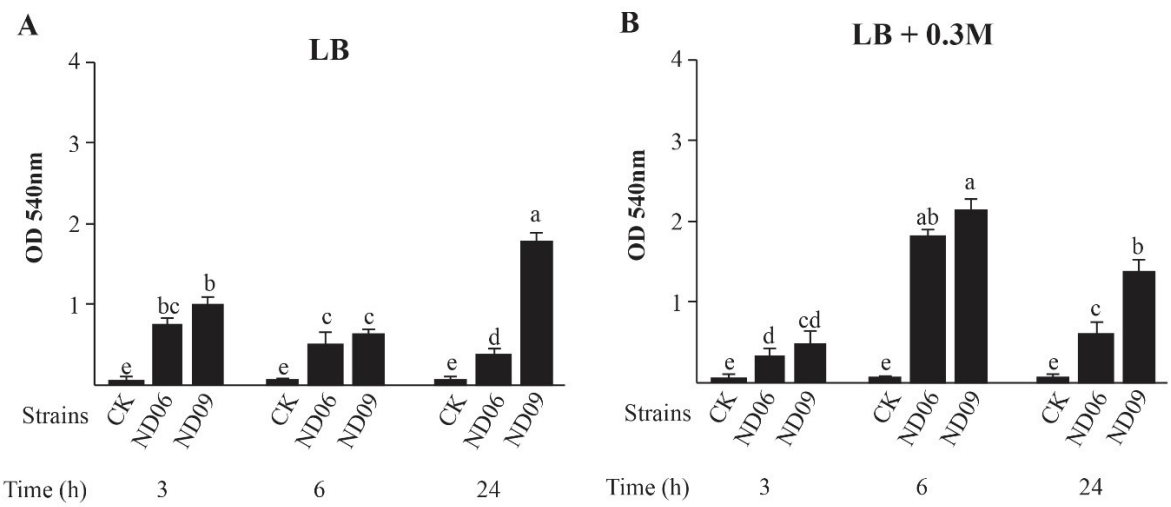


Figure 1: Biofilm formation ability of bacterial isolates (ND06 and ND09) in LB (A) and LB + 0.3M NaCl (B) in different periods of time. CK: media only. Plotted data are means ± SD (n = *3).* The same letter(s) are not significantly different as determined by Tukey's honestly significant difference test ($p < 0.05$)

ND09 are full of characteristics such as IAA production, nitrogen fixation, and phosphate solubilization. However, when considering the results of IAA production, salt-resistance, and phosphate solubility index, 2 strains ND06 and ND09 were selected for further experiments. All two strains gave positive PCR results with primers specific for *Pseudomonas*. *Pseudomonas* strains isolated from rhizosphere and agricultural soil samples were assessed to be relatively safe for humans and animals. These two strains were selected for investigating their ability in promoting plant growth development under *in vitro* and *in vivo* conditions.

In this experiment, the potential of bacterial strains in supporting plants to withstand salty stress will be evaluated on agricultural plant models. Compared to corn, which is only sensitive to salty stress at an average of degrees (Zörb et al., 2004), peanut plants are a very sensitive type to salty stress (Goswami et al., 2014; Sharma et al., 2016). Therefore, although 2 strains were selected from the root rhizosphere of corn and peanut, in this experiment peanut seedlings were selected as models to conduct stress response tests.

In *in vitro* experiments, the results were illustrated in Figure 2 and Table 2. The results indicated that under normal conditions, peanut seedlings treated with the ND09 strain showed growth stimulation expressed in an increase in total plant biomass (34.63 %), shoot biomass (35.22 %), and root biomass (32.87 %) compared to the control. In contrast, the ND06 strain presented no difference from the control plant (Figure 2A and Table 2). It is notable that the IAA concentration reaching from 0.1 to 1 μg ml^{-1} could produce beneficial effects on plant growth (Bui, 2016). It implies that if co-inoculation of isolated strains with plants for a long time, these strains could provide enough exogenous IAA for plant growth by increasing root growth through root elongation and reducing ethylene.

However, under salty stress, all seedlings treated with bacteria showed an increase in the biomass of the plants compared to the control. In particular, the seedling treated with the ND09 strain presented the highest efficiency (Figure 2B and Table 2). This might be because the bacterial isolates produced the ACC deaminase under salt stress to degrade ACC (the precursor of ethylene in all higher plants) and hence prevented the over-accumulation of ethylene in plants under salt stress conditions; subsequently enhancing plant development. This suggests that bacteria have the ability to reduce the effect of salty stress on the growth of peanut plants.

The results of greenhouse experiments were consistent with the *in vitro* results and were illustrated in Figure 3 and presented in Table 3. As can be seen, under normal conditions, seedlings treated with the ND09 strain showed an increase in shoot and root biomass respectively 30.37 % and 32.87 % compared to control seedlings. In contrast, seedlings treated with ND06 strain presented a slight decrease in biomass compared to control seedlings (Figure 3A and Table 3). Under salty stress conditions, all seedlings treated with bacteria had higher fresh biomass than control (Figure 3B and Table 3).

The results of the greenhouse experiments showed a match with ones under the *in vitro* conditions. These results indicated that the ND09 strain not only effectively stimulated peanut seedling growth under normal condi-

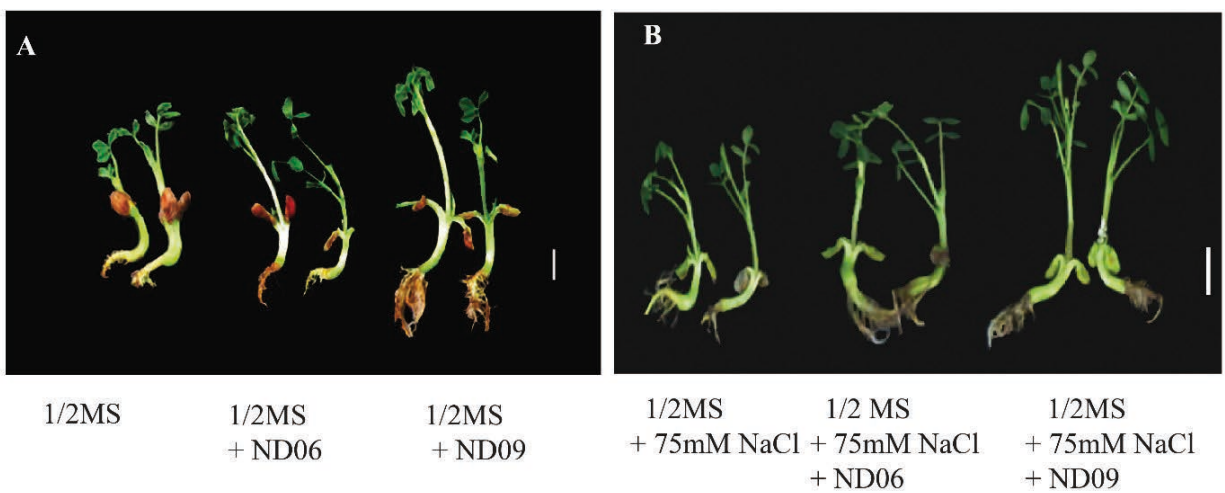


Figure 2: Bacteria isolates (ND06 and ND09) enhances the peanut seedling growth under normal condition (A) and 100 mM NaCl (B) after 4 weeks of sowing under in vitro conditions

Table 2: Effect of selective bacteria on fresh biomass of peanut seedlings grown on different media after 4 weeks under in vitro conditions

	Total plant biomass (mg)		Shoot biomass (mg)		Root biomass (mg)	
Experiments	0 mM NaCl	100 mM NaCl	0 mM NaCl	100 mM NaCl	0 mM NaCl	100 mM NaCl
Peanut seedlings (control)	2213.3 ± 61.2^{b}	1969.5 ± 91.3^{c}	1634.5 ± 101.2^{b}	1612.5 ± 132.1^{c}	579.1 ± 125.0^{b}	356.8 ± 82.3^{c}
Peanut seedlings + ND06	2268.2 ± 75.8^{b}	2410.3 ± 151.3^{b}	1681.7 ± 87.2^{b}	1897.5 ± 121.2^{b}	586.2 ± 72.4^{b}	512.7 ± 64.3^{b}
Peanut seedlings + ND09	2979.7 ± 135.5^{a}	2707.7 ± 35.2^{a}	2210.2 ± 112.3^{a}	2102.3 ± 53.1^{a}	769.5 ± 187.5^{a}	605.3 ± 67.2^{a}

Data are means ± SD (n = 3). Values in the same column with the same letter(s) are not significantly different as determined by Tukey's honestly significant difference test ($p < 0.05$)

tions but also had the potential to improve plant growth under salty tress conditions. Meanwhile, the remaining strain had only a positive effect under salty stresses but not under normal conditions. All of these results suggest the ND09 strain has shown to be a potential strain in the production of probiotic fertilizers that could improve crop yields, whether under normal conditions or salty stress conditions.

These results were in agreement with previous studies that also investigated the alleviation of salt stress by *Pseudomonas* in plant development. Cai et al. (2021) reported that *Chenopodium quinoa* Willd. inoculated with *Pseudomonas* sp. strain M30-35 significantly improved the dry mass of roots by 51.97 % at 150 mM NaCl treatments for 7 d. Another example is the report of Fatima and Arora (2021), who proved that *P. entomophila* PE3 in cobination with 2 % EPS enhanced the growth and resilience of sunflower in saline soil (increment in root and shoot length was 49 % and 85 % respectively in comparison to control). Although each plant species has different selective effects on rhizosphere diversity, *P. fluorescens* and *P. putida* Trevisan, 1889 are still the most dominant species (Egamberdieva, 2015). Many commercial preparations from these two species have been widely used. Several cases of *P. putida* causing disease in humans have been reported, however, these are rare and mostly occur in immunocompromised individuals (Fernández et al., 2015). Another member of this genus, *P. aeruginosa* (Schröter 1872) Migula 1900, has great potential for promoting plant growth and potent antagonism against rhizosphere pathogens. Unlike *P. fluorescens* and *P. putida*, some strains of *P. aeruginosa* are opportunistic pathogens in humans (Fernández et al., 2015). This bacterium is widely distributed in water, soil, and even in some foods. However, the level of risk posed by this bacterium is not large and is only classified as a Class II biosafety risk. Furthermore, not all strains of this species are pathogenic due to the absence of pathogenic genes in the genome. In summary, PGPR strains belonging to the genus *Pseudomonas* can be widely applied in agricultural practices with low risk and controllability.

4 CONCLUSIONS

In this study, 7 bacteria strains belonging to the *Pseudomonas* genus and capable of living in salty conditions were isolated from soil in Nam Dinh. The two strains of bacteria ND06 and ND09 were selected based on phosphate solubilization, nitrogen fixation, and IAA production. The ND09 strain had the potential to stimulate peanut seedling growth under both normal and salty stress conditions, indicating the potential of this strain in sustainable agricultural practices. However, before being widely adopted or commercialized, the ND09 strain

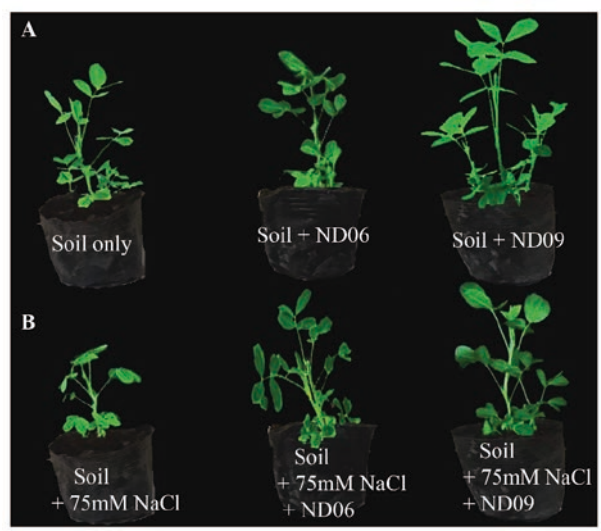


Figure 3: Illustration of a positive effect of bacteria isolates (ND06 and ND09) on the peanut seedling growth under normal condition (A) and 75mM NaCl (B) after 40 days of sowing

Table 3: Bacterial isolate enhance peanut plant growth under salty stress condition after 40 days of Sowing under greenhouse conditions

	Total plant biomass (mg)		Shoot biomass (mg)		Root biomass (mg)	
Experiments	0 mM NaCl	75 mM NaCl	0 mM NaCl	75 mM NaCl	0 mM NaCl	75 mM NaCl
Peanut seedlings (control)	2722.8 ± 31.7^{b}	1559.8 ± 110.1^{c}	2173.7 ± 102.3^{b}	1231.2 ± 111.3^{c}	548.7 ± 62.7^{b}	328.2 ± 101.2^{b}
Peanut seedlings + ND06	2634.2 ± 56.2^{b}	2302.5 ± 102.1^{b}	2091.2 ± 35.7^{b}	1497.3. ± 109.1^{b}	542.6 ± 61.2^{b}	803.7 ± 113.7^{a}
Peanut seedlings + ND09	5396.1 ± 52.1^{a}	2688.9 ± 91.1^{a}	3372.5 ± 115.7^{a}	1895.5 ± 115.7^{a}	2023.4 ± 94.5^{a}	792.7 ± 82.3^{a}

Data are means ± SD (n = 3). Values in the same column with the same letter(s) are not significantly different as determined by the Tukey's honestly significant difference test ($p < 0.005$)

should be evaluated on the risk of disease in humans and animals, as well as the impact on the ecological environment.

5 ACKNOWLEDGEMENTS

This research has been done under the research project QG.21.59 "Study on phosphorus adsorption and proposing solutions to improve phosphorus fertilizer efficiency in rice cultivation in the Red River delta" of Vietnam National University Hanoi. The Vietnam National University Hanoi provided funds for collecting samples and buying chemicals for experiments and supported experimental facilities to carry out experiments.

6 REFERENCES

Bui, T.V. (2016). *Plant physiology*. Ho Chi Minh National University Publishing House, p. 198. (in Vietnamese)

Botelho, G.R., Mendonça-hagler, L.C. (2006). Fluorescent *Pseudomonas* associated with the rhizosphere of crops- An overview. *Brazilian Journal of Microbiology*, *37*, 401-416. https://doi.org/10.1590/S1517-83822006000400001

Kang, S.M., Joo, G.J., Hamayun, M., Na, C.I., Shin, D.H., Kim, Y.K., Hong, J.K., Lee, I.J. (2009). Gibberellin production and phosphate solubilization by newly isolated strain of *Acinetobacter calcoaceticus* and its effect on plant growth. *Biotechnology Letters*, *31*(2), 277-281. https://doi.org/10.1007/s10529-008-9867-2

Cai, D., Xu, Y., Zhao, F., Zhang, Y., Duan, H., Guo, X. (2021). Improved salt tolerance of *Chenopodium quinoa* Willd. contributed by *Pseudomonas* sp. strain M30-35. *Peer Journal, 9*, e10702. https://doi.org/10.7717/peerj.10702

Chu, T.N., Tran, B.T.H., Bui, V.L., Hoang, M. (2019). Plant growth-promoting rhizobacterium *Pseudomonas* PS01 induces salt tolerance in *Arabidopsis thaliana*. *BMC Research Notes*, *12*(1), 1-7. https://doi.org/10.1186/s13104-019-4046-1

Costa-Gutierrez, S.B., Raimondo, E.E., Lami, M.J., Vincent, P.A., Espinosa-Urgel, M., de Cristóbal, R.E. (2020a). Inoculation of *Pseudomonas* mutant strains can improve growth of soybean and corn plants in soils under salt stress. *Rhizosphere*, *16*, 100255. https://doi.org/10.1016/j.rhisph.2020.100255

Costa-Gutierrez, S.B., Lami, M.J., Caram-Di Santo, M.C., Zenoff, A.M., Vincent, P.A., Molina-Henares, M.A., Espinosa-Urgel, M., de Cristóbal, R.E. (2020b). Plant growth promotion by *Pseudomonas putida* KT2440 under saline stress: Role of *eptA*. *Applied Microbiology and Biotechnology*, *104*, 4577-4592. https://doi.org/10.1007/s00253-020-10516-z

Costa-Gutierrez, S.B., Caram-Di Santo, M.C.d.V., Zenoff, A.M., Espinosa-Urgel, M., de Cristóbal, R.E., Vincent, P.A. (2021). Isolation of *Pseudomonas* strains with potential for protection of soybean plants against saline stress. *Agronomy*, *11*, 2236. https://doi.org/10.3390/agronomy11112236

Damodaran, T., Sah, V., Rai, R.B., Sharma, D.K., Mishra, V.K., Jha, S.K., Kannan, R. (2013). Isolation of salt tolerant endophytic and rhizospheric bacteria by natural selection and screening for promising plant growth-promoting rhizobacteria (PGPR) and growth vigour in tomato under sodic environment. *African Journal of Microbiology Research*, *7*(44), 5082-5089.

Egamberdieva, D. (2009). Alleviation of salt stress by plant growth regulators and IAA producing bacteria in wheat. *Acta Physiologiae Plantarum*, *31*(4), 861-864. doi: 10.1007/s11738-009-0297-0. https://doi.org/10.1007/s11738-009-0297-0

Egamberdieva, D. (2011). Survival of *Pseudomonas extremorientalis* TSAU20 and *P. chlororaphis* TSAU13 in the rhizosphere of common bean (*Phaseolus vulgaris*) under saline conditions. *Plant, Soil and Environment*, *57*(3), 122–127. https://doi.org/10.17221/316/2010-PSE

Egamberdieva, D. (2015). *The role of phytohormone producing bacteria in alleviating salt stress in crop plants*. In: *Biotechnological techniques of stress tolerance in plants* (Editors: M. Miransari), Stadium Press LLC, USA, p. 20–39.

El-Nahrawy, S., Yassin, M. (2020). Response of different cultivars of wheat plants (*Triticum aestivum* L.) to inoculation by *Azotobacter* sp. under salinity stress conditions. *Journal of Advances in Microbiology*, *20*, 59-79. https://doi.org/10.9734/jamb/2020/v20i130209

Fatima, T., Arora, N.K. (2021). *Pseudomonas entomophila* PE3 and its exopolysaccharides as biostimulants for enhancing growth, yield and tolerance responses of sunflower under saline conditions. *Microbiological Research*, *244*, 126671. https://doi.org/10.1016/j.micres.2020.126671

Fernández, M., Porcel, M., de la Torre, J., Molina-Henares, M.A., Daddaoua, A., Llamas, M.A., Roca, A., Carriel, V., Garzón, I., Ramos, J.L., Alaminos, M., Duque, E. (2015). Analysis of the pathogenic potential of nosocomial *Pseudomonas putida* strains. *Frontiers in Microbiology*, *6*, 871. https://doi.org/10.3389/fmicb.2015.00871

Goswami, D., Dhandhukia, P., Patel, P., Thakker, J.N. (2014). Screening of PGPR from saline desert of Kutch: Growth promotion in *Arachis hypogea* by *Bacillus licheniformis* A2. *Microbiological Research*, *169*(1), 66-75. https://doi.org/10.1016/j.micres.2013.07.004

Glickmann, E., Dessaux, Y. (1995). A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Applied and Environmental Microbiology*, *61*(2), 793-796. https://doi.org/10.1128/aem.61.2.793-796.1995

Kim, J., Mele, P., Crowley, D. (2013). Application of PCR primer sets for detection of *Pseudomonas* sp. functional genes in the plant rhizosphere. *Journal of Agricultural Chemistry and Environment*, *2*(1), 8-15. https://doi.org/10.4236/jacen.2013.21002

Malik, D.K., Sindhu, S.S. (2011). Production of indole acetic acid by *Pseudomonas* sp.: Effect of co-inoculation with *Mesorhizobium* sp. on nodulation and plant growth of chickpea (*Cicer arietinum*). *Physiology and Molecular Biology of Plants*, *17*(1), 25-32. https://doi.org/10.1007/s12298-010-0041-7

O'Toole, G.A., Kolter, R. (1998). Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: A genetic analysis. *Molecular Microbiology*, *28*, 449-61. https://doi.org/10.1046/j.1365-2958.1998.00797.x.

Pikovskaya, R.I. (1948). Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. *Mikrobiologiya*, *17*, 362–370.

Sharma, S., Kulkarni, J., Jha, B. (2016). Halotolerant rhizobacteria promote growth and enhance salinity tolerance in peanut. *Frontiers in Microbiology*, *7*, 1600. https://doi.org/10.3389/fmicb.2016.01600

Singh, R.P., Jha, P., Jha, P.N. (2015). The plant-growth-promoting bacterium *Klebsiella* sp. SBP-8 confers induced systemic tolerance in wheat (*Triticum aestivum*) under salt stress. *Journal of Plant Physiology*, *184*, 57-67. https://doi.org/10.1016/j.jplph.2015.07.002

Shafi, J., Tian, H., Ji, M. (2017). *Bacillus* species as versatile weapons for plant pathogens: a review. *Biotechnology & Biotechnological Equipment*, *31*, 446–459. https://doi.org/10.1080/13102818.2017.1286950

Sharma, S.B., Sayyed, R.Z., Trivedi, M.H., Gobi, T.A. (2013). Phosphate solubilizing microbes: Sustainable approach for managing phosphorus deficiency in agricultural soils. *Springerplus*, *2*(1), 1-14. https://doi.org/10.1186/2193-1801-2-587

Saravanakumar, D., Samiyappan, R. (2007). ACC deaminase from *Pseudomonas fluorescens* mediated saline resistance in groundnut (*Arachis hypogea*) plants. *Journal of Applied Microbiology*, *102*(5), 1283-1292. https://doi.org/10.1111/j.1365-2672.2006.03179.x

Sheehy, R.E., Honma, M., Yamada, M., Sasaki, T., Martineau, B., Hiatt, W. (1991). Isolation, sequence, and expression in *Escherichia Coli* of the *Pseudomonas* sp. strain ACP gene encoding 1-aminocyclopropane-1-carboxylate deaminase. *Journal of Bacteriology*, *173*(17), 5260-5265. https://doi.org/10.1128/jb.173.17.5260-5265.1991

Upadhyay, S.K., Singh, D.P. (2015). Effect of salt-tolerant plant growth-promoting rhizobacteria on wheat plants and soil health in a saline environment. *Plant Biology*, *17(1)*, 288-293. https://doi.org/10.1111/plb.12173

Widmer, F., Seidler, R.J., Gillevet, P.M., Watrud, L.S., Giovanni, G.D. (1998). A highly selective PCR protocol for detecting 16S rRNA genes of the genus *Pseudomonas* (sensu stricto) in environmental samples. *Applied and Environmental Microbiology*, *64*(7), 2545-2553. https://doi.org/10.1128/AEM.64.7.2545-2553.1998

Wright, S.F., Weaver, R.W. (1981). Enumeration and identification of nitrogen-fixing bacteria from forage grass roots. *Applied and Environmental Microbiology*, *42*(1), 97-101. https://doi.org/10.1128/aem.42.1.97-101.1981

Yadav, S., Yadav, S., Kaushik, R., Saxena, A.K., Arora, D.K. (2014). Genetic and functional diversity of fluorescent *Pseudomonas* from rhizospheric soils of wheat crop. *Journal of Basic Microbiology*, *54*(5), 425-437. https://doi.org/10.1002/jobm.201200384

Zörb, C., Schmitt, S., Neeb, A., Karl, S., Linder, M., Schubert, S. (2004). The biochemical reaction of maize (*Zea mays* L.) to salt stress is characterized by a mitigation of symptoms and not by a specific adaptation. *Plant Science*, *167*(1), 91-100. https://doi.org/10.1016/j.plantsci.2004.03.004

Zörb, C., Geilfus, C.M., Dietz, K.J. (2019). Salinity and crop yield. *Plant Biology*, *21*, 31-38. https://doi.org/10.1111/plb.12884

doi:10.14720/aas.2022.118.3.2333 Original research article / izvirni znanstveni članek

Quantification of apocarotenoids in commercial Indian (Kashmiri) saffron using UV-Vis spectroscopy and HPLC analysis

Tahir ul Gani MIR [1], Jaskaran SINGH [2], Saurabh SHUKLA [1, 3]

Received September 05, 2021; accepted July 10, 2022.
Delo je prispelo 5. septembra 2021, sprejeto 10. julija 2022

Quantification of apocarotenoids in commercial Indian (Kashmiri) saffron using UV-Vis spectroscopy and HPLC analysis

Abstract: Saffron is considered as the most expensive spice in the world. Due to low production, high demand and high cost, saffron is very prone to adulteration for economic benefits while putting public health at risk. The most significant characteristic for determining the quality of the saffron is coloring strength (crocin content), which is determined by measuring UV-Vis absorption at 440 nm in the aqueous preparations of this spice. Picrocrocin and safranal are other key components used to determine saffron quality. This article aims to examine the quality of commercial saffron obtained from various geographical locations of Kashmir (India) by determining their apocarotenoid content using UV-Vis spectrophotometry followed by high-performance liquid chromatography (HPLC) to determine the concentration of saffron metabolites (crocin, picrocrocin and safranal). A total of 31 samples from different origins were used in this study. The UV-Vis spectrophotometric results showed that among 31, only 14 samples fell into grade I, while 9 samples fell in grade II and 5 samples fell in grade III of the ISO category. The remaining 3 samples could not satisfy ISO standards, which indicates that these samples were adulterated. The determination of apocarotenoid content using HPLC analysis varied significantly among samples. These variations may be due to different drying and storage conditions or adulteration.

Key words: saffron; adulteration; crocin; safranal; picrocrocin; UV-Vis spectroscopy; HPLC

Količinsko ovrednotenje apokarotenoidov v komercialnih vzorcih indijskega (kašmirskega) žafrana z analizo UV-Vis spektroskopije in HPLC

Izvleček: Žafran velja za najdražjo začimbo v svetovnem merilu. Zaradi majhne pridelave, velikega povpraševanja in visoke cene je zaradi ekonomskih koristi podvržen ponarejanju, kar povzroča zdravstvena tveganja. Najznačilnejša lastnost za določanje kakovosti žafrana je njegova sposobnost obarvanja (vsebnost krocina), ki se določa z merjenjem UV-Vis absorbcije pri 440 nm v vodnih pripravkih te začimbe. Pikrokrocin in safranal sta ostali klučni komponenti, ki se uporabljata za določanje kakovosti žafrana. V raziskavi smo preučevali kakovost tržnega žafrana pridobljena iz različnih geografskih območij Kašmirja (Indija) z določanjem vsebnosti apokarotenoidov z UV-Vis spektroskopijo, ki ji je sledila analiza z visokotlačno tekočinsko kromatografijo (HPLC), kjer smo v vzorcih žafrana določali koncentracije metabolitov kot so krocin, pikrokrocin in safranal. V raziskavi je bilo analiziranih 31 vzorcev različnega izvora. Rezultati analize z UV-Vis spektroskopijo so pokazali, da se je med 31 vzorci samo 14 uvrstilo v kvaliteto I, 9 vzorcev seje uvrstilo v kvaliteto II in 5 vzorcev v kvaliteto III, glede na ISO kategorije. Preostali 3 vzorci niso izpolnjevali ISO standardov, kar kaže, da so bili ponarejeni. Vsebnost apokarotenoidov v vzorcih se je pri analizi s HPLC značilno razlikovala, kar bi lahko bila posledica različnega sušenja, shranjevanja ali ponarejanja.

Ključne besede: žafran; ponarejanje; krocin; safranal; pikrokrocin; UV-Vis spektroskopija; HPLC

1 Department of Forensic Science, School of Bioengineering & Biosciences, Lovely Professional University, Phagwara, India
2 Department of Forensic Science, Geeta University, Naultha, Panipat, India
3 Corresponding author, e-mail: sau47bt@gmail.com

1 INTRODUCTION

Saffron, often referred to as red gold, is obtained from the stigma of *Crocus sativus* L. (Saxena, 2010). *Crocus sativus* is an angiosperm plant, member of the Asparagales family. The flower of *Crocus sativus* is solitary, purple, with six petals, three stamens, one style, and three reddish-orange stigmas. Saffron crocus grows throughout the Mediterranean–Europe, and Western Asia. It is commonly cultivated in Iran, Greece, Spain, Italy, Afghanistan and India (Kashmir) (Kahriz, 2020). Saffron is quite costly because it is used mainly as a flavoring and aromatizing agent (Mir et al., 2022a; Mzabri et al., 2019). Saffron contains approximately 300 volatile and non-volatile metabolites, including crocin, safranal, picrocrocin, monoterpenes, aldehydes, and various other carotenoids of therapeutic potential (Pandita, 2021). It is a well-known spice that is used to cure a variety of medical conditions, including depression (Siddiqui et al., 2018), cardiovascular illness (Kamalipour & Akhondzadeh, 2011), menstrual irregularities (Beiranvand et al., 2016), asthma (Zilaee et al., 2019)lipid profiles, basophils, eosinophils and clinical symptoms in patients with allergic asthma.\nSTUDY DESIGN: Our study was a clinical trial.\nMETHODS: Subjects (N = 80, 32 women and 48 men, 41.25 ± 9.87 years old, insomnia (Taherzadeh et al., 2020), and digestive problems (Khorasany & Hosseinzadeh, 2016). Crocin is a carotenoid chemical compound responsible for the golden yellow-orange color of saffron; picrocrocin gives bitter flavor, and safranal is responsible for the characteristic aroma of saffron. Saffron is currently known as a flavoring agent and a potent natural agent with many health advantages (Azami et al., 2021; Basker & Negbi, 1983; Bolhassani et al., 2014; Mir et al., 2022b). The potential of saffron and its constituents to protect against natural and artificial poisons has enhanced its significance. Due to the high price of saffron and its great demand in the pharmaceutical industry, illegal trafficking and adulteration are prevalent nowadays (Alonso et al., 1998). The common adulterants used in saffron include maize silk, marigold floret, horsehair, wool, saffron stamens, red dried silk fiber, and safflower (*Carthamus tinctorium* L.). Mixing low-grade saffron with high-grade saffron or old stored saffron that has lost its quality with freshly harvested saffron is also a common method of adulteration in saffron (Kumari et al., 2021; Lowell, 1964; Marieschi et al., 2012; Sereshti et al., 2018). The sale and mixing of high-grade Kashmiri saffron with lower-cost Iranian imports is common in India; the resulting mixes are then sold as pure Kashmiri saffron. This trend has deprived saffron cultivators of Kashmir of a significant portion of their revenue (Hussain, 2005). Dyes such as erythrosine, tartrazine, amaranth, sunset yellow, carmoisine, picric acid, ponceau S, methyl orange, and Sudan red are also used as adulterants in saffron (Lozano et al., 1999; Patel et al., 2019; Petrakis et al., 2015).

Internationally, the grading of saffron is based on the standards formulated by the International Organization for Standardization (ISO). The ISO (ISO 3632-1:2011) certification ensures customers that the saffron they purchase is authentic and safe to consume. ISO 3632 has classified saffron into three grades (Grade I, II and III) based on the concentration of crocin, picrocrocin and safranal present in saffron [Table 1]. A greater concentration of these chemicals indicates a better quality of saffron. A quartz cell with a 1 cm pathway is used to measure $E_{1\%}$ at 440, 330, and 257 nm wavelengths. The results are obtained by measuring the absorption at three wavelengths using the equation; $E_{1\,\%}\ 1\ cm = (A \times 10000]/(M \times (100\text{-}H)]$, where $E_{1\%}$ is the specific extinction coefficient, *1 cm* is the path length, *A* is the absorbance, *M* is the mass in grams of the saffron sample, *H* is the moisture and volatile sample material. The moisture and volatile content of the saffron is determined after drying the samples and represented as a mass fraction using the formula: *[(initial mass-constant mass)/initial mass] × 100* (Hadizadeh et al., 2007; ISO - 3632-1:2011).

Despite international standards, various methods have been reported to detect adulteration and determine the quality of saffron viz UV-Vis spectroscopy (Zalacain et al., 2005; Zougagh et al., 2005) HPLC (Haghighi et al., 2007; Hajimahmoodi et al., 2013; Lozano et al., 1999), micellar liquid chromatography, FTIR (Karimi et al., 2016; Ordoudi et al., 2018), H-NMR (Petrakis et al., 2015), gel-electrophoresis (Paredi et al., 2016). Several factors, including geographical conditions, harvesting period, drying procedure employed, temperature and oxygen exposure during storage and adulteration, all have a significant impact on the quality of saffron (Caballero-Ortega et al., 2004). The primary objective of this research was to estimate the quality range and apocarotenoid content of commercial saffron in Kashmir using UV-Vis spectrophotometry and HPLC analysis.

2 MATERIAL AND METHODOLOGY

Saffron in India is cultivated and commercialized in Kashmir. The main local commercial zones of saffron in Kashmir are Srinagar, Pampore and Budgam. Besides local markets, the saffron in Kashmir is also commercialized by government-operated commercial emporiums (e.g., Government Kashmir Art Emporiums). Twenty-four samples of saffron were collected from Kashmir, among which six samples were collected from Government operated commercial emporiums (KAE), six sam-

Table 1: Grades of saffron based on ISO 3632-1:2011

Component	λ_{max}	Category I	Category II	Category III
Crocin	440 nm	> 200	170-200	120-170
Safranal	257 nm	20-50	20-50	20-50
Picrocrocin	330 nm	> 70	55-70	40-55
Moisture and volatile matter % (m/m),	-	10	10-12	10-12

ples were collected from the local market of Srinagar (SXR), six samples were collected from Pampore (PAM) district, and six samples were collected from Budgam (BUD). The samples collected were supposed to be yielded from the crop year 2019 and processed in 2020 as per their packing. Besides these samples, four samples were collected from Afghanistan (AFG), and two samples were collected from Iran (IRN). The samples collected from KAE, AFG and IRN had an origin certificate and were assured free from any adulteration. One sample was collected from Sigma Aldrich (SIG). A total of 31 samples were used for this study. Crocin and safranal standards were purchased from Sigma Aldrich. Picrocrocin was obtained from BioMall. HPLC-grade reagents (methanol, acetonitrile) were obtained from Loba Chemie.

2.1 DETERMINATION OF FLORAL WASTE CONTENT

About 1 g of each sample was taken, and each filament was spread on the paper. With the help of forceps, different floral waste components were separated, and the samples were weighed again. The floral waste was taken in shoe glass and weighed. The floral waste content of the sample (*wF)* was expressed as per ISO guidelines as a percentage by mass, using the relation:

$$wF = (m_2 - m_1)\ x\ 100/m_o\ \%$$

Where m_0 is the mass, in grams, of the test portion; m_1 is the mass, in grams, of the shoe glass; m_2 is the mass, in grams, of the shoe glass containing the floral waste.

2.2 DETERMINATION OF MOISTURE AND VOLATILE CONTENT

The samples collected needed to be examined for their authenticity. For such purposes, ISO 3632 has provided guidelines for conducting UV-Vis spectroscopy. To calculate E_1 %, first, the moisture content of all the samples was calculated. One gram of saffron from each sample was placed in a Petri dish and kept in the oven for 18 hours at 70 ^{0}C. After that, samples were weighed again to measure the moisture and volatile matter content (*wMV)* and is expressed as:

$$wMV = (m_0 - m_1)\ x\ 100/m_0\ \%$$

where m_0 is the mass, in grams, of the test portion; m_1 is the mass, in grams, of the dry residue.

2.3 UV-VIS SPECTROSCOPY

The UV-Vis spectroscopy for samples was performed according to ISO guidelines with slight modifications in order to get a greater yield of apocarotenoid compounds. Briefly, 100 mg mass of dried saffron samples was extracted with 5 ml cold 50 % (v/v) ethanol in mortar and pastel. The extract was then transferred to a screw-capped 50 ml tube, and a total amount of 20 ml 50 % (v/v) ethanol was added. Tubes were sonicated for 20 minutes on ice, centrifuged for 15 minutes at 4000 rpm, and washed twice with 5 ml of 50 % (v/v) ethanol. Spectrophotometric technique was employed to analyze the supernatant. For analysis, the supernatant (1 ml) was diluted to 5 ml with 50 percent (v/v) ethanol. The absorption of crocin, safranal, and picrocrocin at 440 nm, 330 nm, and 257 nm, respectively, was used to create a standard curve. The sample supernatants were diluted 100 times, and direct absorbance readings were obtained using a Shimadzu spectrophotometer (1 cm pathway quartz cell) at 440 nm, 330 nm, and 257 nm, respectively. A UV-Vis scan was also obtained to observe peaks in samples of different geographical locations. The results obtained were used to measure E_1 % of aqueous saffron extract using the following relation:

$$E_{1\%}\ 1\ cm = (A \times 10000/\ [(M \times (100\text{-}H)]$$

2.4 HPLC ANALYSIS

For HPLC analysis, 50 mg of powdered saffron samples were extracted with 10 ml of 50 % methanol-water (v/v) and magnetically stirred for 24 hours at 4 °C in the

dark. The samples were then centrifuged for 30 minutes at 5000 rpm. The supernatant was collected and filtered through 0.2 µm syringe filters. For quantitative analysis of crocin, picrocrocin and safranal, 1 ml of 2-nitroaniline was added as an internal standard to each sample before analysis (Caballero-Ortega et al., 2007). The analysis was carried out in a Shimadzu HPLC equipped with quaternary pumps; coupled to a photo-diode-array detector. Ethanol (50 %, v/v) and acetonitrile (15 %, v/v) were used as the mobile phase. Detection was carried out with an injection volume of 20 µl, a flow rate of 1 ml min^{-1} with 35-40 min of run time. Crocin was detected at 440 nm, picrocrocin at 250 nm and safranal at 330 nm. A calibration curve was constructed for internal standard using concentrations of 0.125, 0.25, 0.5, and 1.0 mg ml^{-1}. Quantitative analysis was carried out in accordance with the molecular absorption coefficient of each peak obtained at the wavelength of maximum absorbance of the components. The R2 values ranged from 0.9722to 0.9890, and results were expressed in milligrams per gram of saffron stigmas.

2.5 STATISTICAL ANALYSIS

One-way ANOVA was used to compare means and Duncan's Multiple Range Test (DMRT) was used to assess significance using IBM SPSS (version-20). Two tailored Pearson correlations between apocarotenoid levels with floral waste content and moisture levels were done using IBM SPSS (v. 20). The results were also analyzed using the multivariate analysis technique principal component analysis (PCA) using Origin-2021b (version-9.8b). PCA is a dimensionality-reduction technique often used to decrease the dimensionality of big data sets by converting a large collection of variables into a smaller one that still retains most of the information in the large set.

3 RESULTS AND DISCUSSION

The determination of floral waste in the samples was performed by physical separation of floral waste and then measuring its weight. The floral waste in the samples varied in range, with samples obtained from SXR and BUD showing a high range of floral waste. KAE samples and sigma samples showed the lowest range of floral waste, while IRN and AFG samples showed a medium to low range of floral waste. Floral waste in the Sigma sample was not detected (Table 2).

The moisture/volatile matter content was performed to analyze if the samples had been properly dried and processed. The average moisture level in KAE samples was found to be 6.26 %. Samples from SXR, BUD and PAM showed high levels of moisture and volatile content matter (12.45 %, 7.90 %, and 7.26 %, respectively). The average moisture and volatile content in the AFG and IRN samples was 6.35 % and 542 %, respectively, while in the SIG sample, it was found to be 4.49 % (Table 2).

Apocarotenoid content (E_1 %) was determined using UV-Vis spectrophotometry. The main objective of this measure analysis was to analyze the quality range of commercial saffron sold in Kashmir. The saffron samples were evaluated in accordance with the ISO 3632-2:2010 guidelines. One-way ANOVA and DMRT were used to compare means and assess the level of significance. The results showed significant variation in all the samples (Table 2). Results showed that average crocin content varied from 198.5 in KAE samples, 135.16 in SXR samples, 184.5 in PAM samples, 166 in BUD samples, 197.25 in AFG samples, 200.5 in IRN samples and 203 in sigma samples. Based on crocin content, it was found that among thirty-one samples, fourteen samples fell in category I, nine fell into category II, five fell in category III and three were counterfeit or adulterated samples as they showed E1 % less than 110. Similarly, picrocrocin expressed as direct reading of the absorbance at 257 nm showed an average concentration of 36.5 in KAE samples, 23.83 in SXR, 33 in PAM, 28.16 in BUD, 34 in AFG, 38.5 in IRN and 32 in SIG sample (Table 3). The safranal content in twenty-nine samples was found to be above 20, thus falling in the optimum range under ISO criteria. Three samples resulted in a safranal content range below 20, which is not optimal as per ISO guidelines. The floral waste and moisture/volatile content in saffron samples were negatively correlated with crocin content values (-0.87, -0.81, respectively). The results were analyzed using PCA analysis. PC1 (76.23 %) and PC2 (18.06 %) accounted for 94.29 % of the total variance of the data. The coefficient for both the principal components is given in Table 5. A biplot of samples was obtained to distinguish between adulterated and pure saffron (Figure 1).

HPLC analysis provides quick and simple measurement of the three major saffron components, with excellent linearity, selectivity, sensitivity, and accuracy. The crocetin, picrocrocin, and safranal were determined by HPLC at three wavelengths 440, 250, and 330 nm, respectively. The results were analyzed by one-way ANOVA to compare means, and Duncan's Multiple Range Test (DMRT) was used to assess significance. The concentration of these metabolites varied significantly (Table 2). The variations may be attributable to the geographical origin of samples, different drying procedures, storage conditions and adulteration (Biancolillo et al., 2020; Delgado et al., 2005; Maghsoodi et al., 2012). The average concentration of crocin varied from 40.64 mg g^{-1} in

Table 2: Floral waste percentage (wF %), moisture and volatile percentage (wMV %), UV-Vis analysis, HPLC analysis of saffron samples

			UV-Vis Analysis			HPLC analysis		
Sample	wF %	wMV %	Crocin (E1 %) 440 nm	Picrocrocin (E1 %) 257 nm	Safranal (E1 %) 330 nm	Crocin (mg g^{-1})	Picrocrocin (mg g^{-1})	Safranal (mg g^{-1})
KAE1	0.77	5.26	205^{a}	85^{a}	42^{a}	39.32^{a}	5.36^{a}	0.26^{bc}
KAE2	0.41	6.10	212^{a}	86^{a}	44^{a}	43.51^{a}	5.89^{a}	0.31^{a}
KAE3	0.66	5.15	178^{a}	62^{b}	28^{c}	45.36^{a}	6.21^{a}	0.26^{bc}
KAE4	2.73	7.09	203^{a}	74^{a}	35^{a}	39.95^{a}	6.01^{a}	0.29^{a}
KAE5	1.43	6.43	188^{a}	69^{b}	31^{ab}	42.29^{a}	4.03^{b}	0.3^{a}
KAE6	6.17	7.53	205^{a}	79^{a}	39^{a}	33.43^{ab}	4.91^{a}	0.27^{abc}
SXR1	11.51	9.34	134^{b}	56^{c}	24^{c}	38.49^{a}	4.02^{b}	0.28^{ab}
SXR2	4.93	6.73	179^{a}	59^{c}	27^{c}	26.36^{b}	3.16^{c}	0.17^{c}
SXR3	5.61	10.3	184^{a}	62^{b}	28^{c}	32.41^{ab}	3.98^{b}	0.2^{c}
SXR4	20.49	14.68	80^{b}	43^{c}	23^{c}	ND	ND	0.18^{c}
SXR5	9.49	11.35	162^{b}	57^{c}	25^{c}	34.24^{ab}	3.23^{c}	0.29^{a}
SXR6	25.3	22.35	72^{b}	40^{c}	16^{c}	18.26^{b}	2.79^{c}	0.23^{c}
PAM1	4.53	12.08	195^{a}	69^{b}	32^{ab}	33.32^{ab}	3.63^{b}	0.29^{a}
PAM2	5.68	7.84	152^{b}	65^{b}	29^{b}	38.43^{a}	3.23^{c}	0.26^{bc}
PAM3	7.85	7.63	201^{a}	74^{a}	34^{bc}	42.45^{a}	4.02^{b}	0.28^{ab}
PAM4	0.60	6.26	163^{b}	72^{ab}	33^{bc}	30.44^{ab}	3.62^{bc}	0.32^{a}
PAM5	1.93	8.23	189^{a}	67^{b}	30^{b}	29.4^{ab}	3.14^{c}	0.26^{bc}
PAM6	6.25	5.39	207^{a}	82^{a}	40^{a}	33.31^{ab}	4.11^{b}	0.27^{abc}
BUD1	2.48	6.19	202^{a}	79^{a}	38^{a}	32.43^{ab}	3.89^{b}	0.29^{a}
BUD2	2.80	7.26	185^{a}	60^{c}	29^{b}	26.38^{b}	3.77^{b}	0.27^{abc}
BUD3	3.91	8.15	149^{b}	56^{c}	25^{c}	27.39^{b}	3.56^{c}	0.28^{ab}
BUD4	16.60	10.28	105^{b}	53^{c}	24^{c}	20.2^{b}	3.04^{c}	0.21^{c}
BUD5	11.29	5.33	181^{a}	59^{c}	27^{c}	30.3^{ab}	3.69^{b}	0.23^{c}
BUD6	10.24	6.35	176^{a}	58^{c}	26^{c}	32.41^{ab}	3.51^{c}	0.25^{bc}
AFG1	1.56	5.68	201^{a}	78^{a}	37^{a}	41.34^{a}	5.1^{a}	0.27^{abc}
AFG2	1.34	5.34	208^{a}	81^{a}	39^{a}	30.49^{ab}	4.25^{b}	0.3^{a}
AFG3	1.97	6.63	192^{a}	68^{b}	31^{b}	31.42^{ab}	4.07^{b}	0.29^{a}
AFG4	3.81	7.76	188^{a}	64^{b}	29^{bc}	38.38^{a}	3.81^{b}	0.25^{bc}
IRN1	2.10	5.26	206^{a}	84^{a}	41^{a}	35.42^{ab}	5.33^{a}	0.26^{bc}
IRN2	1.92	5.59	195^{a}	75^{a}	36^{a}	35.12^{ab}	5.1^{a}	0.29^{a}
SIGMA	ND*	4.49	203^{a}	72^{ab}	32^{ab}	34.41^{ab}	4.46^{ab}	0.31^{a}

Means followed by the same letter within the columns are not significantly different ($p < 0.05$) using DMRT
*ND- Not Detected

KAE samples, 29.952 mg/g in SXR samples, 34.55mg/g in PAM samples, 28.18 mg/g in BUD samples, 35.40 mg g^{-1} in AFG samples, 35.27 mg g^{-1} in IRN samples and 34.41 mg g^{-1} in sigma sample. Safranal, one of the main components responsible for the fragrance of the spice, is soluble in polar solvents and poorly soluble in nonpolar solvents. The safranal content as per the ISO 3632 (2011) method cannot be categorized in any grade as the ISO

Table 3: Quality characteristics of saffron obtained from different geographical locations using the ISO-3632 method

Sample Origin	ISO Category	Crocin (E1 %) 440 nm	Safranal (E1 %) 257 nm	Picrocrocin (E1 %) 257 nm
KAE	I (4)	203-212	28-31	62-69
	II (2)	168-178	35-42	74-84
SXR	II (2)	179-184	27-28	59-62
	III (2)	134-162	24-25	56-57
	IV* (2)	72-80	16-23	40-43
PAM	I (3)	195-207	32-40	64-82
	II (1)	189	30	67
	III (2)	152-163	29-33	65-73
BUD	I (1)	202	38	79
	II (3)	176-185	26-29	58-60
	III (1)	149	25	56
	IV* (1)	105	24	53
AFG	I (3)	192-208	31-39	68-81
	II (1)	188	29	64
IRN	I (2)	36-41	75-24	75-24
SIG	I (1)	203	32	72

*Highly adulterated or counterfeit saffron samples

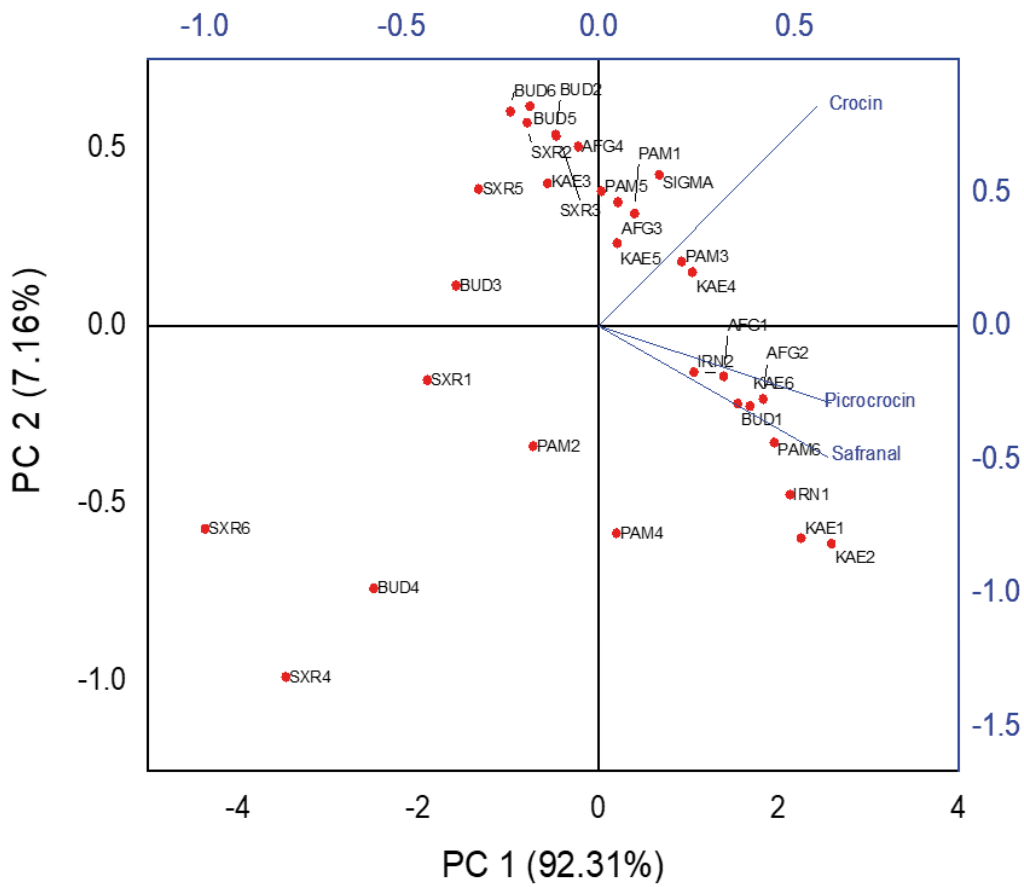


Figure 1: PCA analysis (biplot) of saffron samples (UV-Vis analysis)

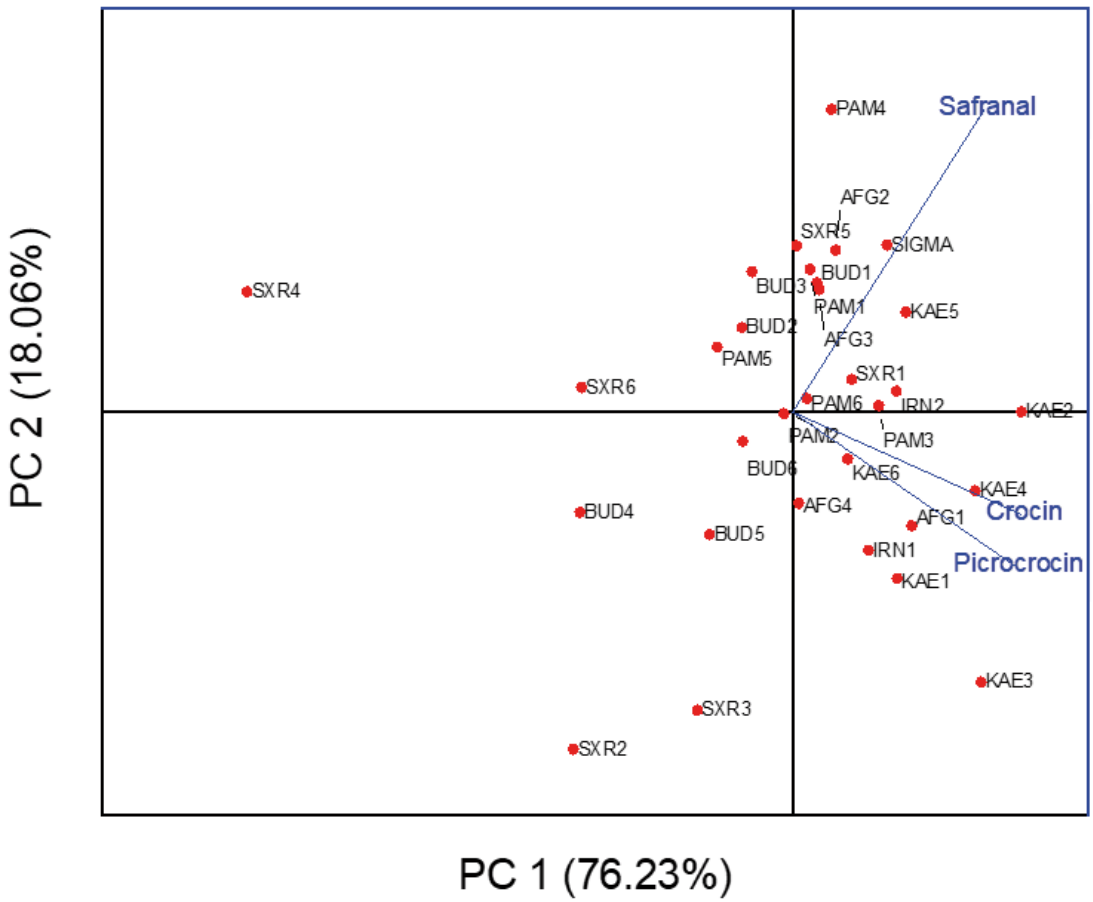


Figure 2: PCA analysis (biplot) of saffron samples (HPLC analysis)

method doesn't provide a precise classification of grades of saffron based on safranal content. The average safranal content differed significantly across KAE (0.28 mg g^{-1}), SXR (0.22 mg g^{-1}), PAM (0.28 mg g^{-1}), BUD (0.25 mg g^{-1}), AFG (0.27 mg g^{-1}), IRN (0.27 mg g^{-1}), and sigma samples (0.31 mg g^{-1}). Meanwhile, average picrocrocin content ranged from 5.40 mg g^{-1} in KAE, 3.43 mg g^{-1} in SXR, 3.62 mg g^{-1} in PAM, and 3.57 mg g^{-1} in BUD samples, 4.30 mg g^{-1} in AFG, 5.21 mg g^{-1} in IRN and 4.46 mg g^{-1} in SIG sample (Table 4). Crocin and picrocrocin were not detected in SXR4 sample. A biplot of samples was obtained to analyze the relation between metabolites in samples using PCA (Figure 2). PC1 (92.31 %) and PC2 (7.16 %) accounted for 99.47 % of the total variance of the data. The coefficient for both the principal components is given in Table 5.

The results obtained from UV-Vis spectroscopy showed that saffron samples from KAE were of the highest grade compared to other saffron obtained from other commercial sites. The saffron from PAM and BUD commercial sites showed a moderate range of quality. The samples from AFG and IRN fell in grade I and II as per ISO parameters. The saffron from SXR markets showed the lowest grade compared to other samples. The HPLC analysis showed a higher concentration of apocarotenoid content in KAE samples, followed by AFG and IRN samples. The SXR samples showed the lowest quality and apocarotenoid content, which indicates an indication of adulteration.

4 CONCLUSION

The evaluation of the quality of saffron selections was done by UV-Vis Spectroscopy according to the limit set by the ISO 3632, and the determination of apocarotenoid content was analyzed by HPLC analysis. The UV-Vis spectrophotometric results categorized the sample into different grades as per standards formulated by ISO. Only fourteen samples were identified as Grade I, and 13 samples were either grade II or grade III. The remaining 3 samples were found to be highly adulterated. The results obtained from HPLC analysis showed significant variation. The highest concentration of apocarotenoids

Table 4: HPLC-based concentration range of crocin, safranal and picrocrocin of saffron samples obtained from different geographical locations

Sample Origin	Crocin (mg g^{-1})	Safranal (mg g^{-1})	Picrocrocin (mg g^{-1})
KAE	33.43-45.36	0.26-0.31	4.03-6.21
SXR	18.26-38.49	0.17-0.29	2.79-4.02
PAM	29.40-42.45	0.26-0.32	3.14-4.11
BUD	20.20-32.43	0.21-0.29	3.04-3.89
AFG	30.49-41.34	0.25-0.30	3.81-5.10
IRN	35.12-35.42	0.26-0.29	5.10-5.33
SIG	34.41	0.31	4.46

Table 5: Loading of the first two principal components (PC's) for concentration of metabolites

Variable	Coefficients of PC1	Coefficients of PC2
UV-Vis analysis		
Crocin	0.5555	0.82244
Safranal	0.58313	-0.49034
Picrocrocin	0.59278	-0.28836
HPLC analysis		
Crocin	0.61407	-0.28918
Safranal	0.51423	0.85245
Picrocrocin	0.59875	-0.43554

was found in KAE samples, followed by AFG, IRN, PAM and BUD samples. Samples from SXR showed the least concentration of apocarotenoids, indicating a high level of adulteration. Adulteration in saffron is a big concern and needs to be addressed scientifically. It has an adverse effect on the saffron industry since counterfeit or adulterated saffron accounts for considerable market share. Although various instrumental methods (HPLC, GC-MS, FTIR, Raman Spectroscopy, etc.) for detecting adulteration in saffron, these methods are laboratory-based and cannot be used on a large sample size. Consequently, improvements to the existing ISO methods are suggested, and the development of a technique for on-the-spot detection of adulteration in saffron is recommended for future studies.

5 REFERENCES

Alonso, G. L., Salinas, M. R., & Garijo, J. (1998). Method to determine the authenticity of aroma of saffron (*Crocus sativus* L.). *Journal of Food Protection*, *61*(11), 1525–1528. https://doi.org/10.4315/0362-028X-61.11.1525

Azami, S., Shahriari, Z., Asgharzade, S., Farkhondeh, T., Sadeghi, M., Ahmadi, F., Vahedi, M. M., & Forouzanfar, F. (2021). Therapeutic potential of saffron (*Crocus sativus* L.) in ischemia stroke. *Evidence-Based Complementary and Alternative Medicine*, *2021*, e6643950. https://doi.org/10.1155/2021/6643950

Basker, D., & Negbi, M. (1983). Uses of saffron. *Economic Botany*, *37*(2), 228–236. https://doi.org/10.1007/BF02858789

Beiranvand, S. P., Beiranvand, N. S., Moghadam, Z. B., Birjandi, M., Azhari, S., Rezaei, E., Salehnia, A. N., & Beiranvand, S. (2016). The effect of *Crocus sativus* (saffron) on the severity of premenstrual syndrome. *European Journal of Integrative Medicine*, *8*(1), 55–61. https://doi.org/10.1016/j.eujim.2015.06.003

Biancolillo, A., Foschi, M., & D'Archivio, A. A. (2020). Geographical classification of Italian saffron (*Crocus sativus* L.) by multi-block treatments of UV-Vis and IR spectroscopic data. *Molecules*, *25*(10), 2332. https://doi.org/10.3390/molecules25102332

Bolhassani, A., Khavari, A., & Bathaie, S. Z. (2014). Saffron and natural carotenoids: Biochemical activities and anti-tumor effects. *Biochimica et Biophysica Acta (Bba)-Reviews on Cancer*, *1845*(1), 20–30. https://doi.org/10.1016/j.bbcan.2013.11.001

Caballero-Ortega, H., Pereda-Miranda, R., Riverón-Negrete, L., Hernández, J.M., Medécigo-Ríos, M., Castillo-Villanueva, A. and Abdullaev, F.I. (2004). Chemical composition of saffron (*Crocus sativus* L.) from four countries. *Acta Horticulturae*, *650*, 321–326. https://doi.org/10.17660/ActaHortic.2004.650.39

Delgado, M., Zalacain, A., Pardo, J., López, E., Alvarruiz, A., & Alonso, G. (2005). Influence of different drying and aging conditions on saffron constituents. *Journal of Agricultural and Food Chemistry*, *53*, 3974–3979. https://doi.org/10.1021/jf0404748

Hadizadeh, F., Mahdavi, M., Emami, S. A., Khashayarmanesh, Z., Hassanzadeh, M., Asili, J., Seifi, M., Nassirli, H., Shariatimoghadam, A., & Noorbakhsh, R. (2007). Evaluation of iso method in saffron qualification. *Acta Horticulturae*, *739*, 405–410. https://doi.org/10.17660/ActaHortic.2007.739.53

Haghighi, B., Feizy, J., & Kakhki, A. H. (2007). LC determination of adulterated saffron prepared by adding styles colored with some natural colorants. *Chromatographia*, *66*(5), 325. https://doi.org/10.1365/s10337-007-0321-8

Hajimahmoodi, M., Afsharimanesh, M., Moghaddam, G., Sadeghi, N., Oveisi, M. R., Jannat, B., Pirhadi, E., Zamani Mazdeh, F., & Kanan, H. (2013). Determination of eight synthetic dyes in foodstuffs by green liquid chromatography. *Food Additives & Contaminants: Part A*, *30*(5), 780–785. https://doi.org/10.1080/19440049.2013.774465

Hussain, A. (2005, January 28). Saffron industry in deep distress. *BBC NEWS*. http://news.bbc.co.uk/2/hi/south_asia/4216493.stm

ISO - ISO 3632-1 (2011). *Spices—Saffron (Crocus sativus L.)—Part 1: Specification*. https://www.iso.org/standard/44523.html

Kahriz, P. P. (2020). Saffron (*Crocus sativus* L.) cultivation in Turkey. In *Saffron* (pp. 33–43). Elsevier. https://doi.org/10.1016/B978-0-12-818462-2.00003-6

Kamalipour, M., & Akhondzadeh, S. (2011). Cardiovascular ef-

fects of saffron: An evidence-based review. *The Journal of Tehran Heart Center*, *6*(2), 59.

Karimi, S., Feizy, J., Mehrjo, F., & Farrokhnia, M. (2016). Detection and quantification of food colorant adulteration in saffron sample using chemometric analysis of FT-IR spectra. *RSC Advances*, *6*(27), 23085–23093. https://doi.org/10.1039/C5RA25983E

Khorasany, A. R., & Hosseinzadeh, H. (2016). Therapeutic effects of saffron (*Crocus sativus* L.) in digestive disorders: A review. *Iranian Journal of Basic Medical Sciences*, *19*(5), 455–469.

Kumari, L., Jaiswal, P., & Tripathy, S. (2021). Various techniques useful for determination of adulterants in valuable saffron: A review. *Trends in Food Science & Technology*, *111*. https://doi.org/10.1016/j.tifs.2021.02.061

Lowell, G. (1964). Saffron adulteration. *Journal of Association of Official Agricultural Chemists*, *47*(3), 538. https://doi.org/10.1093/jaoac/47.3.538

Lozano, P., Castellar, M. R., Simancas, M. J., & Iborra, J. L. (1999). A quantitative high-performance liquid chromatographic method to analyse commercial saffron (*Crocus sativus* L.) products. *Journal of Chromatography A*, *830*(2), 477–483. https://doi.org/10.1016/S0021-9673(98)00938-8

Maghsoodi, V., Kazemi, A., & Akhondi, E. (2012). Effect of different drying methods on saffron (*Crocus sativus* L) quality. *Iranian journal of chemistry & chemical engineering-international english edition*, *31*, 85–89.

Marieschi, M., Torelli, A., & Bruni, R. (2012). Quality control of saffron (*Crocus sativus* L.): Development of SCAR markers for the detection of plant adulterants used as bulking agents. *Journal of Agricultural and Food Chemistry*, *60*(44), 10998–11004. https://doi.org/10.1021/jf303106r

Mir, T. ul G., Wani, A. K., Singh, J., & Shukla, S. (2022a). Therapeutic application and toxicity associated with *Crocus sativus* (saffron) and its phytochemicals. *Pharmacological Research - Modern Chinese Medicine*, *4*, 100136. https://doi.org/10.1016/j.prmcm.2022.100136

Mir, T. U. G., Malik, A. Q., Singh, J., Shukla, S., & Kumar, D. (2022b). An overview of molecularly imprinted polymers embedded with quantum dots and their implementation as an alternative approach for extraction and detection of crocin. *Chemistry Select*, *7*(21), e202200829. https://doi.org/10.1002/slct.202200829

Mzabri, I., Addi, M., & Berrichi, A. (2019). Traditional and modern uses of Saffron (*Crocus Sativus*). *Cosmetics*, *6*(4), 63. https://doi.org/10.3390/cosmetics6040063

Ordoudi, S. A., Staikidou, C., Kyriakoudi, A., & Tsimidou, M. Z. (2018). A stepwise approach for the detection of carminic acid in saffron with regard to religious food certification. *Food Chemistry*, *267*, 410–419. https://doi.org/10.1016/j.foodchem.2017.04.096

Pandita, D. (2021). Saffron (Crocus sativus L.): Phytochemistry, therapeutic significance and omics-based biology. In *Medicinal and Aromatic Plants* (pp. 325–396). Elsevier. https://doi.org/10.1016/B978-0-12-819590-1.00014-8

Paredi, G., Raboni, S., Marchesani, F., Ordoudi, S. A., Tsimidou, M. Z., & Mozzarelli, A. (2016). Insight of saffron proteome by gel-electrophoresis. *Molecules*, *21*(2), 167. https://doi.org/10.3390/molecules21020167

Patel, K., Maguigan, K., & Shoulders, B. (2019). 648: Evaluation of beta-lactam concentrations associated with extended daily dialysis. *Critical Care Medicine*, *47*(1), 304. https://doi.org/10.1097/01.ccm.0000551400.16360.6c

Petrakis, E. A., Cagliani, L. R., Polissiou, M. G., & Consonni, R. (2015). Evaluation of saffron (*Crocus sativus* L.) adulteration with plant adulterants by 1H NMR metabolite fingerprinting. *Food Chemistry*, *173*, 890–896. https://doi.org/10.1016/j.foodchem.2014.10.107

Saxena, R. B. (2010). Botany, taxonomy and cytology of *Crocus sativus* series. *AYU (An International Quarterly Journal of Research in Ayurveda)*, *31*(3), 374. https://doi.org/10.4103/0974-8520.77153

Sereshti, H., Poursorkh, Z., Aliakbarzadeh, G., & Zarre, S. (2018). Quality control of saffron and evaluation of potential adulteration by means of thin layer chromatography-image analysis and chemometrics methods. *Food Control*, *90*, 48–57. https://doi.org/10.1016/j.foodcont.2018.02.026

Siddiqui, M. J., Saleh, M. S. M., Basharuddin, S. N. B. B., Zamri, S. H. B., Mohd Najib, M. H. bin, Che Ibrahim, M. Z. bin, binti Mohd Noor, N. A., Binti Mazha, H. N., Mohd Hassan, N., & Khatib, A. (2018). Saffron (*Crocus sativus* L.): As an antidepressant. *Journal of Pharmacy & Bioallied Sciences*, *10*(4), 173–180. https://doi.org/10.4103/JPBS.JPBS_83_18

Taherzadeh, Z., Khaluyan, H., Iranshahy, M., Rezaeitalab, F., Ghalibaf, M. H. E., & Javadi, B. (2020). Evaluation of sedative effects of an intranasal dosage form containing saffron, lettuce seeds and sweet violet in primary chronic insomnia: A randomized, double-dummy, double-blind placebo controlled clinical trial. *Journal of Ethnopharmacology*, *262*, 113-116.

Zalacain, A., Ordoudi, S. A., Blázquez, I., Díaz-Plaza, E. M., Carmona, M., Tsimidou, M. Z., & Alonso, G. L. (2005). Screening method for the detection of artificial colours in saffron using derivative UV-Vis spectrometry after precipitation of crocetin. *Food Additives & Contaminants*, *22*(7), 607–615. https://doi.org/10.1080/02652030500150051

Zilaee, M., Hosseini, S. A., Jafarirad, S., Abolnezhadian, F., Cheraghian, B., Namjoyan, F., & Ghadiri, A. (2019). An evaluation of the effects of saffron supplementation on the asthma clinical symptoms and asthma severity in patients with mild and moderate persistent allergic asthma: A double-blind, randomized placebo-controlled trial. *Respiratory Research*, *20*(1), 39. https://doi.org/10.1186/s12931-019-0998-x

Zougagh, M., Ríos, A., & Valcárcel, M. (2005). An automated screening method for the fast, simple discrimination between natural and artificial colorants in commercial saffron products. *Analytica Chimica Acta*, *535*(1), 133–138. https://doi.org/10.1016/j.aca.2004.11.060

doi:10.14720/aas.2022.118.3.2529 Original research article / izvirni znanstveni članek

Seed priming with ZNPs reduced expression of salinity tolerance genes in *Glycine max* L. and improved yield traits

Reda Mohamed GAAFAR [1, 2], Mohamed Lotfi HALAWA [1], Adel Ramadan EL-SHANSHORY [1], Abdelhamid Abdelrahim EL-SHAER [3], Rana Hosny DIAB [1], Marwa Mahmoud HAMOUDA [1]

Received January 18, 2022; accepted July 17, 2022.
Delo je prispelo 18. januarja 2022, sprejeto 17. julija 2022

Seed priming with ZNPs reduced expression of salinity tolerance genes in *Glycine max* L. and improved yield traits

Abstract: Little has been done to evaluate the molecular role of ZnO nanoparticles (ZNPs) in regulating biochemical processes and plant yield in response to salt-induced stress. In this study, the molecular response of salt-stressed soybean ('Giza111') was assessed under different concentrations of ZNPs (25, 50, 100, and 200 mg l^{-1}) by measuring some osmolytes, yield parameters, and Na^+ and K^+ content. The impact of salinity on the mRNA expression levels of three key salt-tolerance related genes *(GmCHX1, GmPAP3,* and *GmSALT3)* using qRT-PCR was also determined. The high level of salinity (250 mM NaCl) led to a significant increase in Na^+ content, total soluble proteins, and total soluble carbohydrates and significantly upregulated gene expression of *GmCHX1, GmPAP3,* and *GmSALT3*, while reducing K^+ content, K^+/Na^+ ratio and all yield parameters compared to control plants. Soaking soybean seeds in various ZNP concentrations, on the other hand, increased K^+ content and K^+/Na^+ ratio while decreasing Na^+ content, total soluble proteins, and total soluble carbohydrates in stressed plants, particularly at 50 mg l^{-1} ZNPs. Furthermore, *GmCHX1, GmPAP3,* and *GmSALT3* expressions were all downregulated at 50 mg l^{-1} ZNPs, which ultimately improved soybean yield parameters. Accordingly, these results recommend the application of 50 mg l^{-1} ZNPs for improving the productivity of soybean cultivated in saline soils.

Key words: ZnO; nanoparticles; salinity; soybean; gene expression; qRT-PCR ; productivity

Predtretiranje semen s cinkovimi nano delci je zmanjšalo izražanje genov tolerance na slanost pri soji (*Glycine max* L.) in izboljšalo lastnosti pridelka

Izvleček: Malo je bilo narejenega za ovrednotenje molekularne vloge nano delcev ZnO (ZNPs) pri uravnavanju biokemičnih procesov in pridelka rastlin kot odziva na slanostni stres. V tej raziskavi je bil ocenjen molekularni odziv na solni stres pri soji ('Giza111') pri uporabi različnih koncentracij ZNPs (25, 50, 100, in 200 mg l^{-1}) z meritvami nekaterih osmotikov, parametrov pridelka in vsebnosti Na^+ in K^+. Vpliv slanosti na količino mRNK treh ključnih s toleranco na slanost povezanih genov *(GmCHX1, GmPAP3,* in *GmSALT3)* je bil določen z uporabo qRT-PCR metode. Velika slanost (250 mM NaCl) je vodila k znatnemu povečanju vsebnosti Na^+, celokupnih topnih beljakovin, celokupnih topnih ogljikovih hidratov in značilno povečala izražanje genov *GmCHX1, GmPAP3,* in *GmSALT3*, med tem ko, je zmanjšala vsebnost K^+, razmerja K^+/Na^+ in vse parameter pridelka v primerjavi s kontrolo. Namakanje semen soje v različnih koncentracijah ZNP je povečalo vsebnost K^+ in razmerje K^+/Na^+ v rastlinah pod stresom in hkrati zmanjšalo vsebnost Na^+, celokupnih topnih beljakovin in celokupnih topnih ogljikovih hidratov, še posebej pri uporabi 50 mg l^{-1} ZNPs. Dodatno je bilo pri tem obravnavanju zmanjšano izražanje genov *GmCHX1, GmPAP3,in GmSALT3,* kar je na koncu izboljšalo parametre pridelka soje. Skladno s temi rezultati priporočamo uporabo 50 mg l^{-1} ZNPs za izboljšanje pridelka soje, gojene na slanih tleh.

Ključne besede: ZnO; nano delci; slanost; soja; izražanje genov; qRT-PCR; produktivnost

1 Botany Department, Faculty of Science, Tanta University, Egypt
2 Corresponding author, e-mail: redagaafar@science.tanta.edu.eg
3 Physics Department, Faculty of Science, Kafrelsheikh University, Kafrelsheikh, Egypt

1 INTRODUCTION

Soybean (*Glycine max* L.) is one of the important food and industrial crops worldwide because of its content of cholesterol-free oil (30 %) and proteins (40 %), which are similar in their nourishing value to animal proteins (Van Zanten et al., 2016). The fractions and derivatives of soybean seeds have major economic importance in a wide range of industrial, food, pharmaceutical, and agricultural products (Chen et al., 2012).

Salinity of the soil is a serious problem all over the world. It has been estimated that around 954 million hectares are already salinized (Qadir et al., 2014). It usually causes a reduction of water potential, ion imbalances or disturbances in ion homeostasis, resulting in a reduction of plant growth and crop productivity (Han et al., 2019). Mittler (2002) observed that the oxidative demolition of the cell (oxidative stress) occurs by injuring membranes (lipid peroxidation), proteins, RNA, and DNA molecules as a result of elevated ROS levels in the cells. DNA damage is caused by $OH^{\cdot}$ and O^{2-} radicals, and this damage results in heritable changes (Fatima et al., 2017). Moreover, these signals play an important role in the adaptation process of plants to abiotic stress (Choudhury et al., 2017).

Plant tolerance to salinity stress includes physiological and molecular changes such as accumulation of organic solutes, antioxidant enzymes, and inorganic ions as well as gene expression responses (Ahanger et al., 2017). These alterations include either the induction of some polypeptides, the disappearance of others, or the overexpression of other sets of proteins (El-Mashad et al., 2012). Therefore, linking the expression of a gene to a higher degree of tolerance within a genotype offers an imperative argument for a role in plant adaptation (Abreu et al., 2013). Numerous reports suggest that the harmful effect of salinity stress was manifested by relatively higher expression of salt-related genes in soybean, such as *GmP5CS*, *GmDREB1a*, *GmGOLS*, *GmBADH* and *GmNCED1* (Liu et al., 2017), *GmERF3* (Zhang et al., 2009), *GmMYB* genes, *GmMYB76*, *GmMYB92* and *GmMYB177* (Liao et al., 2008), *GmPAP3* (Liao et al., 2003), *GmCHX1* (Patil et al., 2016) and *GmSALT 3* (Guan et al., 2014).

Previous studies in soybean determined that a QTL on chromosome 3 is the major genomic region that dictates salinity tolerance in soybean (Patil et al., 2016; Chen et al., 2018). This gene locus carries the dominant functional sodium/hydrogen exchanger family gene in wild (*GmCHX1*) and cultivated soybean (*GmNcl/GmSALT3*), which explains more than 64 % of the phenotypic variation (Qi et al., 2014). Normally, the *GmCHX1* gene is expressed under high salt conditions in root stellar cells and limits salt transport to shoot tissues (Guan et al., 2014). It has been described that the full-length *GmSALT3* protein is closely correlated to the *Arabidopsis thaliana AtCHX20* (a Cation/Proton Exchanger), which is a functionally characterized member of the *CPA2* (Cation/Proton Antiporter2) family of transporters (Padmanaban et al., 2007; Qu et al., 2020). Functional studies of *AtCHXs* have shown that they might play a role in modulating cation and pH homeostasis within the endomembrane system (Chanroj et al., 2011). The ER-localized AtCHX20 was suggested to be an endomembrane K^+ transporter involved in the osmoregulation of guard cells (Padmanaban et al., 2007). Purple acid phosphatases (PAPs) represent a diverse group of acid phosphatases in animals, microorganisms, and plants (Vogel et al., 2001; Olczak et al., 2003). The primary biochemical reaction of PAPs is to catalyze the hydrolysis of phosphate esters and anhydrides. The physiological role of *GmPAP3* might be related to the adaptation of soybean to NaCl stress, possibly through its involvement in reactive oxygen species (ROS) forming and/or scavenging or stress-responding signal transduction pathways (Liao et al., 2003; Soleimani et al., 2017).

Zinc (Zn) is a metallic cofactor for more than 300 enzymes. The Zn-finger proteins that attach to deoxyribonucleic acid (DNA) are clear evidence of the usefulness of Zn in biological systems (Hezaveh et al., 2019). Zinc is a structural component of ribosomes and is essential for their structural integrity. On the other hand, it has other indirect effects on the control of stomatal opening and closing and ROS detoxification (Haliloglu et al., 2020). Currently, nanotechnology has broad perspectives in all fields of science (Dewdar et al., 2018). The application of nanoparticles to plants can be beneficial for growth and development due to their greater absorbance and high reactivity (Fraceto et al., 2016). ZnO nanoparticles (ZNPs) are one of the most frequently used nanoproducts (Samei et al., 2019). Interestingly, priming of seeds with ZNPs positively affected the yield traits in salt-stressed plants, whereas ZNPs stimulated natural auxin (IAA), thus activating cell division and enlargement and also increasing K^+ ion content, which increases storage of food in seeds (Ali and Mahmoud, 2013), maintaining the structural integrity of biomembranes (He et al., 2015), improving protein synthesis and DNA replication (Landa et al., 2015), scavenging free oxygen radicals and decreasing the uptake of excess Na^+ and Cl^- (Farhangi-Abriz and Torabian, 2018), as well as augmentation of photosynthesis, total soluble proteins, total soluble carbohydrates, and total phenols in stressed plants (Abdel Latef et al., 2017).

Transcription factors are the primary regulators of gene expression in a variety of genes that are involved in reducing and/or protecting against cellular stress damage

(Linh et al., 2020). The catalytic activity of RNA polymerases, which is essential for gene expression, is well known to require Zn^{2+} ions. Zn stabilizes several structural motifs in transcriptional regulatory proteins, such as Zn finger domains (Albert et al., 1998). Zn has been shown to upregulate gene expression, particularly in Zn-controlled genes, in numerous studies. Plants treated with ZnO, for example, had the highest *OsZIP1* expression in their roots after 7 days when compared to no-zinc controls (Selvaraj and Dananjeyan, 2016). Recently, ZNPs boosted the expression of the wheat drought-tolerance genes *DREB2* and *Wdhn13*, catalase activity (CAT1), proline biosynthesis (*P5CS*), and proline biosynthesis (*P5CS*) genes (Raeisi Sadati et al., 2022).

It was concluded that priming with ZNPs, particularly at 60 mg l^{-1}, improved photosynthetic pigments, altered osmoregulation, and decreased MDA and Na concentrations in lupine plants (Abdel Latef et al., 2017). So, the current study was conducted to investigate the effect of seed-priming using different concentrations of ZNPs on the expression of three salinity-tolerance genes. In addition, their impacts on alleviating salinity stress and improving productivity in soybean plants were assessed.

2 MATERIALS AND METHODS

2.1 PLANT MATERIALS

Seeds of a soybean cultivar ('Giza 111') were provided by the Food and Legumes Research Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt.

2.2 SYNTHESIS AND CHARACTERIZATION OF ZNO NANOPARTICLES

In this study, ZnO nanoparticles were synthesized using the chemical bath deposition (CBD) method as described by El-Shaer et al. (2018). The crystalline structure and optical properties of the prepared ZnO nanostructures were examined with X-ray Diffraction (XRD, Shimadzu 6000), while the samples' morphology was investigated using a scanning electron microscope (SEM, JSM-651OLV). As shown in Fig. 1A, ZnO nanostructures are formed as nano-rods with a hexagonal quartzite crystal structure. These nano-rods accumulate to form the surface morphology of grains, similar to flowers. The XRD pattern of ZnO nano-rods is shown in Fig. 1B. The diffraction peaks at 32°, 34.5°, 36.4°, 47.5°, 57°, 62.7°, 67.9°, and 69.3° correspond to the (100), (002), (101), (102), (110), (103), (112), and (201) lattice planes, respectively (Fig. 1B).

2.3 PLANT GROWTH CONDITIONS

Priming and growing of the soybean seeds ('Giza 111') were performed as described by Gaafar et al. (2020). The seeds were sterilized with 70 % ethanol for 5 min and sodium hypochlorite (10 %) for 10 min, followed by washing several times with distilled water. Four concentrations of ZnO nanoparticles (ZNPs) of 25 (ZNPs25), 50 (ZNPs50), 100 (ZNPs100), and 200 (ZNPs200) mg l^{-1} were used to prime the seeds for two hours at room temperature, and distilled water was used as a control (0). Previous studies have found that low concentrations of

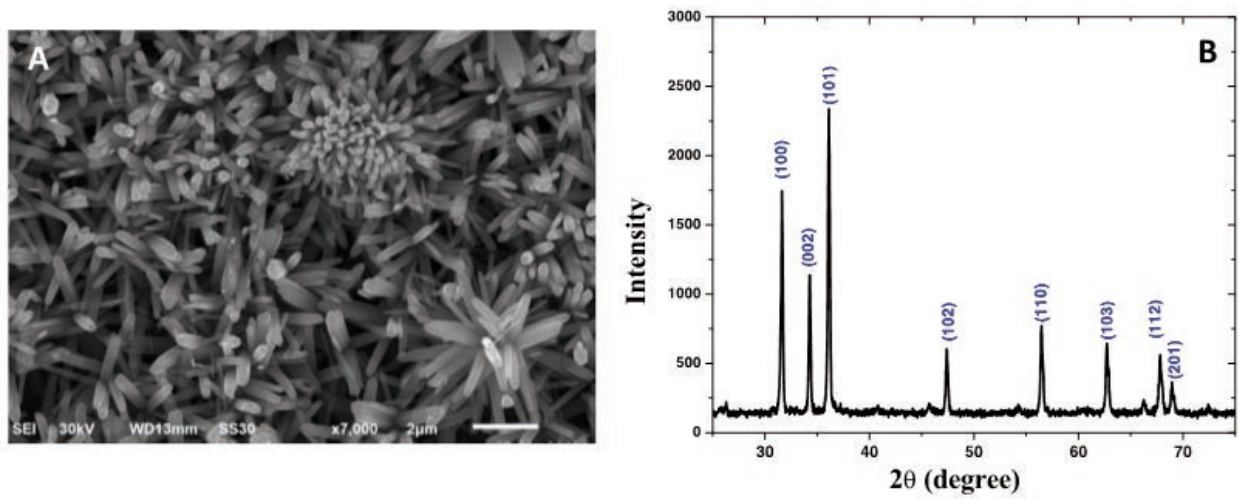


Fig. 1: (A) SEM image and (B) XRD chart of ZnO nanostructures prepared by CBD method

ZnO NPs are beneficial to plant growth, whereas concentrations equal to or greater than 200 mg l^{-1} are detrimental. Therefore, the used ZNP concentrations were chosen (Liu et al., 2015; Abdel Latef et al., 2017). After priming, the seeds were sown (20 seeds/pot) in plastic pots (45 cm x 40 cm) filled with 24 kg of 2:1 (clay: sandy) soil. Based on the preliminary experiment results, 250 mM NaCl (S) was chosen as a sub-lethal salinity level and used in this study.

The pots were irrigated with tap water until seed germination (emergence), then with tap water and with 250 mM NaCl solution to 80 % field capacity for 21 days (seedling stage) and 90 days (yield stage). Three pots were used as replicates for each treatment. The germinated soybean seeds were let to grow in the green house under the following environmental conditions: 29 ± 2 °C/25 ± 2 °C day/night and 16h/8h light/dark regimes. The 21-day-old seedlings of all treatments were collected, washed, and used for further analyses, and the productivity of yielded seeds was determined on 90-day-old plants.

2.4 DETERMINATION OF SODIUM AND POTASSIUM CONTENT

According to Allen et al. (1974), the mixed acid digestion method was used for element determination. The concentration of Na^+ and K^+ (mg g^{-1} d.m.) was determined by using Inductively Coupled Plasma (ICP, STI) at the central laboratory of Tanta University.

2.5 QUANTITATIVE ESTIMATION OF TOTAL SOLUBLE PROTEINS AND TOTAL SOLUBLE CARBOHYDRATES

The total soluble proteins were extracted according to the method described by Naguib et al. (1968). Then the protein content was determined as described by Bradford (1976), and the phenol-sulfuric acid method has been used for estimation of total soluble carbohydrates according to Dubois *et al.* (1956).

2.6 QUANTITATIVE REAL TIME PCR (QRT-PCR) RNA EXTRACTION AND PURIFICATION

For the extraction of total RNA, approximately 100 mg of ground plant fresh leaves were used, and RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The total RNA was then quantified and assessed for quality using a Nanodrop (ScanDrop, Analytik, Jena, Germany). Total RNA samples were kept at -80 °C until further analysis.

2.7 CDNA SYNTHESIS

The cDNA synthesis was performed using the SensiFAST cDNA synthesis kit (Ameridian Life Science, USA) using the protocol of the manufacturer. The cDNA synthesis reaction contained the following components: 1 µg total RNA, 1 µl reverse transcriptase enzyme, 4 µl 5 × Trans Amp buffer, which was completed to a total volume of 20 µl. The conditions for cDNA synthesis were as follows: primer annealing for 10 min at 25 °C, reverse transcription for 15 min at 42 °C and finally 5 min at 85 °C for enzyme inactivation. After being diluted in 10 mM Tris-HCl (pH = 8) and 0.1 mM EDTA, the cDNA reaction products were stored at -20 °C.

2.8 GENE EXPRESSION ANALYSIS (QRT- PCR)

In order to measure the gene expression of the three targeted genes, the reaction mix was prepared by mixing 10 µl of TOP real qPCR2x premix (SYBR Green with low ROX), 1 µl of each of the cDNA template, forward and

Table 1: List of sequences of the primers used for gene expression study by qRT-PCR

Primer name	Sequence (5'→3')	Length (bp)	Annealing temp. (°C)	Reference
GmPAP3F	GTGGCCGGCAGTTGACATCC	20	55.5	Liao et al. (2003)
GmPAP3R	GCTGTGCCCTGGCTCTTCTGTG	22	55.5	
GmCHX1F	GATTTGTTTTCGGGCTAACG	20	49.5	Gutierrez Gonzalez et al. (2010)
GmCHX1R	ATCCACCACGCTTCGTAACT	20	49.5	
GmSALT3F	CGGTTGATGAAGGGAAAAC	19	48.5	Hu et al. (2009)
GmSALT3R	TCCTTGACGCTTGGAGTGTT	20	48.5	
GmTublinF	GAGAAGAGTATCCGGATAGG	20	50	Gutierrez Gonzalez et al. (2010)
GmTublinR	GTTTCCGAACACTCAAGCTC	20	50	

reverse primer (10 pmol µl^{-1}) and was completed up to 20 µl. The Rotor-Gene Q5 plex (Qiagen, Germany) was used, and the PCR conditions were as follows: an initial denaturation step at 95 °C for 10 min; a denaturation step at 95 °C for 10 s; an annealing step at 60 °C for 15 s; and an elongation step at 72 °C for 15 s. The thermal cycler steps were repeated 35 times. The sequences of the primers used for qRT-PCR analysis are shown in Table 1. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method according to Livak and Schmittgen (2001).

2.9 YIELD TRAITS

The yield parameters, including length of pods/plant, mass of pods/plant, the mass of 1000 seeds, the number of pods/plant, the number of seeds/pod, the mass of seeds/pod, and the mass of seeds/plant, were determined at the end of the growing season. (approximately 3 months from cultivation). The maturity (number of viable - nonviable seeds in pods * 100) and the productivity of soybean (weight of the yielded seeds/pot in grams) were also calculated.

2.10 STATISTICAL ANALYSIS

The statistical analyses were carried out according to a completely randomized design (CRD) using analysis of variance. The significance was determined using LSD values at $p = 0.05$ and 0.01 according to Bishop (1983). The results were analyzed using a one-way ANOVA test to determine the degree of significance. The statistical analyses were performed using CoStat Software version 6.311 (CoHort Software, CA, USA). The heatmap of the gene expression data and Pearson correlation were constructed using R software (ver. 4.1.1).

3 RESULTS

3.1 SODIUM AND POTASSIUM CONTENT

The results in Table 2 show the effect of salinity stress on mineral ion content (Na^+, K^+, and K^+/Na^+) in 21-day old soybean seedlings after soaking of soybean seeds in different concentrations of ZNPs (0, 25, 50, 100, and 200 mg l^{-1}). The salinity stress (250 mM NaCl) severely decreased the content of potassium by 68 % compared to control. Similarly, it reduced the K^+/Na^+ ratio by 90 % compared to control. In contrast, the content of Na^+ was highly increased by 2.16-fold compared to control. On the other hand, the combination of ZNPs50+S (50 mg l^{-1} + 250 mM NaCl) significantly increased the content of potassium by 1.67-fold compared to salt-stressed seedlings and ameliorated the harmful effect of salinity

Table 2: Effect of salinity (S = 250 mM NaCl) on the content of Na^+, K^+ and K^+/Na^+ ratio of 21-day old soybean ('Giza 111') seedlings grown in clay-sandy soil (2:1 w/w) after soaking of soybean seeds in four different concentrations of ZnO nanoparticles (ZNPs) (ZNPs25 = 25, ZNPs50 = 50, ZNPs100 = 100, and ZNPs200 = 200 mg/L)

Treatments		K^+ (mg g-1 d. m.)	Na^+ (mg g-1 d. m.)	K^+/Na^+ ratio
Salinity level (0 mM NaCl)	Control	4.287 ± 0.04 ^{a}	2.346 ± 0.0008 ^{f}	1.827
	ZNPs25	4.043 ± 0.04 ^{b}	2.160 ± 0.007 ^{f}	1.871
	ZNPs50	4.242 ± 0.01 ^{a}	1.761 ± 0.022 ^{g}	2.409
	ZNPs100	3.942 ± 0.04 ^{b}	2.247 ± 0.009 ^{f}	1.754
	ZNPs200	3.756 ± 0.04 ^{c}	2.286 ± 0.048 ^{b}	1.642
Salinity level (S = 250 mM NaCl)	Salinity (S)	1.336 ± 0.01 ^{g}	7.419 ± 0.08 ^{a}	0.180
	ZNPs25+S	2.539 ± 0.14 ^{e}	5.425 ± 0.13 ^{d}	0.468
	ZNPs50+S	3.569 ± 0.11 ^{d}	4.061 ± 0.03 ^{e}	0.878
	ZNPs100+S	2.011 ± 0.008 ^{f}	6.629 ± 0.28 ^{c}	0.303
	ZNPs200+S	1.118 ± 0.004 ^{h}	7.120 ± 0.06 ^{b}	0.157
	F-value	689.8	894.8	-
	LSD (0.05)	0.138	0.226	-
	Significance	*		-

Values are the mean of three replicates ± SD. Values within the same column for each factor designated by different letters are significant at $p \leq 0.05$, while values with identical letters are non-significant. *: significant at $p \leq 0.05$

stress. Also, the combination of ZNPs25 + S (25 mg l^{-1} + 250 mM NaCl) increased the content of potassium ions by only 1.07-fold.

Moreover, results indicated that the combination of ZNPs200 + S (200 mg l^{-1} + 250 mM NaCl) exhibited a severe harmful effect compared to other treatments; thus, it reduced the potassium and K^+/Na^+ ratio content by 16 % and 12 %, respectively, compared to salt stressed seedlings (Table 2).

3.2 TOTAL SOLUBLE PROTEINS AND TOTAL SOLUBLE CARBOHYDRATES

The results in Figure 2 (A and B) indicated that high salinity stress (S = 250 mM NaCl) caused a highly significant increase in total soluble carbohydrates and protein content by 75.1 % and 76.1 %, respectively, compared to control. However, the results showed a general decrease in total soluble carbohydrates and protein content for all

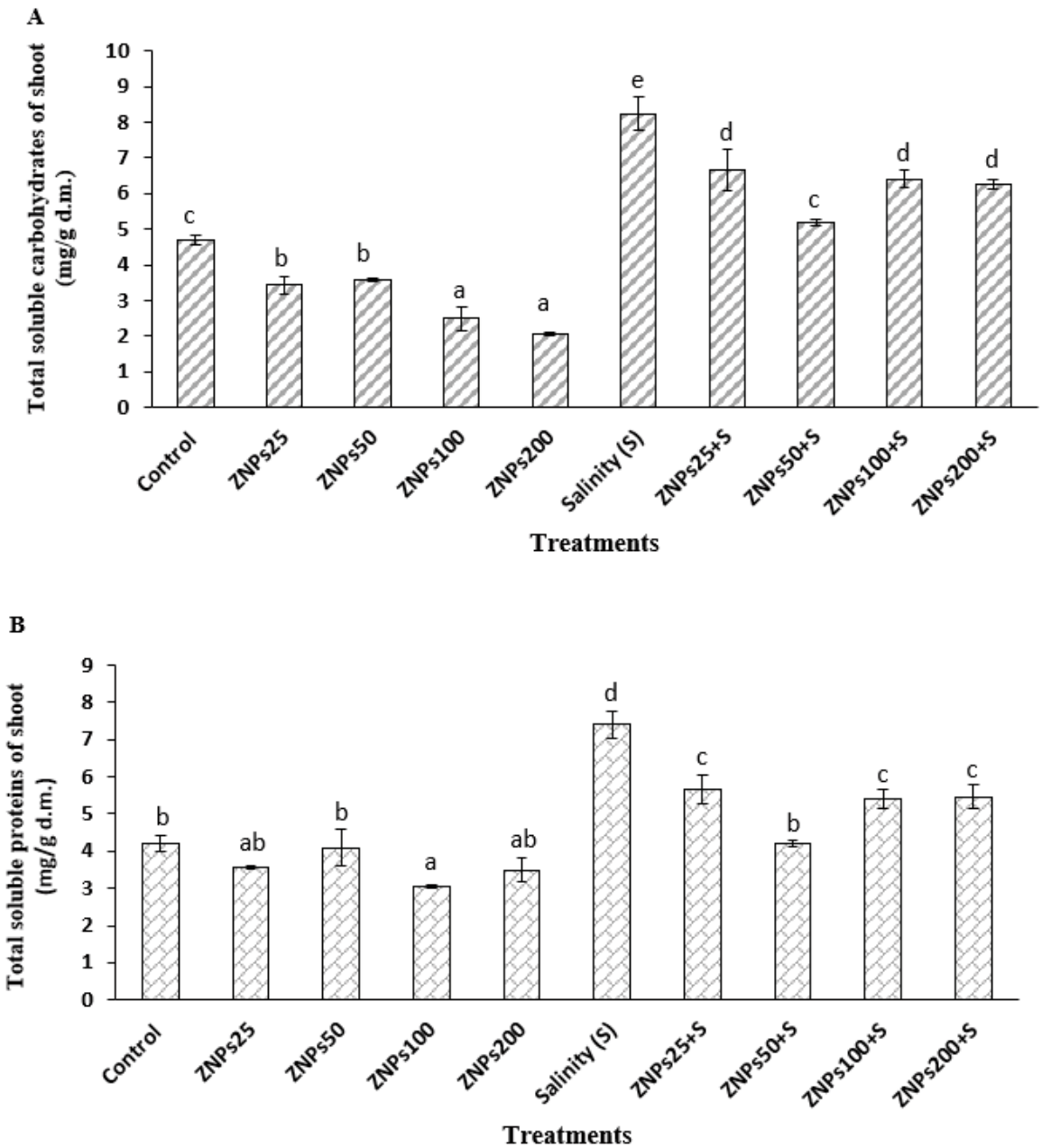


Fig. 2: Effect of NaCl (S = 250 mM) on the total soluble carbohydrates (A) and total soluble proteins (B) of 21-day old soybean ('Giza 111') seedlings grown in clay-sandy soil (2:1 w/w) after soaking of soybean seeds in four different concentrations of ZnO nanoparticles (ZNPs) (ZNPs25 = 25, ZNPs50 = 50, ZNPs100 = 100, and ZNPs200 = 200 mg l^{-1})

ZNPs (25, 50, 100, and 200 mg l^{-1}) combined with salinity, except for the combination of ZNPs25+S (25 mg l^{-1} + 250 mM NaCl), which exhibited the least reduction in total soluble carbohydrates and protein content with a percentage of 10 % and 23 %, respectively, compared to control plants, which were irrigated with water. The highest reduction in total soluble carbohydrates and protein content was recorded in the case of ZNPs50+S (50 mg l^{-1} + 250 mM NaCl) with 29 % and 43 %, respectively, compared to control plants irrigated with salt only (Fig. 2A and B).

3.3 GENE EXPRESSION ANALYSIS (QRT- PCR)

3.3.1 GmCHX1

The results of qRT-PCR analysis showed that *GmCHX1* expression was increased by ZNPs alone and the highest increase was with ZNPs100+S (100 mg l^{-1} + 250 mM NaCl) compared to control (no salinity) by about 1.2-fold (Fig. 3). Also, 250 mM NaCl (S) alone showed the highest increase in *GmCHX1* gene expression by 1.9-fold compared to control (no salinity and no ZNPs). However, ZNPs25+S (25 mg l^{-1} + 250 mM NaCl) decreased gene expression by 0.4-fold, and then it was increased with ZNPs50+S (50 mg l^{-1} + 250 mM NaCl). Also, *GmCHX1* gene expression was decreased by 0.08-fold with ZNPs100+S (100 mg l^{-1} + 250 mM NaCl). In contrast, ZNPs200+S (200 mg l^{-1} + 250 mM NaCl) showed an increase of 1.8-fold, which was similar to that of salinity (S = 250 mM NaCl) (Fig. 3).

3.3.2 GmPAP3

GmPAP3 expression was slightly increased by ZNPs treatments, and the two concentrations (ZNPs100 and ZNPs200 mg l^{-1}) showed the highest increases of about 0.73 and 0.70-fold, respectively, compared to control (no salinity and no ZNPs) (Fig. 3). In the case of salt treatment (S = 250 mM NaCl), *GmPAP3* expression increased by 3-fold. ZNPs25+S (25 mg l^{-1} + 250 mM NaCl), ZNPs50 + S (50 mg l^{-1} + 250 mM NaCl), ZNPs100 + S (100 mg l^{-1} + 250 mM NaCl), and ZNPs200 + S (200 mg l^{-1} + 250 mM NaCl) all reduced *GmPAP3* expression. The highest decrease was by 0.46-fold and was recorded with ZNPs25+S (25 mg l^{-1} + 250 mM NaCl) treatment (Fig. 3).

3.3.3 GmSALT3

In the case of *GmSALT3*, gene expression was increased by ZNP treatment using a concentration of 100 mg l^{-1} compared to control (no salinity and no ZNPs) by about 2-fold, as shown in Fig. 3. However, salinity stress (250 mM NaCl) showed the highest increase in *GmSALT3* gene expression by 7.7-fold. The concentration of 25 mg l^{-1} of ZNPs with salinity decreased gene expression by 1.56-fold, and then it was increased by the concentration of 50 mg l^{-1} of ZNPs with salinity by 4.5-fold. Then, in comparison to the salinity stress alone (250 mM NaCl), gene expression decreased by 3.9-fold with ZNPs100 + S (100 mg l^{-1} + 250 mM NaCl) treatment and by 3.5-fold with ZNPs200 + S (200 mg l^{-1} + 250 mM NaCl). In contrast to the salinity stress alone (250 mM NaCl), which increased gene expression by 8-fold (Fig. 3).

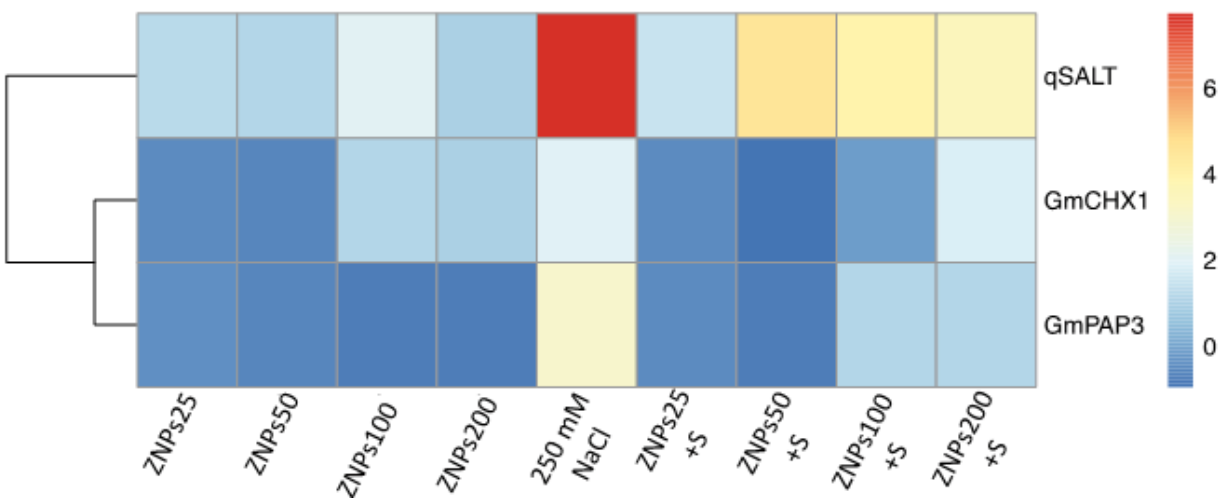


Fig. 3: Heatmap of relative expression of three soybean salinity-linked genes (*GmPAP3*, *GmCHX1*, and *GmSALT3*) in 21-day old soybean ('Giza 111') seedlings after soaking of soybean seeds in four different concentrations of ZnO nanoparticles (ZNPs) (ZNPs25 = 25, ZNPs50 = 50, ZNPs100 = 100, and ZNPs200 = 200 mg l^{-1})

3.4 YIELD PARAMETERS

The results given in Table 3 show the effect of 250 mM NaCl and ZNPs (25, 50, 100, and 200 mg l^{-1}) treatments on yield parameters. These results revealed an observable increase in all measured yield parameters, specifically in the case of ZNPs25 (25 mg l^{-1}) and ZNPs50 (50 mg l^{-1}) treatments without salinity, where these treatments increased the pod length, pod mass, number of pods/plants, and mass of pods/plant, number of seeds/pods, mass of seeds/pods, and mass of seeds/plant. The most significant increase was with ZNPs50 (50 mg l^{-1}) by 29 %, 27.8 %, 62.8 %, 39.9 %, 15.3 %, 47.8 %, and 78.8 %, respectively, compared to control. In contrast, results showed that the application of 200 mg l^{-1} ZNPs (ZNPs200) caused a highly significant decrease in all measured yield parameters: pod length, pod mass, number of pods/plant, mass of pods/plant, number of seeds/pod, mass of seeds/pods, and mass of seeds/plant by 37.8 %, 16.1 %, 13.89 %, 20.9 %, 26.9 %, 11.5 %, and 18.1 %, respectively compared to control.

Similarly, a remarkable increase in all measured yield parameters in the case of treatments ZNPs25 + S, ZNPs50 + S, and ZNPs100 + S was observed (Table 3). The most significant increases in pod length, pod mass, number of pods/plants, and mass of pods/plant, number of seeds/pods, mass of seeds/pods, and mass of seeds/plant were with ZNPs50+S (50 mg l^{-1} + 250 mM NaCl) by 50.11 %, 85.4 %, 42.6 %, 47.7 %, 341.6 %, 100 %, and 119 %, respectively, compared to control. Whereas, a decrease in these yield parameters was observed with ZNPs200+S (200 mg l^{-1} + 250 mM NaCl) by 53.2 %, 15.2 %, 23.2 %, 14.5 %, 100 %, 15.7 %, and 4.7 %, respectively, compared to control.

Similarly, the most significant increases in mass of the seeds (g/plant), mass of 1000 seeds, maturity percentage, and productivity index (g/pot) were with treatment ZNPs50 + S (50 mg l^{-1} + 250 mM NaCl) by 14.8 %, 31.5 %, 32.6 %, and 118.9 %, respectively, compared to control. In contrast, treatment with ZNPs200 + S (200 mg l^{-1} + 250 mM NaCl) decreased these parameters by 3 %, 14.2 %, 30.3 %, and 17.8 %, respectively, compared to control. These results proved the efficiency of 50 mg l^{-1} (ZNPs50) for increasing the productivity of the soybean plant under high salinity levels (S = 250 mM NaCl).

3.5 PEARSON CORRELATION ANALYSIS

As shown in Figure 4, the Na^+ was only positively corrected with Zn ($r = 1.0^*$), total proteins ($r = 0.89^*$), and total carbohydrates ($r = 92^*$), while it was negatively corrected with the rest of the studied characters, with different correlation coefficients. The pod weight was positively correlated with the weight of seeds/plant ($r = 0.9^*$), K^+ ($r = 0.91^*$), K^+/Na^+ ratio ($r = 0.88^*$), and the number of seeds/pod ($r = 0.79^{ns}$).

4 DISCUSSION

As sessile organisms, plants have adapted a variety of signal perception mechanisms as well as pathways to control molecular responses in order to respond effectively to abiotic stress situations (Dudziak et al. 2019). Exposure of soybean seedlings to high salinity stress (250 mM NaCl) imposed a significant depletion in K^+ content and in K^+/Na^+ ratio by 68 % and 90 %, respectively, compared to control. Similar findings were also found by Taffouo et al. (2009) and Khan et al. (2017) in cowpea and soybean, respectively. Contrarily, the amount of Na^+ was 2.16-fold more than it was in the control plants. It is possible that high salinity promoted the uptake of Na^+ due to its adverse effects on membrane integrity. In this regard, a similar conclusion was also made by Abdel Latef et al. (2017) on lupine plants.

According to reports, K^+ is required for maintaining osmotic balance and is an essential co-factor for many enzymes. Therefore, K^+ reduction negatively affects the growth and productivity of plants (Hauser and Horie, 2010). The results of this study indicated that K^+ content in soybean seedlings showed a highly significant reduction under acute salinity stress. However, treatment with ZNPs increased K^+ by 1.67-fold and decreased Na^+ by 45 % compared to salt-stressed seedlings, which is comparable to the findings of Abdel Latef et al. (2017) on lupine plants (*Lupine termis* L.). This is due to the fact that Zn^{+2} helps in maintaining the structural and functional integrity of root cell membranes and therefore controls the influx and efflux of Na^+ across the plasma membranes (Rezaie and Abbasi, 2014).

The application of ZnO is associated with a remarkable increase in K^+ uptake from soil to roots (Weisany et al., 2012; Soliman et al., 2015). As a consequence of enhanced K^+ uptake, plants treated with ZnO had greater K^+/Na^+ ratios than those under salinity stress alone. A high K^+/Na^+ ratio is often reported as a good indicator of a high tolerance to salt stress conditions (Khan et al., 2017). Thus, applications of ZNPs could be a useful strategy for achieving increased macronutrient uptake by plants (Dimkpa and Bindraban, 2016), which is similar to what was observed in this study, where ZNPs50+S (50 mg l^{-1}+ 250 mM NaCl) treatment significantly increased the content of potassium by 1.67-fold compared to salt-stressed seedlings and ameliorated the harmful effect of salinity stress.

Table 3: Effect of salinity (S = 250 mM NaCl) on the yield parameters of 90-day old soybean (cv. Giza 111) plants grown in clay-sandy soil (2:1 w/w) after soaking of soybean seeds in four different concentrations of ZnO nanoparticles (ZNPs) (ZNPs25 = 25, ZNPs50 = 50, ZNPs100 = 100, and ZNPs200 = 200 mg l^{-1}

Treatments		Pod length (cm)	Pod mass (g)	No. of pods/ plant	M. of pods/ plant (g)	No. of seeds/ pod	M. of seeds/ pod (g)	M. of seeds/ plant (g)	M. of 1000 seed (g)	Percentage of maturity (%)	Productivity index (g/pot)
Salinity level (0 mM NaCl)	Control	6.71 ± 0.12 ^{e}	2.41 ± 0.05 ^{d}	5.11 ± 0.44 ^{d}	8.42 ± 0.17 ^{d}	5.2 ± 0.18 ^{c}	1.38 ± 0.12 ^{e}	134.72 ± 0.22 ^{c}	61.12 ± 4.5 ^{h}	36 ± 4.1 ^{f}	5.42 ± 0.17 ^{d}
	ZNPs25	7.34 ± 0.26 ^{c}	2.92 ± 0.05 ^{b}	6.16 ± 0.44 ^{c}	10.66 ± 0.25 ^{b}	5.8 ± 0.16 ^{ab}	1.58 ± 0.03 ^{d}	154.22 ± 0.13 ^{b}	92.33 ± 4.4 ^{b}	55.8 ± 1.6 ^{b}	8.37 ± 0.11 ^{b}
	ZNPs50	8.68 ± 0.17 ^{a}	3.08 ± 0.13 ^{a}	8.32 ± 0.68 ^{a}	11.78 ± 0.16 ^{a}	6.0 ± 0.27 ^{a}	2.04 ± 0.12 ^{a}	175.8 ± 0.15 ^{a}	94.65 ± 4.8 ^{a}	69.9 ± 3.2 ^{a}	10.48 ± 0.14 ^{a}
	ZNPs100	7.72 ± 0.20 ^{b}	2.12 ± 0.15 ^{e}	4.6 ± 0.93 ^{e}	6.30 ± 0.32 ^{f}	4.6 ± 0.43 ^{d}	1.68 ± 0.06 ^{c}	124.4 ± 0.20 ^{d}	74.24 ± 4.8 ^{d}	29.4 ± 3.0 ^{g}	4.35 ± 0.11 ^{g}
	ZNPs200	4.17 ± 0.10 ^{i}	2.02 ± 0.06 ^{f}	4.4 ± 0.44 ^{f}	6.66 ± 0.41 ^{e}	3.8 ± 0.18 ^{e}	1.22 ± 0.06 ^{f}	117.0 ± 0.12 ^{f}	66.34 ± 4.4 ^{f}	27.1 ± 3.4 ^{h}	4.64 ± 0.17 ^{f}
Salinity level (S=50 mM NaCl)	Salinity (S)	4.51 ± 0.11 ^{h}	1.31 ± 0.1 ^{h}	4.43 ± 0.44 ^{f}	6.62 ± 0.11 ^{e}	1.2 ± 0.12 ^{g}	0.95 ± 0.11 ^{h}	106.2 ± 0.42 ^{h}	70.12 ± 0.63 ^{e}	41.9 ± 2.6 ^{c}	3.65 ± 0.36 ^{h}
	ZNPs25+S	5.22 ± 0.12 ^{g}	1.87 ± 0.1^{g}	3.46 ± 0.44 ^{h}	8.46 ± 0.13 ^{d}	4.3 ± 0.15 ^{de}	1.01 ± 0.02 ^{gh}	113.4 ± 0.20 ^{g}	74.23 ± 0.48 ^{d}	39.3 ± 3.0 ^{d}	5.35 ± 0.41 ^{e}
	ZNPs50+S	6.77 ± 0.15 ^{d}	2.43 ± 0.11 ^{c}	6.32 ± 0.68 ^{b}	9.78 ± 0.16 ^{c}	5.3 ± 0.11 ^{bc}	1.9 ± 0.15 ^{b}	122.0 ± 0.72 ^{e}	92.22 ± 0.40 ^{c}	55.6 ± 3.3 ^{b}	7.99 ± 0.46 ^{c}
	ZNPs100+S	5.55 ± 0.11 ^{f}	1.15 ± 0.12 ^{i}	3.6 ± 0.93 ^{g}	4.30 ± 0.33 ^{h}	4.6 ± 0.13 ^{d}	1.08 ± 0.03 ^{g}	107.0 ± 0.30 ^{h}	66.1 ± 1.0 ^{g}	37.6 ± 3.4 ^{e}	4.64 ± 0.47 ^{f}
	ZNPs200+S	2.11 ± 0.13 ^{j}	1.11 ± 0.12 ^{j}	3.4 ± 0.44 ^{h}	5.66 ± 0.23 ^{g}	2.4 ± 0.18 ^{f}	0.80 ± 0.04 ^{i}	103.0 ± 0.31 ^{i}	60.1 ± 0.31 ^{i}	29.2 ± 0.31 ^{g}	3.0 ± 0.31 ^{i}
	F-value	114655.2	14568.5	1794.3	8091.5	62.9	257.5	4036.3	252664.1	5384.5	15567.3
	LSD (0.05)	0.017	0.017	0.108	0.077	0.562	0.077	1.08	0.077	0.562	0.056
	Significance						*				

Values are the mean of three replicates ± SD. Values within the same column for each factor designated by different letters are significant at $p \leq 0.05$, while values with identical letters are non-significant

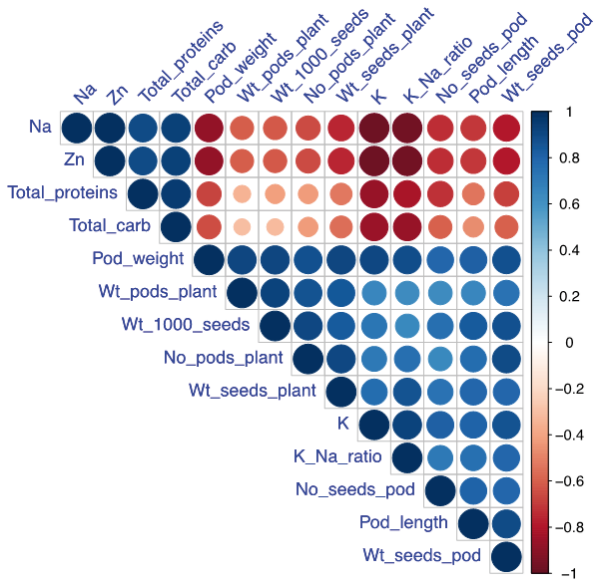


Fig. 4: Correlogram based Pearson correlation analysis of Na^+, K^+, K^+/Na^+, Zn and yield parameters of 90-day old soybean ('Giza 111') plants grown in clay-sandy soil (2:1 w/w) after soaking of soybean seeds in different concentrations of ZNPs (25, 50, 100, and 200 mg l^{-1}). On the right hand side of the correlogram, the legend color shows the correlation coefficients and the corresponding colors. The positive correlations are displayed in blue, while the negative correlations are shown in red. The color intensity and the size of the circle are proportional to the correlation coefficients

In this study, high salinity stress (250 mM NaCl) caused a highly significant increase in total soluble proteins and carbohydrates content by 76 % and 75 %, respectively, in salt-stressed soybean seedlings. Similar results were reported by Sadeghipour (2017) in *Vigna unguiculata* (L.), Karimi et al. (2019) in *Vitis vinifera* (L.), and Cardoso et al. (2019) in two varieties of cowpea. It is well known that osmotic stress induced by salt stress leads to the synthesis of proteins, which play an important role in plant salt tolerance through cytosolic calcium signal. This signal activates the calcium sensor protein for activation of the protein kinase to regulate Na^+/H^+ antiporter in plasma membranes and tonoplasts, thus the osmo-sensory histidine kinase regulates osmotic homeostasis and ROS scavenging (Chinnusamy et al., 2005; Abdel Latef et al., 2017). In addition, total soluble carbohydrates are key osmolytes in the osmotic adjustment of all plants, ROS scavenging, and maintaining ion homeostasis under salinity stress (Chen and Jiang, 2010) and have a direct relationship with physiological processes in plants (Tombesi et al., 2019).

In this study, treatment with ZNPs reduced total soluble proteins and total soluble carbohydrates content under salinity stress, and the highest reduction was recorded in the case of ZNPs50+S (50 mg l^{-1} + 250 mM Na Cl) with 43 % and 36 %, respectively. This result indicates that ZNPs alleviated the harmful impacts of salinity stress. The ZNPs treatment might cause an inhibition of oxidative stress, decreasing the content of Na^+ in the shoot tissues (Haidera et al., 2019). Indeed, ZNPs have been shown to increase CO_2 fixation, photosynthetic pigments, photosynthetic efficiency, and plant growth restoration in response to salt stress (Soliman et al., 2015; Kasim et al., 2017; Mathur et al., 2019).

In addition, in this study, the expression levels of three key salt-tolerance related genes (*GmCHX1*, *GmPAP3*, and *GmSALT3*) were determined under 250 mM of NaCl salt alone. The gene expression was increased for all three genes (*GmCHX1*, *GmPAP3*, and *GmSALT3*) by 1.9-, 3-, and 7.7-fold, respectively. Generally, stress results in changes in the cellular program that involve significant transcriptional alterations aimed at increasing the chances of survival (Diédhiou et al., 2008). A study by Dang et al. (2014) proved that overexpression of *GmPAP3* improved rice salt tolerance by increasing the ROS-scavenging ability and decreasing oxidative damage. Similarly, a possible tolerance role of *GmPAP3* under oxidative stress was demonstrated in soybean, indicating that the *GmPAP3* gene expression is regulated by salinity, osmotic, and oxidative stresses (Liao et al., 2003; Li et al., 2008b; Soleimani et al., 2017). It can be concluded that salinity induces the formation of ROS, which in turn activates *GmPAP3*, leading to an increase in ROS degradation till reaching the proper level in the mitochondria, at which point the activity of the *GmPAP3* gene is decreased (Francisca, 2005; Li et al., 2008a).

As mentioned above, the results of this study revealed that *GmCHX1* expression was increased under salinity stress by 1.9-fold, which is parallel to the results of Patil et al. (2016), who reported that salinity stress (200 mM NaCl) significantly induced the expression of the *GmCHX1* gene in soybean, maintaining ion homeostasis by lowering the Na^+/K^+ ratio. This result is also consistent with data from this study, which showed a high reduction in K^+/Na^+ ratio by 90 % compared to control. Furthermore, the *GmCHX1* gene was highly expressed in the leaves and roots of soybean seedlings in response to salinity stress (Do et al., 2016). It was reported that low Na^+ accumulation in shoot tissues of soybean plants may be due to the powerful function of the *GmCHX1* gene, which was highly expressed in salt-stressed soybean roots, forming Na^+ exclusion proteins in root tissues and preventing Na^+ entrance from soil to roots (Guan et al., 2014; Qu et al., 2020). This function of the *GmCHX1* gene has been documented in other plant species such as cotton (Wu et al., 2004), rice (Ren et al., 2005), *Arabidopsis* (Møller et al., 2009) and wheat (Munns et al., 2012).

Moreover, the results of this study indicated that *GmSALT3* gene expression was significantly increased in response to salinity stress by 7.7-fold in salt-stressed soybean seedlings. It was reported that the *GmSALT3* gene is the major salt tolerance gene in soybean belonging to the cation/H^+ exchanger (CHX) family (Patil et al., 2016), which is mainly expressed in root cells associated with the phloem and xylem, leading to limiting the accumulation of sodium ions in leaves (Pardo et al., 2006), which improved the physiological and morphological parameters and ultimately increased soybean yield under saline conditions (Do et al., 2016). As *GmSALT3* is localized in the endoplasmic reticulum (ER), it plays a direct role in the retrieval of salt from the xylem (Padmanaban et al., 2007; Cao et al., 2019). It has been reported that *GmSALT3* exerts a positive effect on soybean salt tolerance by exclusion of Na^+ in plant shoots and therefore prevents the toxic accumulation of Na^+ in photosynthetic tissues (Maathuis et al., 2014). Furthermore, Do et al. (2016) suggest that *CHX1/GmSALT3* controls Na^+, K^+, and Cl^- accumulation and may function as a cation-chloride co-transporter.

Application of nanoparticles alters the levels of expression of certain transcription factors, making it possible to modify plant tolerance to salinity stress (Yamaguchi et al., 2013). In particular, application of ZNPs could upregulate or downregulate the stress-tolerant genes depending on their function by cascade reactions, thereby enhancing salt tolerance (Jonak et al., 2002).

The results of this study showed that application of ZNPs in combination with salt-stress downregulated the expression of the three studied salinity-tolerant genes in soybean seedlings compared to salt-stressed ones. The expression of *GmCHX1, GmPAP3,* and *GmSALT3* was decreased by 0.4, 0.46, and 1.56-fold, respectively, particularly with 25 mg l^{-1} ZNPs in combination with high salinity stress (250 mM NaCl). Interestingly, this finding confirms the ameliorative role of ZNPs in improving soybean plant tolerance in response to salinity, which was reflected in enhancement effects on mineral uptake, total soluble proteins, total soluble carbohydrates, and yield characteristics. This finding is in accordance with that of Almutairi (2019) and Alharby et al. (2016) in tomato plants, where ZNPs imposed a positive response on plant metabolism under salt stress. It was reported that the differential response of *GmPAP3* expression in soybean to different ZNPs treatments under salinity stress could be as a result of reverted effects caused by NPs (Zhang et al., 2020) by excluding sodium ions from the roots, thus preventing the accumulation of toxic concentrations in the stem and leaves (Munns and Tester, 2008; Zhang et al., 2020).

5 CONCLUSIONS

The results of the present study indicated the importance of Zn^{+2} in increasing soybean tolerance to salt stress. Soaking seeds of soybean cultivar Giza 111 in ZNPs at 50 mg l^{-1} reduced oxidative damage caused by salinity stress, downregulated salt-tolerant gene expression, and increased soybean plant yield under high salinity stress (250 mM NaCl). Additionally, gene expression analysis of *GmCHX1, GmPAP3,* and *GmSALT3* confirmed their roles in salt tolerance in the soybean cultivar Giza 111. Moreover, the significant downregulation of these genes under combined treatments (250 mM NaCl and ZNPs) suggests that soybean plants favor ZNPs as an antioxidant. Therefore, application of low concentrations of ZNPs, particularly 50 mg l^{-1}, is recommended before planting as a nano-fertilizer stimulator and could be a strategy to energize the growth and economic yield in plants growing in salinized soils, where it increases the adaptation capability of soybeans under such conditions.

6 REFERENCES

Abdel Latef, A. A., Alhmad, M. F. A. and Abdelfattah, K. E. (2017). The possible roles of priming with ZnO nanoparticles in mitigation of salinity stress in lupine (*Lupinus termis*) plants. *Journal of Plant Growth Regulation*, *36*, 60-70. https://doi.org/10.1007/s00344-016-9618-x

Abreu, I. A., Farinha, A. P., Negrão, S., Gonçalves, N., Fonseca, C., Rodrigues, M. and Oliveira, M. M. (2013). Coping with abiotic stress: proteome changes for crop improvement. *Journal of Proteomics*, *93*, 145-168. https://doi.org/10.1016/j.jprot.2013.07.014

Ahanger, M. A., Tomar, N. S., Tittal, M., Argal, S. and Agarwal, R. M. (2017). Plant growth under water/salt stress: ROS production; antioxidants and significance of added potassium under such conditions. *Physiology and Molecular Biology of Plants*, *23*(4), 731-744. https://doi.org/10.1007/s12298-017-0462-7

Albert, I.L.; Nadassy, K.; Wodak, S.J. (1998). Analysis of zinc binding sites in protein crystal structures. *Protein Science*, *7*, 1700-1716. https://doi.org/10.1002/pro.5560070805

Alharby, H. F., Metwali, E. M., Fuller, M. P. and Aldhebiani, A. Y. (2016). The alteration of mRNA expression of SOD and GPX genes, and proteins in tomato (*Lycopersicon esculentum* Mill.,) under stress of NaCl and/or ZnO nanoparticles. *Saudi Journal of Biological Sciences*, *23*(6), 773-781. https://doi.org/10.1016/j.sjbs.2016.04.012

Ali, E. A. and Mahmoud, A. M. (2013). Effect of foliar spray by different salicylic acid and zinc concentration on seed yield components of mungbean in sandy soil. *Asian Journal of Crop Science*, *5*(1), 33-40. https://doi.org/10.3923/ajcs.2013.33.40

Allen, S.G., Girmshaw, H.M., Parkinson, J.A. and Quarmby, C.

(1974). *Chemical analysis of ecological materials*. Blackwell Scientific Publications, Oxford, London, 565.

Almutairi, Z. M. T. (2019). Plant molecular defense mechanisms promoted by nanoparticles against environmental stress. *International Journal of Agriculture and Biology, 21*, 259-270.

Bishop, O. N. (1983). *Statistics in biology*. Longman Penguin London, 56-63.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1-2), 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Cao, Y., Liang, X., Yin, P., Zhang, M. and Jiang, C. (2019). A domestication-associated reduction in K^+-preferring HKT transporter activity underlies maize shoot K^+ accumulation and salt tolerance. *New Phytologist*, *222*(1), 301-317. https://doi.org/10.1111/nph.15605

Cardoso, K. P. S., Conceição, S. S. and de Araújo Brito, A. E. (2019). Biochemical metabolism of two cultivars of cowpea treated with 24-Epibrassinolide and subjected to saline stress. *Australian Journal of Crop Science*, *13*(3), 444. https://doi.org/10.21475/ajcs.19.13.03.p1390

Chakraborty, K., Basak, N., Bhaduri, D., Ray, S., Vijayan, J., Chattopadhyay, K. and Sarkar, R. K. (2018). "Ionic basis of salt tolerance in plants: Nutritional homeostasis and oxidative stress tolerance," in *Plant Nutrients and Abiotic Stress Tolerance*, eds M. Hasanuzzaman, M. Fujita, H. Oku, K. Nahar, and B. Hawrylak-Nowak (Berlin: Springer), 325-362. https://doi.org/10.1007/978-981-10-9044-8_14

Chanroj, S., Lu, Y., Padmanaban, S., Nanatani, K., Uozumi, N., Rao, R., et al.(2011) Plant-specific cation/H^+ exchanger 17 and its homologs are endomembrane K^+ transporters with roles in protein sorting. *Journal of Biological Chemistry*, *286*, 33931-33941. https://doi.org/10.1074/jbc.M111.252650

Chen, C., Yu, Y., Ding, X., Liu, B., Duanmu, H., Zhu, D. and Zhu, Y. (2018). Genome-wide analysis and expression profiling of PP2C clade D under saline and alkali stresses in wild soybean and *Arabidopsis*. *Protoplasma*, *255*(2), 643-654. https://doi.org/10.1007/s00709-017-1172-2

Chen, H. and Jiang, J. G. (2010). Osmotic adjustment and plant adaptation to environmental changes related to drought and salinity. *Environmental Reviews*, *18*, 309-319. https://doi.org/10.1139/A10-014

Chen, K. I., Erh, M. H., Su, N. W., Liu, W. H., Chou, C. C. and Cheng, K. C. (2012). Soy foods and soybean products: from traditional use to modern applications. *Applied microbiology and biotechnology*, *96*(1), 9-22. https://doi.org/10.1007/s00253-012-4330-7

Chinnusamy, V., Jagendorf, A. and Zhu, J. K. (2005). Understanding and improving salt tolerance in plants. *Crop Science*, *45*(2), 437-448. https://doi.org/10.2135/cropsci2005.0437

Choudhury, F. K., Rivero, R. M., Blumwald, E. and Mittler, R. (2017). Reactive oxygen species, abiotic stress and stress combination. *The Plant Journal*, *90*(5), 856-867. https://doi.org/10.1111/tpj.13299

Dang, Z. H., Qi, Q., Zhang, H. R., Li, H. Y., Wu, S. B. and Wang, Y. C. (2014). Identification of salt-stress-induced genes from the RNA-Sequencing data of *Reaumuria trigyna* using differential-display reverse transcription PCR. *International Journal of Genomics*, *1-7*. https://doi.org/10.1155/2014/381501

Dewdar, M., Abbas, M., Hassanin, A. and Aleem, H. (2018). Effect of nano micronutrients and nitrogen foliar applications on sugar beet (*Beta vulgaris* L.) of quantity and quality traits in marginal soils in Egypt. *International Journal of Current Microbiology and Applied Sciences*, *7*(8), 4490-4498. https://doi.org/10.20546/ijcmas.2018.708.475

Diédhiou, C. J., Popova, O. V., Dietz, K. J. and Golldack, D. (2008). The SNF1-type serine-threonine protein kinase SAPK4 regulates stress-responsive gene expression in rice. *Biomed Central Plant Biology*, *8*(1), 1-13. https://doi.org/10.1186/1471-2229-8-49

Dimkpa, C. O. and Bindraban, P. S. (2016). Fortification of micronutrients for efficient agronomic production: a review. *Agronomy for Sustainable Development*, *36*(1), 7. https://doi.org/10.1007/s13593-015-0346-6

Do, T. D., Chen, H., Hien, V. T. T., Hamwieh, A., Yamada, T., Sato, T. and Xu, D. (2016). Ncl synchronously regulates Na^+, K^+, and Cl^- in soybean and greatly increases the grain yield in saline field conditions. *Scientific Reports*, *6*, 1-10. https://doi.org/10.1038/srep19147

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. T. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350-356. https://doi.org/10.1021/ac60111a017

El-Mashad, A. A. and Mohamed, H. I. (2012). Brassinolide alleviates salt stress and increases antioxidant activity of cowpea plants (*Vigna sinensis*). *Protoplasma*, *249*(3), 625-635. https://doi.org/10.1007/s00709-011-0300-7

El-Shaer, A., Abdelfatah, M., Basuni, A. and Mosaad, M. (2018). Effect of KOH molarity and annealing temperature on ZnO nanostructure properties. *Chinese Journal of Physics*, *56*(3), 1001-1009. https://doi.org/10.1016/j.cjph.2018.03.015

Farhangi-Abriz, S. and Torabian, S. (2018). Nano-silicon alters antioxidant activities of soybean seedlings under salt toxicity. *Protoplasma*, *255*(3), 953-962. https://doi.org/10.1007/s00709-017-1202-0

Fatima, U., Khan, M. F., Fatima, J., Shahab, U., Ahmad, S. and Yusuf, M. A. (2017). DNA damage, response, and repair in plants under genotoxic stress: stress signaling in plants. *Genomics and Proteomics Perspective, 2*, 151-171. https://doi.org/10.1007/978-3-319-42183-4_7

Fraceto, L. F., Grillo, R., de Medeiros, G. A., Scognamiglio, V., Rea, G. and Bartolucci, C. (2016). Nanotechnology in agriculture. *Frontiers in Environmental Science, 4*, 20. https://doi.org/10.3389/fenvs.2016.00020

Francisca, L. W. Y. (2005). *Characterization and Functional Studies of GmPAP3, a Novel Purple Acid Phosphatase-like Gene in Soybean Induced by NaCl Stress but not Phosphorus Deficiency* (Doctoral dissertation, The Chinese University of Hong Kong).

Gaafar R.M., Diab R., Halawa M., Elshanshory A., El-Shaer A., Hamouda A. (2020). Role of zinc oxide nanoparticles in ameliorating salt tolerance in soybean. *Egyptian Journal of Botany, 60*(3), 733-747. https://doi.org/10.21608/ejbo.2020.26415.1475

Guan, R., Chen, J., Jiang, J., Liu, G., Liu, Y., Tian, L. and Qiu, L. J. (2014). Mapping and validation of a dominant salt tolerance gene in the cultivated soybean (*Glycine max*) variety Tiefeng 8. *The Crop Journal*, *2*(6), 358-365. https://doi.org/10.1016/j.cj.2014.09.001

Haider, M. S., Jogaiah, S., Pervaiz, T., Yanxue, Z., Khan, N. and Fang, J. (2019). Physiological and transcriptional variations inducing complex adaptive mechanisms in grapevine by salt stress. *Environmental and Experimental Botany*, *162*, 455-467. https://doi.org/10.1016/j.envexpbot.2019.03.022

Haliloglu, K., Hosseinpour, A., Cinisli, K. T., Ozturk, H. I., Ozkan, G., Pour-Aboughadareh, A. and Poczai, P. (2020). Investigation of the protective roles of zinc oxide nanoparticles and plant growth promoting bacteria on DNA damage and methylation in tomato (*Solanum lycopersicum* L.) under salinity stress. *Horticulture Environment and Biotechnology*. *7*(2), 23-41.

Han, G., Yuan, F., Guo, J., Zhang, Y., Sui, N. and Wang, B. (2019). *AtSIZ1* improves salt tolerance by maintaining ionic homeostasis and osmotic balance in Arabidopsis. *Plant Science*, *285*, 55-67. https://doi.org/10.1016/j.plantsci.2019.05.002

Hauser, F. and Horie, T. (2010). A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K^+/Na^+ ratio in leaves during salinity stress. *Plant, Cell and Environment*, *33*(4), 552-565. https://doi.org/10.1111/j.1365-3040.2009.02056.x

He, X. C., Lin, M., Li, F., Sha, B. Y., Xu, F., Qu, Z. G. and Wang, L. (2015). Advances in studies of nanoparticle-biomembrane interactions. *Nanomedicine*, *10*(1), 121-141. https://doi.org/10.2217/nnm.14.167

Hezaveh, T. A., Pourakbar, L., Rahmani, F. and Alipour, H. (2019). Interactive effects of salinity and ZnO nanoparticles on physiological and molecular parameters of rapeseed (*Brassica napus* L.). *Communications in Soil Science and Plant Analysis*, *50*(6), 698-715. https://doi.org/10.1080/00103624.2019.1589481

Jonak, C., Ökrész, L., Bögre, L. and Hirt, H. (2002). Complexity, cross talk and integration of plant MAP kinase signaling. *Current Opinion in Plant Biology*, *5*(5), 415-424. https://doi.org/10.1016/S1369-5266(02)00285-6

Karimi, R., Mohammadparast, B. and Minazadeh, R. (2019). Phytochemical responses and antioxidant activity of potassium-treated grapevines (*Vitis vinifera* L.) in salinity stress condition. *Journal of Plant Process and Function*, 245-260.

Kasim, W. A. E. A., AboKassem, E. M. and Ragab, G. A. A. (2017). Ameliorative effect of yeast extract, IAA and green-synthesized nano zinc oxide on the growth of Cu-stressed *Vicia faba* seedlings. *Egyptian Journal of Botany*, 1-16. https://doi.org/10.21608/ejbo.2017.885.1065

Khan, M. S. A., Karim, M. A., Haque, M. M., Karim, A. J. M. S. and Mian, M. A. K. (2017). Effect of salt and water stress on gas exchange, dry matter production and K^+/Na^+ ions selectivity in soybean. *Bangladesh Journal of Agricultural Research*, *42*(3), 487-501. https://doi.org/10.3329/bjar.v42i3.34507

Landa, P., Prerostova, S., Petrova, S., Knirsch, V., Vankova, R. and Vanek, T. (2015). The transcriptomic response of *Arabidopsis thaliana* to zinc oxide: A comparison of the impact of nanoparticle, bulk, and ionic zinc. *Environmental Science and Technology*, *49*(24), 14537-14545. https://doi.org/10.1021/acs.est.5b03330

Li, C., Fang, G., Li, J., Ai, L., Dong, B. and Zhao, X. (2008a). Effect of seed layer on structural properties of ZnO nanorod arrays grown by vapor-phase transport. *The Journal of Physical Chemistry C*, *112*(4), 990-995. https://doi.org/10.1021/jp077133s

Li, W. Y. F., Shao, G. and Lam, H. M. (2008b). Ectopic expression of *GmPAP3* alleviates oxidative damage caused by salinity and osmotic stresses. *New Phytologist*, *178*(1), 80-91. https://doi.org/10.1111/j.1469-8137.2007.02356.x

Liao, H., Wong, F. L., Phang, T. H., Cheung, M. Y., Li, W. Y. F., Shao, G. and Lam, H. M. (2003). GmPAP3, a novel purple acid phosphatase-like gene in soybean induced by NaCl stress but not phosphorus deficiency. *Gene*, *318*, 103-111. https://doi.org/10.1016/S0378-1119(03)00764-9

Liao, Y., Zou, H. F., Wang, H. W., Zhang, W. K., Ma, B., Zhang, J. S. and Chen, S. Y. (2008). Soybean GmMYB76, GmMYB92, and GmMYB177 genes confer stress tolerance in transgenic *Arabidopsis* plants. *Cell Research*, *18*(10), 1047-1060. https://doi.org/10.1038/cr.2008.280

Linh TM, Mai NC, Hoe PT, Lien LQ, Ban NK, Hien LTT, Chau NH, Van NT. (2020). Metal-based nanoparticles enhance drought tolerance in soybean. *Journal of Nanomaterials*, 1-13. https://doi.org/10.1155/2020/4056563

Liu, H. R., Sun, G. W., Dong, L. J., Yang, L. Q., Yu, S. N., Zhang, S. L. and Liu, J. F. (2017). Physiological and molecular responses to drought and salinity in soybean. *The Plant Biology, 61*(3), 557-564. https://doi.org/10.1007/s10535-017-0703-1

Liu, X., Wang, F., Shi, Z., Tong R., Shi X. (2015).Bioavailability of Zn in ZnO nanoparticle-spiked soil and the implications to maize plants. *Journal of Nanoparticle Research, 17***,** 175. https://doi.org/10.1007/s11051-015-2989-2

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and $2^{-\Delta\Delta CT}$ method. *Methods*, *25*(4), 402-408. https://doi.org/10.1006/meth.2001.1262

Maathuis, F. J., Ahmad, I. and Patishtan, J. (2014). Regulation of Na^+ fluxes in plants. *Frontiers in Plant Science*,5, 1-9. https://doi.org/10.3389/fpls.2014.00467

Mathur, S., Raikalal, P. and Jajoo, A. (2019). Physiological responses of wheat to environmental stresses. *Wheat Production in Changing Environments*, 31-61. https://doi.org/10.1007/978-981-13-6883-7_2

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*, *7*(9), 405-410. https://doi.org/10.1016/S1360-1385(02)02312-9

Møller, I. S., Gilliham, M., Jha, D., Mayo, G. M., Roy, S. J., Coates, J. C. and Tester, M. (2009). Shoot Na^+ exclusion and increased salinity tolerance engineered by cell type-specific alteration of Na^+ transport in Arabidopsis. *The Plant Cell*, *21*(7), 2163-2178. https://doi.org/10.1105/tpc.108.064568

Munns, R. and Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, *59*, 651-8. https://doi.org/10.1146/annurev.arplant.59.032607.092911

Munns, R., James, R. A., Xu, B., Athman, A., Conn, S. J., Jordans, C. and Plett, D. (2012). Wheat grain yield on saline

soils is improved by an ancestral Na^+ transporter gene. *Nature Biotechnology*, *30*(4), 360-364. https://doi.org/10.1038/nbt.2120

Naguib, M. I., Youssef, E. and Kinawy, M. M. (1968). Effect of kinetin on the nitrogen metabolism of radish plants. *Bulletin of The Faculty of Science, Cairo University*, *42*, 13-22.

Olczak, M., Morawiecka, B. and Watorek, W. (2003). Plant purple acid phosphatases genes, structures and biological function. *Acta Biochimica Polonica*, *50*, 1245-1256. https://doi.org/10.18388/abp.2003_3648

Padmanaban, S., Chanroj, S., Kwak, J. M., Li, X., Ward, J. M. and Sze, H. (2007). Participation of endomembrane cation/H^+ exchanger AtCHX20 in osmoregulation of guard cells. *Plant Physiology*, *144*(1), 82-93. https://doi.org/10.1104/pp.106.092155

Pardo, J. M., Cubero, B., Leidi, E. O. and Quintero, F. J. (2006). Alkali cation exchangers: roles in cellular homeostasis and stress tolerance. *Journal of Experimental Botany*, *57*(5), 1181-1199. https://doi.org/10.1093/jxb/erj114

Patil, G., Do, T., Vuong, T. D., Valliyodan, B., Lee, J. D., Chaudhary, J. and Nguyen, H. T. (2016). Genomic-assisted haplotype analysis and the development of high-throughput SNP markers for salinity tolerance in soybean. *Scientific Reports*, *6*(1), 1-13. https://doi.org/10.1038/srep19199

Qadir, M., Quillérou, E., Nangia, V., Murtaza, G., Singh, M., Thomas, R. J. and Noble, A. D. (2014). Economics of salt-induced land degradation and restoration. *Natural Resources Forum*, *38*(4), 282-295. https://doi.org/10.1111/1477-8947.12054

Qi, X., Li, M. W., Xie, M., Liu, X., Ni, M., Shao, G., Song, C., and Yim, K.Y. (2014). Identification of a novel salt tolerance gene in wild soybean by whole-genome sequencing. *Nature Communications*, *5*, 4340. https://doi.org/10.1038/ncomms5340

Qu, Y., Guan, R., Bose, J., Henderson, S. W., Wege, S., Qiu, L. J. and Gilliham, M. (2020). *GmSALT3* confers shoot Na^+ and Cl^- exclusion in soybean via two distinct processes. *Frontiers in Plant Science*, 1-7.

Raeisi Sadati, S.Y., Godehkahriz S. J., Ebadi A. and Sedghi M. (2022). Zinc Oxide Nanoparticles Enhance Drought Tolerance in Wheat via Physio-Biochemical Changes and Stress Genes Expression. *Iranian Journal of Biotechnology*, *20*(1), e3027.

Ren, Z. H., Gao, J. P., Li, L. G., Cai, X. L., Huang, W., Chao, D. Y. and Lin, H. X. (2005). A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nature Genetics*, *37*(10), 1141-1146. https://doi.org/10.1038/ng1643

Rezaei, M. and Abbasi, H. (2014). Foliar application of nano-chelate and non-nanochelate of zinc on plant resistance physiological processes in cotton (*Gossipium hirsutum* L.). *Iranian Journal of Plant Physiology. 4*, 1137-1144.

Sadeghipour, O. (2017). Nitric oxide increases Pb tolerance by lowering Pb uptake and translocation as well as phytohormonal changes in cowpea (*Vigna unguiculata* (L.) Walp.). *Sains Malaysiana*, *46*(2), 189-195. https://doi.org/10.17576/jsm-2017-4602-02

Samei, M., Sarrafzadeh, M. H. and Faramarzi, M. A. (2019). The impact of morphology and size of zinc oxide nanoparticles on its toxicity to the freshwater microalga, *Raphidocelis subcapitata*. *Environmental Science and Pollution Research*, *26*(3), 2409-2420. https://doi.org/10.1007/s11356-018-3787-z

Selvaraj, K., and Dananjeyan, B. (2016). Expression of Zinc Transporter Genes in Rice as Influenced by Zinc-Solubilizing *Enterobacter cloacae* Strain ZSB14. *Frontiers in Plant Science*, *7*, 446. https://doi.org/10.3389/fpls.2016.00446

Soleimani, V., Ahmadi, J., Sadeghzadeh, B. and Golkari, S. (2017). The expression of *GmP5CS, GmPAP3,* and *GmBZIP50* genes under saline condition in soybean using real-time PCR. *Genetics*, *49*(2), 483-494. https://doi.org/10.2298/GENSR1702483S

Soliman, A. S., El-feky, S. A. and Darwish, E. (2015). Alleviation of salt stress on *Moringa peregrina* using foliar application of nanofertilizers. *Journal of Horticulture and Forestry, 7*, 36-47. https://doi.org/10.5897/JHF2014.0379

Taffouo, V. D., Kouamou, J. K., Ngalangue, L. M. T., Ndjeudji, B. A. N. and Akoa, A. (2009). Effects of salinity stress on growth, ions partitioning and yield of some cowpea (*Vigna unguiculata* L. Walp.) cultivars. *International Journal of Botany*, *5*(2), 135-143. https://doi.org/10.3923/ijb.2009.135.143

Tombesi, S., Cincera, I., Frioni, T., Ughini, V., Gatti, M., Palliotti, A. and Poni, S. (2019). Relationship among night temperature, carbohydrate translocation and inhibition of grapevine leaf photosynthesis. *Environmental and Experimental Botany*, *157*, 293-298. https://doi.org/10.1016/j.envexpbot.2018.10.023

Van Zanten, H. H., Mollenhorst, H., Klootwijk, C. W., Van Middelaar, C. E. and De Boer, I. J. (2016). Global food supply: land use efficiency of livestock systems. *The International Journal of Life Cycle Assessment*, *21*(5), 747-758. https://doi.org/10.1007/s11367-015-0944-1

Vogel, A., Spener, F. and Krebs, B. (2001). Purple acid phosphatase. *Handbook of metalloproteins*. Chichester, 752-766.

Weisany, W., Sohrabi, Y., Heidari, G., Siosemardeh, A. and Ghassemi-Golezani, K. (2012). Changes in antioxidant enzymes activity and plant performance by salinity stress and zinc application in soybean (*Glycine max* L.). *Plant Omics*, *5*(2), 60.

Wu, C. A., Yang, G. D., Meng, Q. W. and Zheng, C. C. (2004). The cotton *GhNHX1* gene encoding a novel putative tonoplast Na^+/H^+ antiporter plays an important role in salt stress. *Plant and Cell Physiology*, *45*(5), 600-607. https://doi.org/10.1093/pcp/pch071

Yamaguchi, T., Hamamoto, S. and Uozumi, N. (2013). Sodium transport system in plant cells. *Frontiers in Plant Science*, *4*, 410. https://doi.org/10.3389/fpls.2013.00410

Zhang, G., Chen, M., Li, L., Xu, Z., Chen, X., Guo, J. and Ma, Y. (2009). Overexpression of the soybean GmERF3 gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic tobacco. *Journal of Experimental Botany*, *60*(13), 3781-3796. https://doi.org/10.1093/jxb/erp214

Zhang, X., Zörb, C. and Geilfus, C. M. (2020). The root as a sink for chloride under NaCl-salinity. *Plant Physiology and Biochemistry*, *155*,161-168. https://doi.org/10.1016/j.plaphy.2020.06.036

doi:10.14720/aas.2022.118.3.2589 Original research article / izvirni znanstveni članek

Genome wide identification of AGC kinase genes and their expression in response to heat and cold stresses in barley

Zohreh HAJIBARAT [1], Abbas SAIDI [1, 2]

Received March 07, 2022; accepted July 26, 2022.
Delo je prispelo 7. marca 2022, sprejeto 26. julija 2022

Genome wide identification of AGC kinase genes and their expression in response to heat and cold stresses in barley

Abstract: AGC kinases are highly conserved regulators in a variety of cellular processes such as differentiation, proliferation, and growth. They are known to play important roles in stress and hormonal responses, including ROS signaling. AGC kinases are the main class of protein kinases in plants, having central functions in different stages of plant growth. In the present study, the analysis of phylogenetic relationships, gene structures, chromosomal locations, synteny analysis, gene ontology, subcellular localization, and gene expression of AGC kinase identified 28 AGC kinase genes in barley. Phylogenetic tree grouped them into seven subfamilies, as supported by exon-intron organization. Gene duplication and synteny indicated that tandom and block duplication events played an essential role in the expansion of AGC kinase gene families in barley. The Real-time quantitative reverse transcription PCR (qRT-PCR) analysis performed for *HvAGC kinase gene* were largely expressed in different tissues of roots, stems, and leaves in Azaran and Jolgeh cultivars under heat and cold stresses. The results of chromosomal localization showed that the AGC kinases were located on all chromosomes of barley except chromosome 1. Genome evolution of species was surveyed using identification of orthologous and paralogous genes. Identifying overlaps between orthologous clusters can enable us to study the function and evolution of proteins in different species. To our knowledge, this is the first detailed report of using AGC kinases for bioinformatics analysis in barley. Results revealed a broad understanding of the AGC kinase gene family in barley, which will be valuable for improving barley varieties' response to heat and cold stresses. Also, *HvNDR6.2* gene can utilized as molecular markers under cold stress in the three organs.

Key words: AGC kinase protein; protein model; synteny; gene duplication

Identifikacija genov na ravni celotnega genoma za kinaze AGC in njihovo izražanje kot odziv na vročinski in hladni stres pri ječmenu

Izvleček: Kinaze AGC so v veliki meri ohranjeni regulatorji različnih celični procesov kot so diferenciacija, proliferacina in rast. Znano je, da imajo pomembne vloge pri stresnih in hormonskih odzivih, vključno s signalizacijo ROS. Kinaze AGC so glavna skupina proteinskih kinaz v rastlinah, ki imajo osrednjo vlogo v razlilčnih fazah rasti rastlin. V tej raziskavi je bilo pri ječmenu na osnovi analize filogenetskih odnosov, genskih struktur, kromosomskih lokacij, analize sintenije in genske ontologije, njihove subcelularne lokalizacije in izražanja genov kinaz AGC identificiranih 28 genov kinaz AGC. Filogenetsko drevo jih je na osnovi organizacije intronov in eksonov porazdelilo v sedem poddružin. Podvojevanje genov in sintenija sta pokazali, da sta imela pri ječmenu tandemsko in bločno podvojevanje odločilno vlogo pri ekspanziji družin genov za kinaze AGC. Analiza kvantitativne reverzne transkripcije PCR v realnem času (qRT-PCR) opravljene za gene *kinase HvAGC je pokazala, da so se ti geni v veliki meri izrazili v različnih tkivih korenin, stebla in listov pri sortah* Azaran in Jolgeh v razmerah vročinskega in hladnega stresa. Rezultati kromosomske lokalizacije so pokazali, da so bili geni za kinase AGC pri ječmenu locirani na vseh kromosomih, razen na kromosomu 1. Evolucija genoma ječmena je bila preučena z identifikacijo ortolognih in paralognih genov. Prepoznavanje prekrivanj med skupinami ortolognih genov omogoča preučevanje funkcije in razvoja proteinov pri različnih vrstah. Glede na vedenje avtorjev je to prvo podrobnejše poročanje o uporabi kinaz AGC z analizo bioinfomatskih pristopov pri ječmenu. Rezulati so odkrili veliki pomen družin genov za kinaze AGC pri ječmenu, kar bo pomembno za izboljšanje sort ječmena pri odzivu na vročinski in hladni stres. Gen *HvNDR6.2* bi lahko uporabili kot molekularni marker odziva pri hladnem stresu v koreninah, steblu in listih.

Ključne besede: AGC proteinske kinaze; nabor proteinov; sintenija; podvajanje genov

1 Department of Plant Sciences and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran
2 Corresponding author, e-mail: abbas.saidi@gmail.com

1 INTRODUCTION

Protein kinases catalyze the transfer of phosphoryl group from adenosine triphosphate (ATP) to some amino acids such as (serine, threonine) in their substrate proteins. Most protein kinases, like AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family) AGC kinase(, have seven subfamilies which have been reported in plants and bacteria. Protein phosphorylation is one of the most important post-translational modifications (PTMs) for cellular signaling, mediated by a group of enzymes called protein kinases (Bradley and Beltrao, 2019). The activity of these enzymes lead to the regulation of almost all cellular processes. This is achieved at any time by a network of different kinases that are transiently active. Therefore, control of cellular systems requires that each kinase targets only a limited set of substrates. AGC kinases are divided into seven groups based on sequence similarity, evolutionary conservation, and known functions (Bradley and Beltrao, 2019). Seven subgroups of AGC kinase (AGC1, AGC2, PDK-1, S6K, IRE, NDR, AGC2 related subfamilies) are common to eukaryotic genomes of animals, plants, and diatoms. AGC kinases are termed after the cyclic AMP dependent kinases (PKA), cGMP-dependent kinases, and the diacylglycerol-activated/phospholipid-dependent kinase PKC. Details and biochemical properties of 28 AGC kinase genes in barley are given in Table 1. The member of the 3-phosphoinositide dependent protein kinase 1 (PDK1) genes are highly conserved among eukaryotes (Mora et al., 2004). Furthermore, orthologous of the p70 ribosomal protein S6 kinase (S6K), the nuclear Dbf2-related (NDR) kinase subfamily identified in *Arabidopsis* (Galvan-Ampudia and Offringa, 2007). Based on amino acid sequence homology, AGC1-4 were placed in AGCVIII kinases in *Arabidopsis,* implicated in the regulation of cell polarity, growth, and cell division. AGCVIII kinases have conserved domains and are most associated to animal PKA and PKC, playing a key role in developmental stages and stress responses. In AGC2 related subfamily, two phototropin genes (PHOT1 and PHOT2) were expressed in plant seeds (Galvan-Ampudia and Offringa, 2007). Most protein kinases regulate cell growth and division in embryo, cotyledons, floral organs, and stress signaling (Rentel et al., 2004). In AGC1 subfamily, PINOID (PID) gene has been revealed to regulate the polarity of auxin transport by phosphorylating the large central hydrophilic loop of auxin efflux carriers (Dhonukshe et al., 2010; Huang et al., 2010). AGC kinases have multiple functions in different biological processes such as pollen germination and development and plant growth and development. Also, AGC kinase genes can be utilized in different abiotic and biotic stresses. Different function of proteins could be due to the presence of conserved domains or gene duplication (Saidi and Hajibarat 2020a). In this study, comprehensive analysis of phylogeny of AGC kinase genes, gene structures, gene duplications, synteny analysis, gene ontology, chromosomal distribution of AGC kinases were further performed. Gene expression of five AGC kinase genes were analyzed in response to heat and cold stresses in three tissues.

2 MATERIAL AND METHODS

2.1 PHYSICOCHEMICAL CHARACTERISTICS, PHYLOGENETIC ANALYSIS, AND GENE STRUCTURE OF AGC KINASE PROTEINS

The ensemble plant database was utilized to download the sequences of AGC kinase family genes from barley (*H. vulgare L), w*ild cabbage *(B. oleraceae L.), r*apeseed *(B. napus L.), f*ield mustard *(B. rapa L.), maize (Z. mays L.), Arabidopsis, and rice (O. sativa L.)*. ExPASy server (https://www.expasy.org/) was used to predict the theoretical isoelectric point (pI) and the molecular mass (Mm) of each of the AGC kinase proteins. AGC kinase protein sequences were aligned in seven species using the *MUSCLE* and phylogenetic tree was drawn using MEGA 7, applying the Neighborjoining algorithm. The Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/) was used to obtain information on the exon – intron of AGC kinase proteins. The prediction subcellular localization of AGC kinase was performed using CELLO database.

2.2 CHROMOSOMAL DISTRIBUTION AND GENE ONTOLOGY (GO) ANALYSIS AND DETECTION OF ORTHOLOGOUS AND PARALOGOUS OF AGC KINASE GENES

For locating the AGC kinase genes on barley chromosome, AGC kinase genes were placed on each chromosome according to the physical location of the gene. The AGC kinase genes were distributed on all chromosomes and depicted with MapChart (Voorrips, 2002). For functional annotation using default parameters, the nucleotide sequences of DEGs were submitted to the online annotation tool of Mapman (http://www.plabipd.de/portal/mercator-sequence-annotation) (Thimm et al., 2004). The orthologous and paralogous genes among species were identified using Blastp of proteins (Altschul et al., 1990). When the protein sequence identity exceeded 70 %, it was considered orthologous genes whereas, when the AGC kinase protein sequences identity exceed-

Table 1: Details and Biochemical properties of AGC kinase genes in barley

Protein Name	Genomic Locus	Duplication	Chromosome location	Number of amino acids	Molecular weight	Theoretical pI	Subcellular localization
HORVU2Hr1G072580	HvAGC1.8		chr2H : 520772898-520775368	384	42.4	5.61	Cytoplasmic
HORVU3Hr1G082260	HvPDPK1		chr3H:598502260-598506773	481	53.7	6.17	Mitochondrial
HORVU3Hr1G024030	HvAGC1.10		chr3H : 90576271-90577182	1315	145	6.36	Cytoplasmic
HORVU4Hr1G062610	HvAGC1.11		chr4H : 523887114-523888691	417	47.7	6.03	Cytoplasmic
HORVU3Hr1G031930	HvPHOT1		chr3H : 160765754-160767689	527	60.4	5.9	Cytoplasmic
HORVU4Hr1G007540	HvIREH1		chr4H : 19756134-19766838	320	45	5.7	Mitochondrial
HORVU4Hr1G062610	HvPK3		chr4H : 523887114-523888691	464	53.8	8.05	Cytoplasmic
HORVU5Hr1G108690	HvAGC1.5	Block duplicate	chr5H : 628434675-628436329	692	75.4	6.39	Cytoplasmic
HORVU5Hr1G041960	HvAGC1.12		chr5H : 318743447-318747958	670	71.6	5.99	Cytoplasmic
HORVU1Hr1G030190	HvNDR6		chr1H : 172906058-172908519	427	45.2	9.09	Cytoplasmic
HORVU1Hr1G077430	HvNDR7		chr1H : 517244865-517251727	281	30	5.8	Cytoplasmic
HORVU1Hr1G031420	HvAGC1.7		chr1H : 188829523-188831273	470	51.6	9.78	Cytoplasmic
HORVU2Hr1G072580	HvKIPK		chr2H : 520772898-520775368	465	50.6	9.64	Cytoplasmic
HORVU2Hr1G093580	HvAGC1.3.1		chr2H : 658836435-658839125	123	13.7	9.99	Cytoplasmic
HORVU5Hr1G035610	HvKIPK.1		chr5H : 248570403-248571724	337	37.3	9.19	Cytoplasmic
HORVU5Hr1G072930	HvAGC2.4		chr5H : 537104020-537105417	512	55.2	9.27	Cytoplasmic
HORVU0Hr1G005020	HvAGC2.2		chr6h : 28702476-28703321	337	37.3	9.19	Cytoplasmic
HORVU6Hr1G062330	HvAGC1.3		chr6H : 417602550-417605458	340	38	9.2	Cytoplasmic
HORVU5Hr1G009390	HvPID		chr5H : 21969701-21971152	555	60.3	6.23	Cytoplasmic
HORVU6Hr1G054770	HvAGC2.1		chr6H : 347811467-347812777	783	85.9	9.25	Cytoplasmic
HORVU7Hr1G050240	HvAGC2.4		chr7H : 180272159-180273571	790	86	9.3	Cytoplasmic
HORVU7Hr1G050240	HvAGC2.3		chr7H : 180272159-180273571	529	58.3	7.1	Cytoplasmic
HORVU3Hr1G020020	HvNDR4	Block duplicate	chr3H : 60417083-60425196	525	57	9.22	Cytoplasmic
HORVU3Hr1G020020	HvNDR6.1	Block duplicate	chr3H : 60417083-60425196	527	56.5	8.07	Cytoplasmic
HORVU3Hr1G031930	HvAGC1.6		chr3H : 160765754-160767689	525	56.9	9.22	Cytoplasmic
HORVU4Hr1G050660	HvS6K2		Chr3 : 2648625-2650407	483	52	9.46	Mitochondrial
HORVU3Hr1G031930	HvAGC1.5.1		chr3H : 160765754-160767689	783	85.4	9.3	Cytoplasmic
HORVU1Hr1G027770	HvNDR6.2	Block duplicate	chr1H : 143842779-143854406	350	37.6	6.33	Cytoplasmic

ed 85 %, it was considered paralogous genes. The analysis of synteny of AGC kinase genes were performed using Circos program (http://mkweb.bcgsc.ca/tableviewer/visualize/). PLAZZA was used to detect the duplication patterns containing segmental/tandem duplications (Wang et al., 2009). Identification of orthologous clustering between AGC kinase members of *Arabidopsis thaliana*, (L.) Heynh. rice, and barley was performed using OrthoVenn2 (https://orthovenn2.bioinfotoolkits.net/home) webserver.

2.3 BARLEY GROWTH UNDER HEAT AND COLD TREATMENTS

This study was done based on a randomized complete block design (RCBD) with three replications at the Seed and Plant Improvement Institute, Karaj (latitude 35 °, longitude 50 ° and altitude 1313 m above sea level) during 2021. Azaran and Jolgeh cultivars were cultured in pots at 25 °C for two weeks. Young roots, stems, and leaves from 2-week-old seedlings were harvested for tissue-specific

expression analysis under cold or heat stress, treated for four hours at 4 °C or 42 °C, respectively.

2.4 RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR (QRT-PCR) ANALYSIS

Total RNA was extracted from root, stem, and leaf under stress conditions using RNA-Plus kit (*Sinaclone*) based on the manufacturer's instructions. For the preparation of tissue-specific RNA, the organ samples were collected separately two week-old seedlings under cold (4 °C) and heat (42 °C) stresses for 4 hrs. To remove residual genomic DNA contamination in RNA samples, DNase I (Fermentase Company) was utilized. The purity and concentration of RNA were determined by nanodrop as well as the quality of which was validated using 1 % agarose gel analysis. Then, cDNA synthesis was performed according to Easy cDNA Synthesis Kit instructions. Three replications were performed for the analysis of each gene, with the barley Actin gene utilized as the reference gene. All primers used in gene expression analysis are listed in Table 2. Primers were designed using the Oligo program. Real time (qPCR) was done on ABI 7500 using SYBR Green Supermix as described in the manufacture's guidelines. Relative expression was determined via $2^{-\Delta\Delta Ct}$ technique after normalization of the Ct value for individual genes versus Actin as the reference gene. Analysis of gene expression was performed using the REST software (according to the Pfaffl method). RT-qPCR was conducted to determine the expression profile for the five AGC kinase genes using various tissues under heat and cold stresses. RT-qPCR expression analysis was carried out using our established protocol.

Table 2: Primers used for AGC kinase genes in this study

No.	Primer Name	Sequence 5'→ 3'
1	HvNDR4F	TGGCTTCGTCTGGACAACCTGCT
	HvNDR4R	CTTGGTTGGAACACGCTACACC
2	HvNDR6F	GAGGAGTTGTATACACCACC
	HvNDR6R	ACGTCGCATCCCTTTGCTCA
3	HvNDR6.2F	CCGATGAGTCCATAACCCGGCG
	HvNDR6.2R	GGATTTCTACAAACTGCTCGCC
4	HvAGC1.5F	AAGCAACACCCCTTCTTCGAG
	HvAGC1.5R	GTTCTAGAAATACTCGAACTGGCC
5	HvAGC1.5.1F	AGATCAAGCAGCACCCCTTC
	HvAGC1.5.1R	CGCTGTGGTCTAAAAGAACTCG
6	Actin	F: GGTCCATCCTAGCCTCACTC
	Actin	R: GATAACAGCAGTGGAGCGCT

3 RESULTS AND DISCUSSION

In the present study, AGC kinase genes in barley were surveyed using genome-wide identification, chromosomal distribution, evolutionary relationships, synteny analysis, and gene structure. Detailed information including the biochemical properties of the 28 AGC kinase genes are listed in Table 1. Sequence analysis showed that the lengths of the deduced AGC kinase proteins varied from 123 amino acids (HvAGC1.3.1) to 1315 amino acids (HvAGC1.10). The predicted molecular weights (MW) and isoelectric points (pI) ranged from 13.7 kDa (HvAGC1.3.1) to 85.9 kDa (HvAGC2.1) and from 5.61 (HvAGC1.8) to 9.99 (HvAGC1.3.1) (Table 1). AGC kinase proteins grouped into the same subfamily exhibited similar motif distributions, suggesting functional similarities for the members in the same subfamily. In addition, same roles of AGC kinases in various plant species that showed differential aspects of AGC kinase functionality in species. For instance, in contrast to the reported conserved functions of many AGC kinases, INCOMPLETE ROOT HAIR ELONGATION (IRE), a kinase of the "AGC other" group, seems to have acquired a new function in *Medicago Truncatula* Gaertn.. In *Arabidopsis,* IRE kinase has been revealed to control root hair elongation, while in*Medicago* a role in the formation of nodules has been indicated (Pislariu and Dickstein, 2007; Oyama et al., 2002; Saidi and Hajibarat, 2021a). Most of AGC kinase genes were located in the cytoplasm, but HvPDPK1, HvIREH1 and HvS6K2 genes were located in the mitochondria.

3.1 PHYLOGENETIC ANALYSIS AND GENE STUCRURE

To assess the evolutionary relationships of AGC kianse proteins in *H. vulgare, B.oleraceae, B.napus. B.rapa, Z.mays, Arabidopis, and O.sativa.* used to discribe phylogenetic analysis using MEGA7 based on protein sequences (Figure 1). The phylogenetic relationships and exon/intron analysis of the barley were showed in Figure 1. The HvAGC from barley was distributed in all groups, proposing that the expansion of HvAGC occurred in barley genome. Many researchers have revealed that the AGC kinase genes from monocots, based on their domains, could be grouped into 7 subfamilies (Kong et al., 2021). The genes within each subfamily showed similar gene structures. Gene structure of AGC kinase genes in barley was grouped into 7 subfamilies, with the largest cluster related to AGC-1 subfamily. The smallest cluster was related to PDK-1 and SK6 subfamilies. The num-

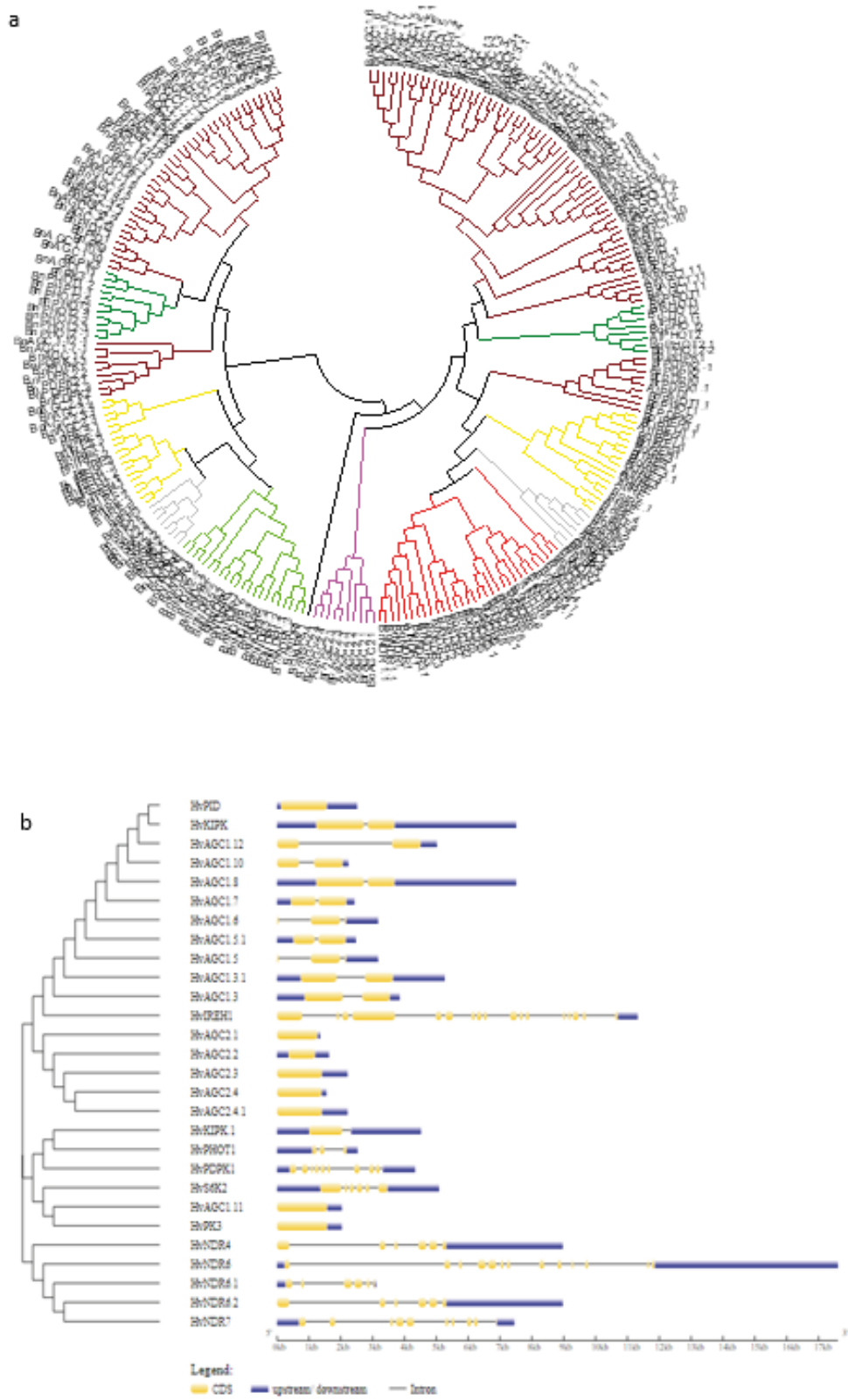


Figure 1: Phylogenetic tree of AGC kinase genes created by the neighbor-joining (NJ) method (a) in MEGA7.0 software in *Arabidopsis,* rice, barley, *Brassica napus, Brassica rapa,* and *Brassica oleracea*. The tree was constructed using the MEGA 6.0 software by the Neighbor joining method. Distributions of the exon- intron pattern in AGC kinase proteins in barley (b)

ber of exons in AGC kinase genes ranged from one to 17. The HvNDR6 and HvIREH1 genes had the highest number of exons. Some AGC kinases such as HvAGC2.1 and HvKIPK.1 genes contained only one exon, indicating that these genes have conserved domains. The gene structure of the AGC kinase genes in the same subfamily is highly consistent (Figure 1b). Most of the AGC kinase genes were grouped in the same subfamily. Most genes had many exons with introns, indicating that patterns of exons and introns, which correlate well with the phylogenetic tree, support their close evolutionary relationships between the AGC kinase genes within the same subfamilies.

The AGC2.1, also known as OXI1, was revealed to be a prerequisite for ROS-mediated responses in *Arabidopsis* like root hair elongation and for resistance to biotrophic pathogens. The activity of OXI1 was enhanced through H_2O_2, wounding, and various elicitor treatments mimicking pathogen attack (Rentel et al., 2004; Petersen et al., 2009). Also, as *oxi1* mutant plants are impaired in the activation of mitogen-activated protein kinase (MPK) and MPK6 in response to cellular injury and oxidative stress, OXI1 is a regulator of stress-responsive MPKs although its mechanism is still unclear. *PDK1* is a key factor involved in stress signaling (Petersen et al., 2009). *PDK1* encodes a gene of the AGC protein kinase family and a significant regulator of AGC kinases. PDK1, detected in mammalian cells, has an essential role in relating lipid signaling to a comprehensive range of cellular signaling and processes (Kyoko et al., 1989). Also, it is involved in the advancement of cell proliferation and survival and is overexpressed in many different tumors (Toda et al., 1988; Saidi and Hajibarat, 2021b). PDK1 contains an N-terminal kinase domain and a C-terminal pleckstrin homology (PH) domain through which it binds to phospholipids (Krupnick et al., 1998).

3.2 CHROMOSOMAL DISTRIBUTION AND DUPLICATION OF AGC KINASE GENES

Anslysis of physical locations on barley chromosomes preseneted that 28 AGC kinase genes were drawn using Mapchart software (Figure 2). In barley, three AGC kinase genes were located on both chromosomes 2 and 6. Six and 10 HvAGC were located on the chromo-

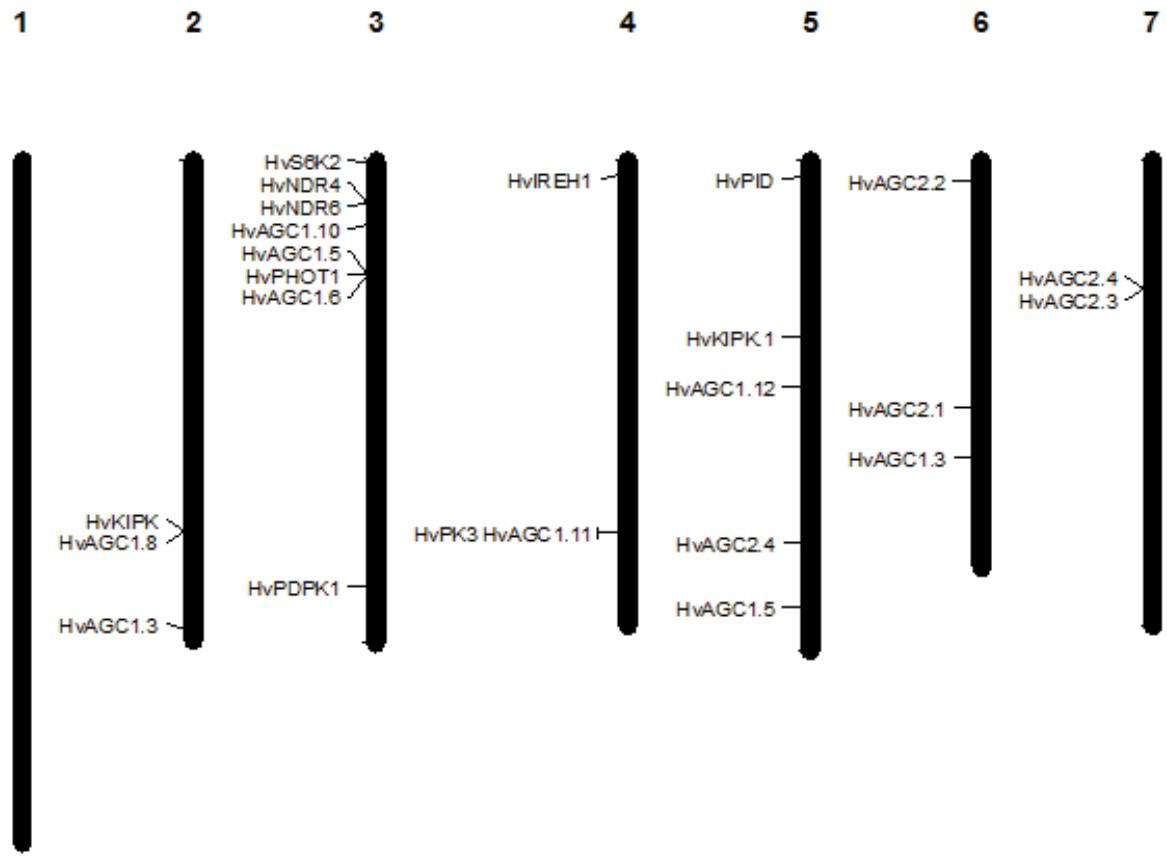


Figure 2: Chromosomal distribution and expansion patterns of AGC kinase genes in barley, drawn using Mapchart software

somes 2 and 3, respectively. Finally, nine HvAGC were distributed on chromosome 5. Our findings showed that the *Hv*AGC genes are unevenly distributed on different chromosomes. Based on the research findings on rice, *Arabidopsis* and *Brachypodium distachyon* (L.) P.Beauv., it has been shown that AGC kinase gene families mainly expanded through whole-genome and chromosomal segment duplications (Xue et al., 2008; Yang et al., 2008). Genes located within a distance of less than 200 kb on the same chromosome are defined as tandem duplications, otherwise they are segmental duplications (Cheung et al., 2003). In barley, 11 pairs of HvAGC duplication genes were involved in tandem duplication events and no gene segmental duplication pairs were found (Figure 2).

3.3 ORTHOLOGOUS AND PARALOGOUS GENES STUDY IN AGC KINASE

In this study, a comparative analysis was done to detect the orthologous of AGC kinase genes in barley genome (Figure 3). Based on the gene identity, orthologous (exceeding 70 %) and paralogous (exceeding 85 %) gene pairs were revealed. Among 28 genes, one gene was paralogous. According to our results, high similarity in barley genes suggests genome duplication (polyploidy), playing a key role in the evolution of AGC kinase genes. The comparative analysis to identify orthologous of AGC kinase genes in barley genome showed that the HvNDR4 with HvNDR6 genes had high similarity (identity 70 %) was orthologous one gene pair and HvNDR6.2 with HvNDR4 genes was paralogous (Figure 3). The syntenic analysis indicated that duplications as main elements for the diversity in AGC kinase genes, suggesting the structural and functional conservation of the genes underlying the origins of evolutionary with conserved domains (Altenhoff and Dessimoz, 2009). Often orthologous genes have similar expression among various species, while paralogous genes have the same basic but slightly different functions. It has been shown that gene duplication is collectively deemed from single-gene duplications (Saidi et al., 2020b). The structural conservation of NDR proteins indicates that they may had similar functions and regulatory mechanisms in various species. Surveys have suggested that NDRs are the main factors of the signaling mechanism in yeast and human (Hergovich, 2016).

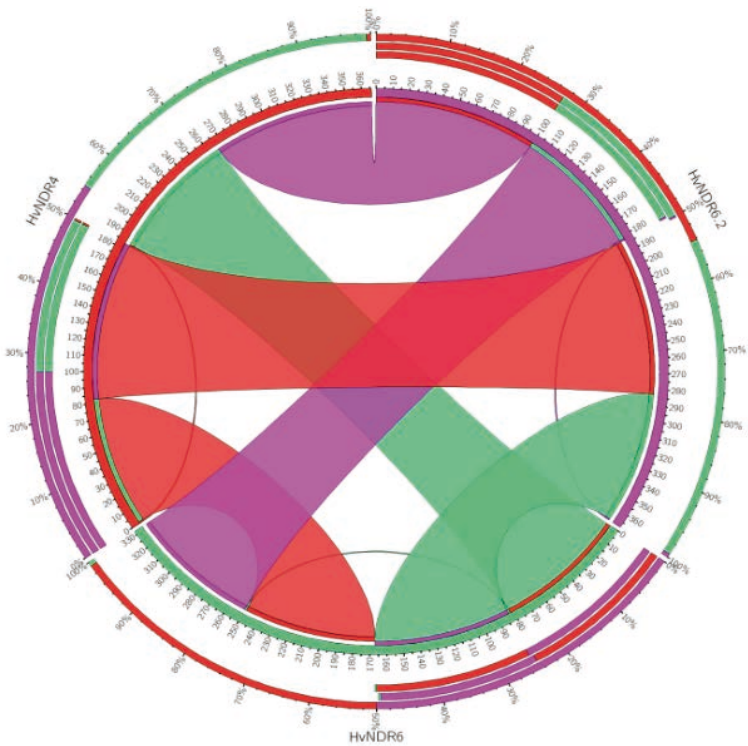


Figure 3: Orthologous and paralogous relationships of AGC kinase genes with three genomes visualized by Circos database in barley

3.4 ORTHOLOGOUS GENE CLUSTERING OF AGC KINASE GENE FAMILY IN *ARABIDOPSIS*, RICE, AND BARLEY

OrthoVenn2 web server was utilized to identify the evolutionary relationship of AGC kinases between dicot (*A. thaliana*) and monocot (*O. sativa*, and *H.vulgare*) plants using orthology analysis. In this study, 86 AGC kinase proteins from three species were clustered in 18 orthologous groups. One monocot-specific cluster was observed containing two AGC kinase proteins, whereas seven AGC kinase proteins were found to be present in two dicot-monocot orthologous clusters in *Arabidopsis* and barley (Fig 4). Also, 18 AGC kinase proteins were found to be present in nine dicot-monocot orthologous clusters in *Arabidopsis* and rice. One cluster containing two AGC kinase proteins has been identified between rice and barley (Figure 4). Interestingly, the OrthoVenn2 analysis also indicated the presence of an *Arabidopsis*-specific orthologous cluster containing two AGC kinases (Figure 7a). A singleton is a rare variant for which genetic difference is performed by a single chromosome in a genome. Among the three species, *Arabidopsis* had the highest number of singletons (54). Singletons usually are dispersed in the genome, indicating that they have been developed independently.

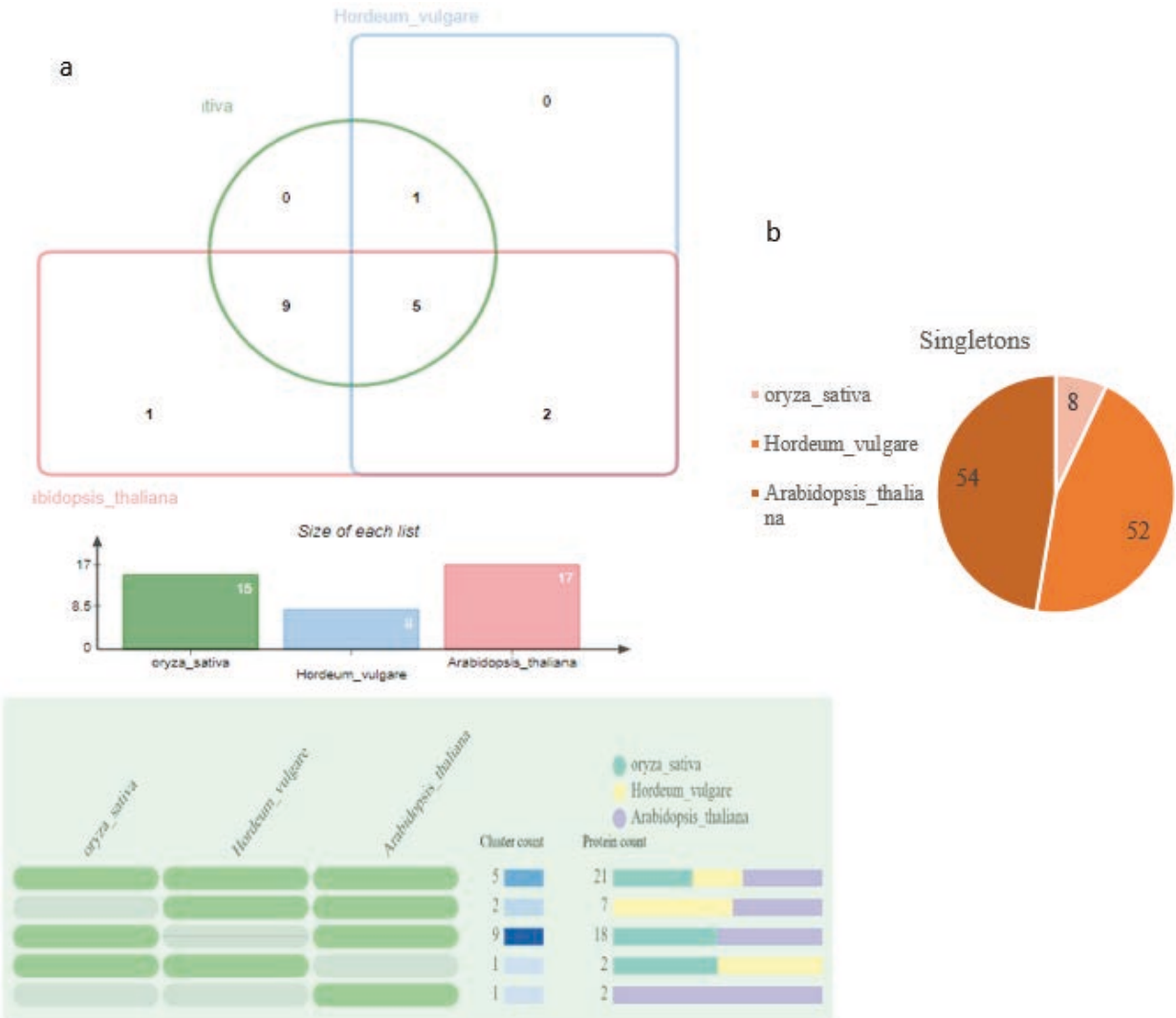


Figure 4 a: Orthologous gene clustering analysis. The orthologous gene clusters among the AGC kinase gene families in *A. thaliana, O. sativa,* and *H. vulgare* were identified and visualized using the OrthoVenn2 web platform. The e-value cut-off 1e-10 was used for the analysis. **b.** Number of singletons identified in *A. thaliana, O. sativa,* and *H. vulgare*

3.5 EXPRESSION PROFILES ANALYSIS OF AGC KIANASE GENES IN SPECIFIC TISSUES UNDER COLD AND HEAT STRESS CONDITIONS

In Azaran cultivar, most of AGC kinase genes showed a wide range of expression levels under heat and cold stress conditions. The expression profile of 5 AGC kinase genes under heat and cold stress conditions were performed using reverse transcription-PCR (qRT-PCR) quantitative analysis in three different tissues: root, stem, and leaf. Most of genes showed increased expression under heat stress as compared to cold stress and were expressed in the leaves showed increased expression, indicating that the AGC kinase genes were involved in leaf development. AGC kinase genes were decreased in cold stress but were increased in response to heat stress. The HvNDR6.2 gene showed high expression in root, shoot, and leaf tissues under heat stress, while it was down-regulated in response to cold (Figure 5a).

In Jolgeh cultivar, AGC kinase genes showed different expressions in response to cold and heat stresses. The HvNDFR4 gene was up-regulated in roots under heat stress but was down-regulated under cold stress condition. In Jolgeh cultivar, most genes were down-regulated in response to heat and cold stresses. Only the HvNDR6.2 gene was up-regulated in leaves and stems under heat stress. The HvNDR6.2 gene showed up-regulated expression in roots, stems, and leaves under cold stress. Most genes showed high levels of relative expression under normal conditions in leaves. But AGC kinase genes was down-regulated under cold stress. The HvNDR4 gene had high expression in roots under heat condition. These results showed that the HvNDR4 gene can be used as a molecular marker in barley root improvement under heat and cold stresses. Also, the HvNDR6.2 gene can be utilized as a molecular marker under cold stress in all three tissues (Figure 5b). In our analysis, the expression profiles of AGC kinase genes under heat and cold stresses revealed differential and overlapping expression patterns. Various expression patterns of AGC kinase genes may suggest various roles in response to heat and cold stress conditions. Based on the synthetic analysis of the AGC kinase genes, the HvNDR4 and HvNDR6 genes were orthologous, indicating that the orthologous genes had similar expression. As a result, the HvNDR4 and HvNDR6 genes can respond almost identically under heat and cold stress conditions.

3.6 GENE ONTOLOGY ANALYSIS

In barley, the largest percentage of detected proteins was involved in protein metabolism (90.91 %) followed by hormonal metabolism (6.06 %) (Figure 6). Most of the AGC kinase genes in the barley were involved in protein metabolism (post-translational modification and protein synthesis) and signaling, where many of the AGC2 kinases were involved in light signaling. Plants possess the same basic AGC kinase subfamilies (PDK1, S6K, and NDR) as other eukaryotes but they do not encode for the AGC kinases, such as PKA and PKC, implicated in the control of cell expansion, proliferation, and auxin polarity in fungi and animals (Zhang and Friml, 2020). One NDR protein, TaAGC1, has been indicated to have bio-

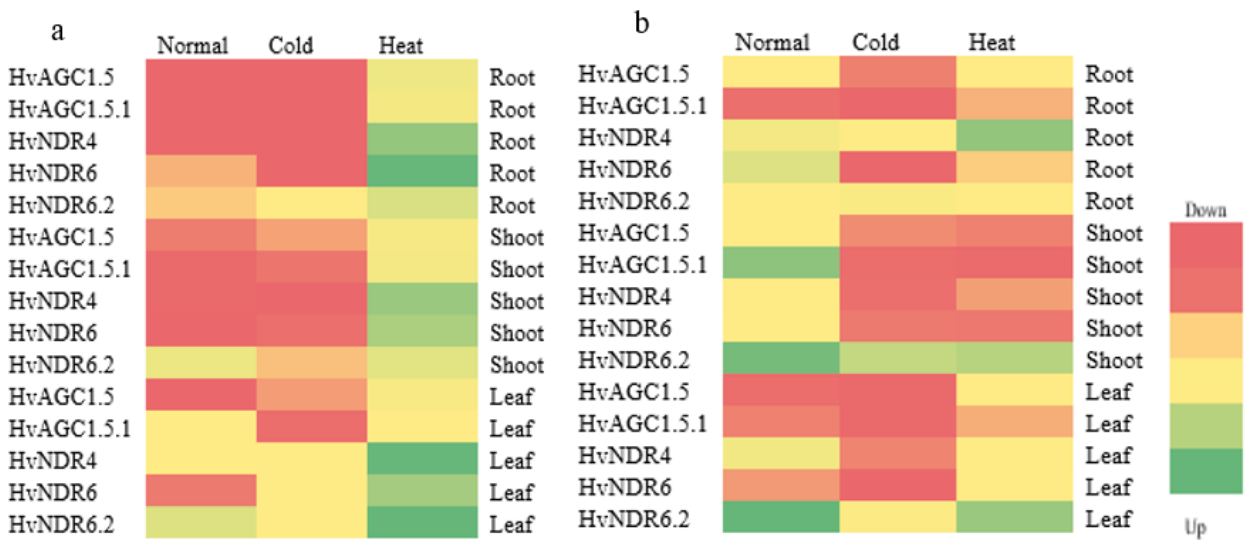


Figure 5: Differential gene expression of Azaran (a) and Jolgeh (b) cultivars under heat and cold stress conditions. Green and red indicate up and down-regulated genes, respectively. Also, yellow color indicates low level expression under both stresses. Gene expression patterns in the three tissues (stem, root, leaf)

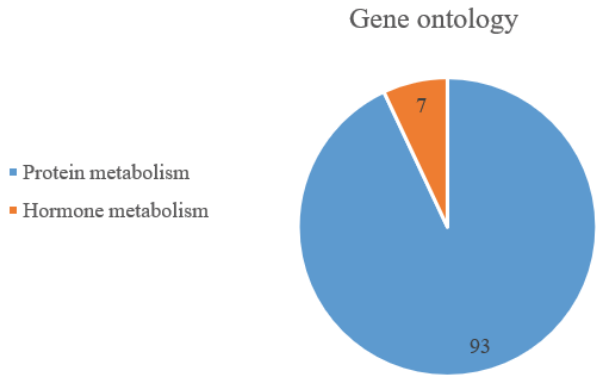


Figure 6: GO classification of the DEGs in barley. Results of significantly enriched pathways involving the AGC kinases genes in barley

logical function, implicated in response to *Rhizoctonia cerealis* E.P. Hoeven used for controlling the induction of ROS-related and defense-related genes in wheat (Zhang and Friml, 2020). Plant responses to external or internal stimuli include rapid protein changes that ultimately lead to the activation of transcriptional processes. Phosphorylation of proteins is commonly used in cellular signaling (protein kinase), where a phosphate group is added to the amino acid side chain. PID and phototropins are main factors in triggering and regulating growth by controlling auxin transport such as PIN. Phototropins have a key role in plant growth and were used directly in polar auxin transport (Galván-Ampudia and Offringa, 2007). The AGC kinase protein kinase family is involved in various signaling pathways, containing light blue and auxin signaling (Christensen et al., 2000; Robert and Offringa, 2008).

4 CONCLUSION

In this study, 28 AGC kinase genes were detected in barley. We studied their phylogenetic relationships, gene structures, chromosomal locations, genes duplication, gene ontology, and clustering of orthologous genes. Further, genome-wide identification of AGC kinase genes were performed. According to structure analysis results, various genes of the same subfamily had similar gene structure, proposing that they have the same evolutionary origin and probably the same functions. These results can provide insights into the functional differences, evolutionary relationships, and comparative genomics analysis of AGC kinases in barley. The HvNDR4 and HvNDR6 genes were both orthologous and were found to serve as molecular signaling in different stresses. AGC kinase genes showed to have different functions, indicating the presence of various conserved domains in these genes. Using this characteristic, candidate genes can be used to genetically improve plants in response to abiotic stresses. Based on the synthetic analysis of the AGC kinase genes, the HvNDR4 and HvNDR6 genes were orthologous, indicating that the orthologous genes show similar expression.

5 CONFLICTS OF INTERESTS

The authors declare that they have no competing interests.

6 REFERENCES

Altenhoff, A.M. & Dessimoz, C. (2009). Phylogenetic and functional assessment of orthologs inference projects and methods. *PLoS Computational Biology*, *5*(1), p.e1000262. https://doi.org/10.1371/journal.pcbi.1000262

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2

Bradley D. & Beltrao, P. (2019). Evolution of protein kinase substrate recognition at the active site. *PLoS Biology*, *17*(6), p.e3000341. https://doi.org/10.1371/journal.pbio.3000341

Cheung, J., Estivill, X., Khaja, R., MacDonald, J.R., Lau, K., Tsui, L.C. & Scherer, S.W. (2003). Genome-wide detection of segmental duplications and potential assembly errors in the human genome sequence. *Genome Biology*, *4*(4), 1-10. https://doi.org/10.1186/gb-2003-4-4-r25

Christensen, S.K., Dagenais, N., Chory, J. & Weigel, D. (2000). Regulation of auxin response by the protein kinase PINOID. *Cell*, *100*(4), 469-478. https://doi.org/10.1016/S0092-8674(00)80682-0

Dhonukshe, P., Huang, F., Galvan-Ampudia, C.S., Mähönen, A.P., Kleine-Vehn, J., Xu, J., Quint, A., Prasad, K., Friml, J., Scheres, B. & Offringa, R. (2010). Plasma membrane-bound AGC3 kinases phosphorylate PIN auxin carriers at TPRXS (N/S) motifs to direct apical PIN recycling. *Development*, *137*(19), 3245-3255. https://doi.org/10.1242/dev.052456

Galván-Ampudia, C.S. & Offringa, R. (2007) Plant evolution: AGC kinases tell the auxin tale. *Trends in Plant Science*, *12*(12), 541-547. https://doi.org/10.1016/j.tplants.2007.10.004

Hergovich, A. (2016). The roles of NDR protein kinases in hippo signalling. *Genes*, *7*(5), 21. https://doi.org/10.3390/genes7050021

Huang, F., Kemel Zago, M., Abas, L., van Marion, A., Galván-Ampudia, C.S. & Offringa, R. (2010). Phosphorylation of conserved PIN motifs directs *Arabidopsis* PIN1 polarity and auxin transport. *The Plant Cell*, *22*(4), 1129-1142. https://doi.org/10.1105/tpc.109.072678

Kong, W., Tan, S., Zhao, Q., Lin, D.L., Xu, Z.H., Friml, J. & Xue, H.W. (2021). mRNA surveillance complex PELOTA–HBS1 regulates phosphoinositide-dependent protein kinase1 and

plant growth. *Plant Physiology*, *186*(4), 2003-2020. https://doi.org/10.1093/plphys/kiab199

Krupnick, J.G. & Benovic, J.L. (1998). The role of receptor kinases and arrestins in G protein–coupled receptor regulation. *Annual Review of Pharmacology and Toxicology*, *38*(1), 289-319. https://doi.org/10.1146/annurev.pharmtox.38.1.289

Kyoko, K., Ohno, S., Serji, M., Ichiro, Y. & Koichi, S. (1989) A novel yeast gene coding for a putative protein kinase. *Gene*, *76*(1), 177-180. https://doi.org/10.1016/0378-1119(89)90021-8

Oyama, T., Shimura, Y. & Okada, K. (2002). The IRE gene encodes a protein kinase homologue and modulates root hair growth in *Arabidopsis*. *The Plant Journal*, *30*(3), 289-299. https://doi.org/10.1046/j.1365-313X.2002.01290.x

Petersen, L.N., Ingle, R.A., Knight, M.R. & Denby, K.J. (2009). OXI1 protein kinase is required for plant immunity against *Pseudomonas syringae* in *Arabidopsis*. *Journal of Experimental Botany*, *60*(13), 3727-3735. https://doi.org/10.1093/jxb/erp219

Pislariu, C.I. & Dickstein, R. (2007). An IRE-like AGC kinase gene, MtIRE, has unique expression in the invasion zone of developing root nodules in *Medicago truncatula*. *Plant Physiology*, *144*(2), 682-694. https://doi.org/10.1104/pp.106.092494

Rentel, M.C., Lecourieux, D., Ouaked, F., Usher, S.L., Petersen, L., Okamoto, H., Knight, H., Peck, S.C., Grierson, C.S., Hirt, H. & Knight, M.R. (2004). OXI1 kinase is necessary for oxidative burst-mediated signalling in *Arabidopsis*. *Nature 427*(6977), 858-861. https://doi.org/10.1038/nature02353

Robert, H.S. & Offringa, R. (2008). Regulation of auxin transport polarity by AGC kinases. *Current Opinion in Plant Biology*, *11*(5),495-502. https://doi.org/10.1016/j.pbi.2008.06.004

Saidi, A. & Hajibarat, Z. (2020a). Computational study of environmental stress-related transcription factor binding sites in the promoter regions of maize auxin response factor (ARF) gene family. *Notulae Scientia Biologicae*, *12*(3), 646-657. https://doi.org/10.15835/nsb12310823

Saidi, A., Hajibarat, Z. & Hajibarat, Z. (2020b). Identification of responsive genes and analysis of genes with bacterial-inducible cis-regulatory elements in the promoter regions in *Oryza sativa* L.. *Acta agriculturae Slovenica*, *116*(1), 115-123. https://doi.org/10.14720/aas.2020.116.1.1035

Saidi, A., Hajibarat, Z. & Hajibarat, Z. (2021a). Phylogeny, gene structure and GATA genes expression in different tissues of Solanaceae species. *Biocatalysis and Agricultural Biotechnology*,102015. https://doi.org/10.1016/j.bcab.2021.102015

Saidi, A., Hajibarat, Z. & Ahmadikhah, A. (2021b). Computational analysis of responsive transcription factors involved in drought and salt stress in rice. *Journal of Applied Biotechnology Reports*, *8*(4), 406-413. doi:10.30491/jabr.2020.243913.1272

Thimm, O., Bläsing, O., Gibon, Y., Nagel, A., Meyer, S., Krüger, P., Selbig, J., Müller, L.A., Rhee, S.Y. & Stitt, M. (2004). MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *The Plant Journal*, *37*(6), 914-939. https://doi.org/10.1111/j.1365-313X.2004.02016.x

Toda, T., Cameron, S., Sass, P. & Wigler, M. (1988). SCH9, a gene of *Saccharomyces cerevisiae* that encodes a protein distinct from, but functionally and structurally related to, cAMP-dependent protein kinase catalytic subunits. *Genes & Development*, *2*(5), 517-527. https://doi.org/10.1101/gad.2.5.517

Voorrips, R.E. (2002). MapChart: software for the graphical presentation of linkage maps and QTLs. *Journal of Heredity*, *93*(1), 77-78. https://doi.org/10.1093/jhered/93.1.77

Wang, L., Yin, H., Qian, Q., Yang, J., Huang, C., Hu, X. & Luo, D. (2009). NECK LEAF 1, a GATA type transcription factor, modulates organogenesis by regulating the expression of multiple regulatory genes during reproductive development in rice. *Cell Research*, *19*(5), 598-611. https://doi.org/10.1038/cr.2009.36

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., de Beer, T.A.P., Rempfer, C., Bordoli, L. & Lepore, R. (2018). SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Research*, *46*(W1), W296-W303. https://doi.org/10.1093/nar/gky427

Xue, Y.J., Tao, L. & Yang, Z.M. (2008). Aluminum-induced cell wall peroxidase activity and lignin synthesis are differentially regulated by jasmonate and nitric oxide. *Journal of Agricultural and Food Chemistry*, *56*(20), 9676-9684. https://doi.org/10.1021/jf802001v

Yang, S., Zhang, X., Yue, J.X., Tian, D. & Chen, J.Q. (2008). Recent duplications dominate NBS-encoding gene expansion in two woody species. *Molecular Genetics and Genomics*, *280*(3), 187-198. https://doi.org/10.1007/s00438-008-0355-0

Zhang, Y. & Friml, J. (2020). Auxin guides roots to avoid obstacles during gravitropic growth. *The New Phytologist*, *225*(3), 1049. https://doi.org/10.1111/nph.16203

Zhou, P.M., Liang, Y., Mei, J., Liao, H.Z., Wang, P., Hu, K., Chen, L.Q., Zhang, X.Q. & Ye, D. (2021). The *Arabidopsis* AGC kinases NDR2/4/5 interact with MOB1A/1B and play important roles in pollen development and germination. *The Plant Journal*, *105*(4), 1035-1052. https://doi.org/10.1111/tpj.15085

Zhu, X., Yang, K., Wei, X., Zhang, Q., Rong, W., Du, L., Ye, X., Qi, L. & Zhang, Z. (2015). The wheat AGC kinase TaAGC1 is a positive contributor to host resistance to the necrotrophic pathogen *Rhizoctonia cerealis*. *Journal of Experimental Botany*, *66*(21), 6591-6603. https://doi.org/10.1093/jxb/erv367

doi:10.14720/aas.2022.118.3.2399 Original research article / izvirni znanstveni članek

Intercropping induces physiological and morphological plasticity in oilseed rape and barley under drought stress

Noushin SADEGHZADEH [1], Roghieh HAJIBOLAND [1, 2], and Charlotte POSCHENRIEDER [3]

Received September 03, 2021; accepted August 03, 2022.
Delo je prispelo 3. septembra 2021, sprejeto 3. avgusta 2022

Intercropping induces physiological and morphological plasticity in oilseed rape and barley under drought stress

Abstract: Intercropping is an agricultural practice that can improve crop yield due to better availability of resources, including water. There are few studies, however, addressing the physiological mechanisms behind this phenomenon. In this work oilseed rape (*Brassica napus* L.) and barley (*Hordeum vulgare* L.) were cultivated either as monocrop (MC) or intercrop (IC) under well-watered (WW) or drought stress (DS) conditions in a growth chamber. After eight weeks DS, the leaf relative water content was higher in the IC compared with the MC plants in both species and the DS-induced senescence of old leaves was considerably postponed in oilseed rape. Intercropped oilseed rape showed elevated levels of leaf photosynthesis rate, superior accumulation of organic osmolytes but higher water loss compared with the MC counterparts under DS conditions. In barley, less transpiration, an increased root : shoot ratio and osmolyte accumulation was observed in the IC compared with MC plants under DS conditions. The water use efficiency was higher in the IC compared to MC barley and the plants yield was higher in the IC than in the MC oilseed rape. Our data showed that intercropping is a reliable practice for cultivation of both species under arid and semi-arid regions or under rainfed conditions.

Key words: drought stress; intercropping; osmotic adjustment; photosynthesis rate;, transpiration: water use efficiency

This paper is part of PhD thesis of N.S under supervision of R.H; C.P was the advisor of this thesis.

Medsetev vzpodbuja fiziološko in morfološko prilagodljivost oljne ogrščice in ječmena v sušnem stresu

Izvleček: Medsetev je način kmetovanja, ki izboljšuje pridelek poljščin zaradi zaradi boljše dostopnosti virov, vključno z vodo. Malo je raziskav, ki bi se ukvarjale s fiziološkimi mehanizmi tega fenomena. V tej raziskavi sta bila v rastni komori gojena oljna ogrščica (*Brassica napus* L.) in ječmen (*Hordeum vulgare* L.) kot monokultura (MC) ali kot mešan posevek (IC) v razmerah dobre preskrbe z vodo (WW) ali v razmerah sušnega stresa (DS). Po osmih tednih rasti v sušnem stresu je bila relativna vsebnost vode pri obeh vrstah večja pri medsetvi kot v monokulturi, pri oljni ogrščici je bilo odmiranje starih listov v razmerah sušnega stresa znatno kasnejše. Oljna ogrščica je imela v medsetvi v razmerah sušnega stresa večjo fotosintezo, večje kopičenje osmotikov, a večjo izgubo vode v primerjavi z gojenjem v monokulturi. Pri ječmenu je bila pri medsetvi v razmerah sušnega stresa manjša transpiracija, povečano razmerje korenina : poganjek, povečana akumulacija osmotikov v primerjavi z rastjo v monokulturi. Učinkovitost izrabe vode je bila pri ječmenu večja v medsetvi kot v monokulturi, v medsetvi je bil večji tudi pridelek oljne ogrščice. Ti podatki kažejo, da je medsetev primeren način gojenja obeh vrst v sušnih in polsušnih območjih v razmerah preskrbe z vodo z deževjem.

Ključne besede: sušni stres; medsetev; osmotska prilagoditev; velikost fotosinteze; transpiracija; učinkovitost izrabe vode

Članek je del disertacije N.S. pod mentorstvom R.H. in svetovanjem C.P.

1 Department of Plant, Cell and Molecular Biology, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran
2 Corresponding author, e-mail: ehsan@tabrizu.ac.ir
3 Plant Physiology Laboratory, Bioscience Faculty, Universidad Autónoma de Barcelona, Spain

1 INTRODUCTION

Drought stress is one of the most important environmental constrains limiting plants production worldwide (Tardieu et al., 2018). Photosynthesis, the key process responsible for growth and dry matter production of plants, decreases under water stress through both stomatal and non-stomatal limitations (Zhou et al., 2013). Nonstomatal factors such as decreased leaf expansion and photosynthetic pigments concentration, leaf senescence and reduced electron transport activities, in combination with stomatal factors, reduce the overall photosynthetic performance of plants under drought stress (Chaves et al., 2009).

Since the CO_2 assimilation is decreased simultaneously with transpiration under water stress, the efficiency of plants for photosynthesis or biomass production at the expense of a given rate of water loss, i.e. water use efficiency (WUE), is an important parameter for plants drought tolerance (Tambussi et al., 2007).

Plants adopt various strategies for confronting drought stress and survive under these conditions. The increased production of low molecular weight organic osmolytes such as free amino acids particularly proline and soluble carbohydrates is crucial for the regulation of cell water content under extreme osmotic environment (Singh et al., 2015). By decreasing the osmotic potential in the cytoplasm, these osmoprotectants help plants to prevent cell dehydration. Moreover, these organic osmolytes contribute to mitigate damage caused by reactive oxygen species (ROS), to prevent membrane injury and to stabilize proteins and enzymes (Krasensky and Jonak, 2012).

Intercropping is establishing two or more crop species together at same field in the same time. Under intercropping conditions, both negative interaction (competition) and positive interaction (facilitation) can occur simultaneously (Brooker et al., 2015). However, by increasing facilitation and decreasing competition between crops, intercropping systems can use environmental resources more effectively. In fact, higher yield has been repeatedly recorded in many intercropping systems compared to monocultures (Martin-Guay et al., 2018). There are evidences showing that biomass and water use efficiency (WUE) of intercropping systems under drought stress are usually greater than that of monocultures (Daneshnia et al., 2015; Chimonyo et al., 2016). There are, however, studies that showed intercropping systems did not increase obviously WUE (Grema and Hess, 1994; Shackel and Hall, 1984), or sometime reduced it (Rees, 1986; Singh et al., 1988; Gao et al., 2009).

Belowground interactions in the ecological and agricultural systems are not restricted to the competition or facilitation mechanisms for nutrient acquisition (Mommer et al., 2016). Increasing evidences obtained from plants co-cultured under laboratory conditions showed considerable influence of both interspecific and conspecific interactions on plants development, metabolism and defense (Schmid et al., 2013; Chen et al., 2018). Almost all of these effects are independent from competition or complementary usage of resources (Semchenko et al., 2014; Kong et al., 2018).

Almost all of previous works on the effect of cropping pattern on plants drought resistance have been undertaken under field conditions with little attention paid to explore the mechanisms behind the improvement of drought tolerance in the intercrop systems. In order to explore the physiological and biochemical effects of belowground root interactions, we cultivated oilseed rape plants and barley under well-watered or drought stress conditions either as monocrop or intercrop and analyzed plants for water content and osmotic parameters. Our working hypothesis is that, the interspecific interactions in the intercrop system may trigger some biochemical and physiological modifications in the co-cultured plants that influence their response to drought.

2 MATERIALS AND METHODS

2.1 PLANTS CULTURE AND TREATMENTS

Seeds of oilseed rape (*Brassica napus* 'Opera') and barley (*Hordeum vulgare* 'Makoui') plants were provided by the Seed and Plant Improvement Institute (Karaj, Iran) and Dryland Agricultural Research Institute (DARI) (Maragheh, Iran), respectively. The seeds were surface sterilised using commercial bleach and germinated in the dark on perlite. After germination, the seedlings were transferred to the light. The 10-day old young oilseed rape seedlings were precultured in the 50 % Hoagland nutrient solution for two weeks before starting the experiment.

Twenty five-day-old oilseed rape together with one-week-old barley seedlings were transferred to 0.8 l plastic pots filled with perlite and cultivated either as monocrop (MC) or intercrop (IC). Since the biomass and leaf area of one barley seedling was a quarter of oilseed rape, 4 barley plants were cultivated with one oilseed rape in the IC pots. In the MC pots either two oilseed rape or 8 barley plants were cultivated.

Two weeks after starting MC/IC treatments, two watering regimes including well-watered (WW) and drought stress (DS) were assigned randomly to the pots. The WW plants were continued to be irrigated to 100 % field capacity (FC) while watering was omitted from DS

pots until they reached the 30 % FC. This was achieved one week after starting the DS treatment.

Everyday throughout the experiment, after weighing, the pots were irrigated with nutrient solution or water as interval. Control and water-stressed plants received the same amount of nutrient solution and the respective FC was achieved by different volumes of water used for irrigation. Water consumption (~water loss; the amount of water needed for adjustment of pots to the respective FC) was recorded daily.

2.2 HARVEST

The plants were harvested eight weeks after reaching the 30 % FC (10 weeks after starting MC/IC treatments). The roots were separated from perlite and washed with distilled water and blotted dry on filter paper. After determination of fresh mass (FM), leaf and root samples were oven-dried at 70 °C for 48 h, and dry mass (DM) was determined. Because of almost complete intertwining the roots in the IC pots under WW conditions, the root mass could not be determined for two species separately.

2.3 MEASUREMENT OF SPAD AND LEAF CHL CONCENTRATION

Leaf greenness was measured daily as the Spectral Plant Analysis Diagnostic (SPAD) index in the second youngest, fully expanded leaf (young leaf) and in the second oldest leaf (old leaf) using a chlorophyll-meter (Minolta, 502). Leaf Chl concentration in the young and old leaves was spectrophotometrically determined after extraction in 70 % aceton for 24 h in the dark at 4 °C (Lichtenthaler and Wellburn, 1985).

2.4 DETERMINATION OF GAS EXCHANGE PARAMETERS AND WATER USE EFFICIENCY

Net CO_2 assimilation rate, transpiration and stomatal conductance to water vapour were measured in the attached young and old leaves with a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) between 10:00 and 13:00 a.m at a photosynthetic photon flux density of 350 μmol m^{-2} s^{-1}.

The instantaneous water use efficiency (iWUE) (μmol $mmol^{-1}$) was defined at leaf scale as the net photosynthesis rate divided by the water transpired in the same time period:

$$iWUE = \frac{Photosynthesis\ rate}{Transpiration\ rate}$$

The biomass WUE (bWUE) (g kg^{-1}) was defined at whole plant scale as the ratio of biomass produced to the rate of water consumed (Tambussi et al., 2007):

$$bWUE = \frac{Shoot\ DM}{Total\ water\ consumption}$$

2.5 MEASUREMENT OF RELATIVE WATER CONTENT AND OSMOTIC POTENTIAL

Relative water content (RWC %) was measured in the leaves harvested 1 h after starting the light period and calculated according to the following equation:

$$RWC\ (\%) = \frac{FM - DM}{TM - DM} \times 100$$

For determination of turgid mass (TM), leaf disks (5 mm diameter) were submerged for 5 h in distilled water, thereafter, they were blotted dry gently on a paper towel and weighed.

Osmotic potential was determined in the leaf and root samples harvested at 1 h after the lights were turned on in the growth chamber. Samples were homogenized in prechilled mortar and pestle and centrifuged at 4000 g for 20 min at 4 °C. The osmotic pressure of the samples was measured by an osmometer (Heman Roebling Messtechnik, Germany), and the miliosmol data were recalculated to MPa.

2.6 DETERMINATIONS OF BIOCHEMICALS

For determination of soluble carbohydrates, leaf and root samples were homogenized in ethanol at 4 °C. After centrifugation at 12,000 *g* for 15 min, an aliquot of the supernatant was mixed with anthrone-sulfuric acid reagent and incubated for 10 min at 100 °C. After cooling, the absorbance was determined at 625 nm (Yemm and Willis, 1954). Glucose (Merck) was used to construct a standard curve. Total soluble proteins were determined using a commercial reagent (Bradford reagent, Sigma) and bovine albumin serum as standard. Proline was extracted and determined by the method of Bates et al. (1973). Leaf tissues were homogenized with 3 % sulfos-

alicylic acid and the homogenate was centrifuged at 3000 g for 20 min. The supernatant was treated with acetic acid and acid ninhydrin, boiled for 1 h, and then absorbance at 520 nm was determined. Proline (Sigma) was used for production of a standard curve. Content of total free a-amino acids was assayed using a ninhydrin colorimetric method (Yemm and Cocking, 1955). Glycine was used for standard curve.

2.7 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

The experimental design was a complete randomised block with four independent pots as four replications. Pairwise comparison of means was performed by the Tukey's test ($p < 0.05$) using Sigma stat (3.02). To assign different physiological parameters to distinct groups, principal component analysis (PCA) was conducted using Minitab 18.

3 RESULTS AND DISCUSSION

3.1 EFFECT OF DS AND IC ON THE BIOMASS AND LEAF AREA

Drought stress (DS) decreased shoot biomass and leaf area of both species (Fig. 1). However, the effects of DS on the shoot biomass and leaf area under MC conditions were not significant in the barley and oilseed rape plants, respectively (Fig. 1). Leaf growth is accomplished through cell division and cell expansion which are both affected by water deficit (Koch et al., 2019). Cell expansion is one of the most drought-sensitive physiological processes because of its dependence on the turgor pressure. Impaired cell division and expansion results in reduced plant height, leaf area and ultimately growth reduction of plant under drought (Skirycz and Inze, 2010). Under long term water deficit as in our work, biomass of plants is also decreased due to the reduced CO_2 assimilation rate (Tardieu and Granier, 2011).

Similar to the shoot growth parameters, root biomass decreased under DS conditions in both species cultivated in the MC pots. The responses of root growth and elongation to drought largely depend on the plant species, the genotype and the severity of drought stress (Sánchez-Blanco et al., 2014). Under mild drought stress root : shoot ratio may increase as the result of a preferential allocation of photosynthates to the roots allowing better water capture as an adaptation to drought (Faroog et al., 2009). Under severe drought, in contrast, root biomass and length is decreased likely because the limited photosynthesis reduces the sucrose export to the roots and ultimately inhibits root growth (Lemoine et al., 2013). Here in our work, root : shoot ratio was not modified by DS in oilseed rape while decreased from 1.0 under WW to 0.71 under DS conditions in barley (Fig. 1).

Intercropped (IC) oilseed rape plants showed higher shoot biomass than the monocropped (MC) counterparts under both well-watered (WW) and DS conditions. Barley, in contrast, produced less shoot biomass when cultivated in the IC pots irrespective the watering regime (Fig. 1). The leaf area also increased under IC conditions in oilseed rape under WW conditions, while this parameter decreased in barley both under WW and DS conditions (Fig. 1). The improvement of shoot growth under intercropping conditions in oilseed rape, but its depression in barley that was observed independent from watering treatments will be discussed below.

Root biomass was not influenced by the IC treatment in oilseed rape, while increased in barley under DS conditions (Fig. 1). The improvement of root biomass in IC barley grown under DS conditions contrasted with the effect of intercropping on shoot biomass and leaf area in this species. This led to an increase in root : shoot ratio from 0.71 in the MC to 3.12 in the IC barley plants, while this ratio was not influenced by intercropping in oilseed rape plants. The effect of IC on WW plants could not be detected because of lacking individual data for each species (see M & M). The total root biomass of plants in the IC pots (1.90 ± 0.22) was significantly higher ($p < 0.05$) than the sum of two MC pots (1.29 ± 0.37) (data not shown).

3.2 EFFECT OF DS AND IC ON THE CHL CONCENTRATION, PHOTOSYNTHESIS AND TRANSPIRATION RATES

The Chl a + b concentration was not influenced by DS in barley but decreased in the old leaves of oilseed rape plants (Table 1). Reduction of Chl under DS is likely the results of higher rates of degradation mainly due to the elevated levels of ROS under these conditions (Noctor et al., 2014). Loss of the balance between the production and scavenging of ROS induces oxidative stress and the accumulated ROS damages proteins, pigments, membrane lipids and other cellular components (Cruz de Carvalho, 2008).

The photosynthesis and transpiration rates decreased significantly by DS in the old leaves of both species and in the young leaves of oilseed rape (Table 1). Reduction of transpiration through stomatal control of water losses has been identified as an early event in plant response to water deficit leading in turn to limitation of

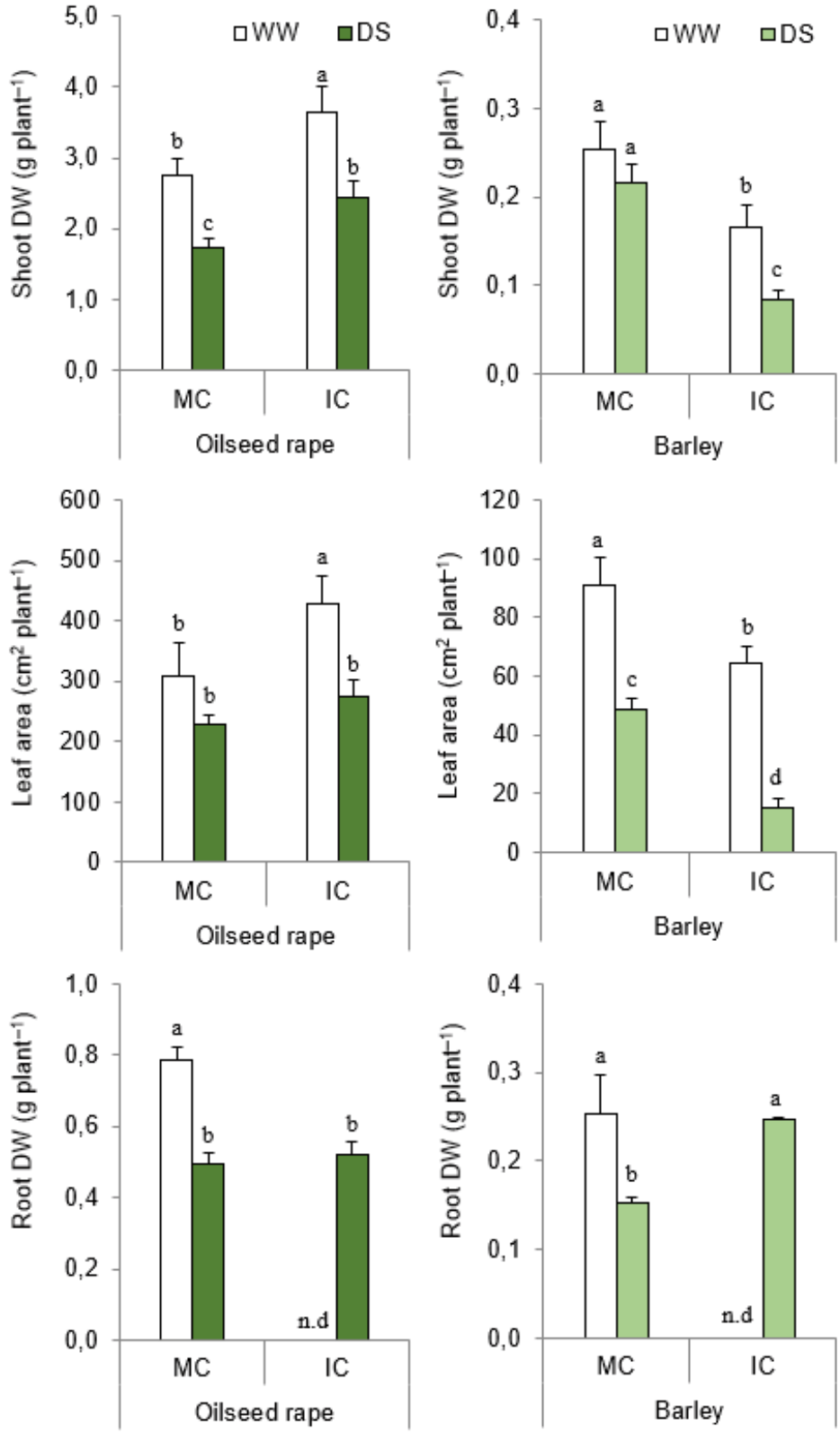


Figure 1: Shoot and root dry biomass and leaf area in oilseed rape and barley cultivated either as monocrop (MC) or intercrop (IC) under well-watered (WW) or drought stress (DS) conditions for eight weeks. Bars indicated by the different letters are significantly different ($p < 0.05$)

CO_2 diffusion into the leaves (Zhou et al., 2013). Since the activity of the photosynthetic electron transport chain is finely tuned to the availability of CO_2 in the chloroplast, restricted CO_2 availability could lead to increased susceptibility to damage to photosynthetic apparatus (Chaves et al., 2002). In addition, reduction in photosynthesis arises by impaired activities of Calvin cycle enzymes and a decline in Rubisco activity (Chaves et al., 2009).

Intercropping did not affect Chl a + b concentration in the young leaves of oilseed rape plants, but increased it in the old leaves of both WW and DS plants. In barley, concentration of Chl a + b was not influenced in either of the leaves under WW or DS conditions (Table 1). In oilseed rape, IC treatment increased photosynthesis rate in the young leaves of DS plants and in the old leaves of both WW and DS plants. In barley, in contrast, leaf photosynthesis rate was not influenced by intercropping (Table 1). Transpiration rate increased by IC treatment in oilseed rape that was significant for the old leaves, while decreased in the young leaves of barley (Table 1).

3.3 EFFECT OF IC ON THE PHENOTYPIC PLASTICITY OF BOTH SPECIES UNDER DS

The cropping pattern influenced plants response to DS differently depending on species. In the DS oilseed rape, IC conditions resulted in a slight increase in the leaf area (Fig. 1), and photosynthesis and transpiration rates per surface area (Table 1). Despite the putatively higher water loss at whole plant level under IC conditions, this strategy may enable this species to keep higher ability for biomass production and synthesis of osmolytes (see below) compared with the MC counterparts. In barley, in contrast, reduction of leaf area and transpiration rate per surface area most likely led to lower water loss at whole plant level accompanied by an increased root biomass and higher root : shoot ratio. Such phenotypic plasticity in response to DS in barley that was observed only under IC conditions may enable this species to capture efficiently water from the dry substrate. Root growth and density, proliferation and size are key responses of plants to drought stress (Farooq et al., 2009). It is well plausible that the belowground root interactions in the IC pots mediate some modifications in the phytohormone balances in plants. In our barely plants, reduction of shoot growth and an increase in the root : shoot ratio under DS conditions are the well-known responses of plants to abscisic acid (Mc Adam et al., 2016). Modification in the levels of phytohormones through root interactions with the neighbor plants has been observed in tobacco (Chen et al., 2018). From an ecological point of view, the ability of plants to plastically adjust to environment play important role in the function of mixed cropping systems (Zhu, 2015).

3.4 EFFECT OF DS AND IC ON WATER STATUS OF THE YOUNG AN OLD LEAVES

Drought stress expectedly decreased RWC in the young and old leaves of both species (Fig. 2). Intercropping did not influence the leaf RWC in the WW plants while significantly increased this parameter in the young

Table 1: Concentrations of chlorophyll (Chl) a + b , photosynthesis and transpiration rates in the young and old leaves of oilseed rape and barley cultivated either as monocrop (MC) or intercrop (IC) under well-watered or drought stress conditions for eight weeks. Data of each column indicated by the different letters are significantly different ($p < 0.05$)

Oilseed rape		Chl a + b (mg g^{-1} FM)		Photosynthesis (µmol m^{-1} s^{-1})		Transpiration rate (mmol m^{-1} s^{-1})	
Young leaf		Old leaf	Young leaf	Old leaf	Young leaf	Old leaf	
Well-watered	MC	6.45 ± 0.85 [a]	2.17 ± 0.32 [c]	4.62 ± 0.12 [ab]	2.43 ± 0.39 [b]	1.41 ± 0.17 [a]	1.22 ± 0.20 [a]
	IC	7.08 ± 0.61 [a]	6.02 ± 0.74 [a]	5.35 ± 0.34 [a]	3.46 ± 0.42 [a]	1.58 ± 0.43 [a]	1.39 ± 0.14 [a]
Drought stress	MC	5.85 ± 0.87 [a]	1.67 ± 0.67 [c]	2.84 ± 0.28 [c]	1.48 ± 0.29 [c]	0.75 ± 0.15 [b]	0.30 ± 0.02 [c]
	IC	6.04 ± 0.37 [a]	4.12 ± 0.39 [b]	4.22 ± 0.60 [b]	2.71 ± 0.19 [b]	1.18 ± 0.08 [ab]	0.67 ± 0.01 [b]
Barley		Chl a + b (mg g^{-1} FM)		Photosynthesis (µmol m^{-1} s^{-1})		Transpiration rate (mmol m^{-1} s^{-1})	
Young leaf		Old leaf	Young leaf	Old leaf	Young leaf	Old leaf	
Well-watered	MC	5.63 ± 0.65 [a]	3.45 ± 0.27 [a]	5.43 ± 0.72 [a]	3.97 ± 0.68 [a]	1.38 ± 0.07 [a]	0.85 ± 0.10 [a]
	IC	5.45 ± 0.31 [a]	3.04 ± 0.36 [a]	5.46 ± 0.44 [a]	3.64 ± 0.54 [ab]	1.30 ± 0.21 [a]	0.73 ± 0.19 [a]
Drought stress	MC	5.21 ± 0.78 [a]	3.26 ± 0.42 [a]	4.36 ± 0.17 [a]	2.71 ± 0.12 [b]	1.30 ± 0.08 [a]	0.35 ± 0.02 [b]
	IC	5.51 ± 0.64 [a]	3.00 ± 0.32 [a]	3.88 ± 0.69 [a]	2.94 ± 0.62 [ab]	0.66 ± 0.15 [b]	0.35 ± 0.05 [b]

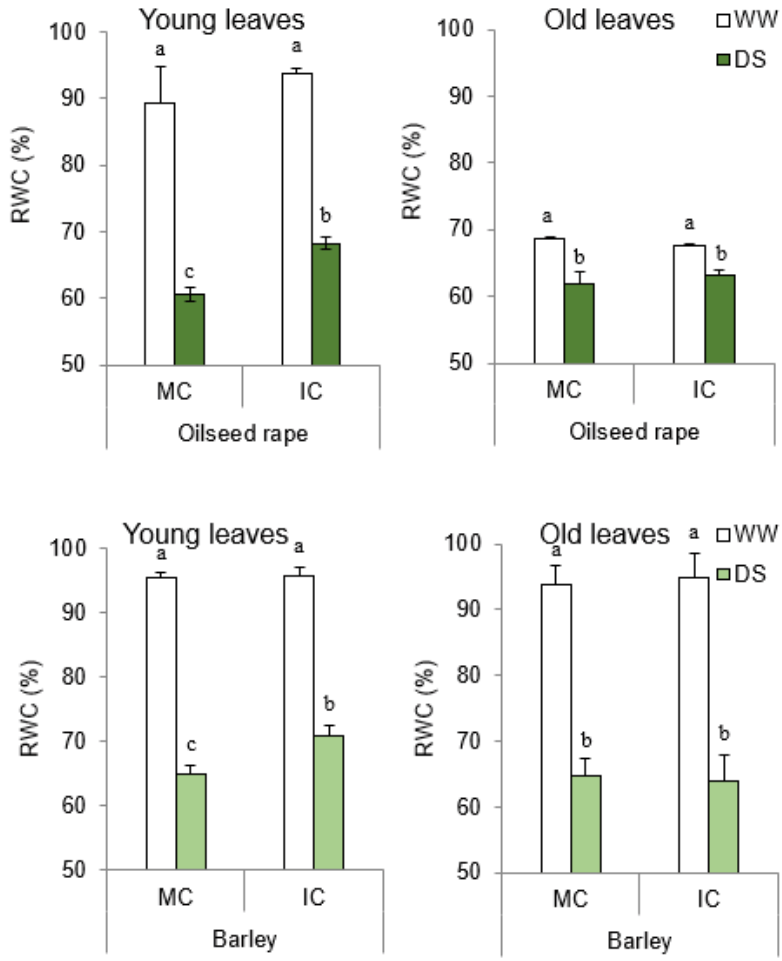


Figure 2: Relative water content (RWC) in the young and old leaves of oilseed rape and barley cultivated either as monocrop (MC) or intercrop (IC) under well-watered (WW) or drought stress (DS) conditions for eight weeks. Bars indicated by the different letters are significantly different ($p < 0.05$)

leaves of both species under DS conditions (Fig. 2). The leaf RWC is a reliable parameter to evaluate the water status of plants that reflects the balance between water supply to the leaf tissue and transpiration rate (Lugojan and Ciulca, 2011). The improvement of RWC in the young leaves of both species upon intercropping in this work is an indication of an interspecific interaction occurred only under water deficit conditions being independent from the effect of IC on biomass production.

3.5 EFFECT OF DS AND IC ON THE WUE AND WATER CONSUMPTION

Instant WUE (iWUE) increased under DS conditions in the old leaves of both species that was observed for both MC and IC plants (Fig. 3). Significant effect of IC on iWUE was observed in the young leaves of barley under DS conditions (Fig. 3). Drought stress increased the biomass WUE (bWUE) too (Fig. 3). This parameter differed also significantly among three culture pots; the lowest bWUE was observed in the MC barley pots both under WW and DS conditions (Fig. 3). Increases in WUE are commonly stated as a response of plants to moderate to severe water deficiency (Tambussi et al., 2007). There are evidences showing that the WUE of intercropping systems are usually greater than that of monoculture (Daneshnia et al., 2015; Chimonyo et al., 2016). There are, however, studies that showed intercropping systems did not increase obviously WUE (Grema and Hess, 1994; Shackel and Hall, 1984), or sometime reduced it (Rees, 1986; Singh et al., 1988; Gao et al., 2009). Here in our work, IC pots have higher bWUE than the MC barley pots both under WW and DS conditions. In oilseed rape, intercropping did not influence bWUE under DS conditions but decreased it under WW conditions (Fig. 3).

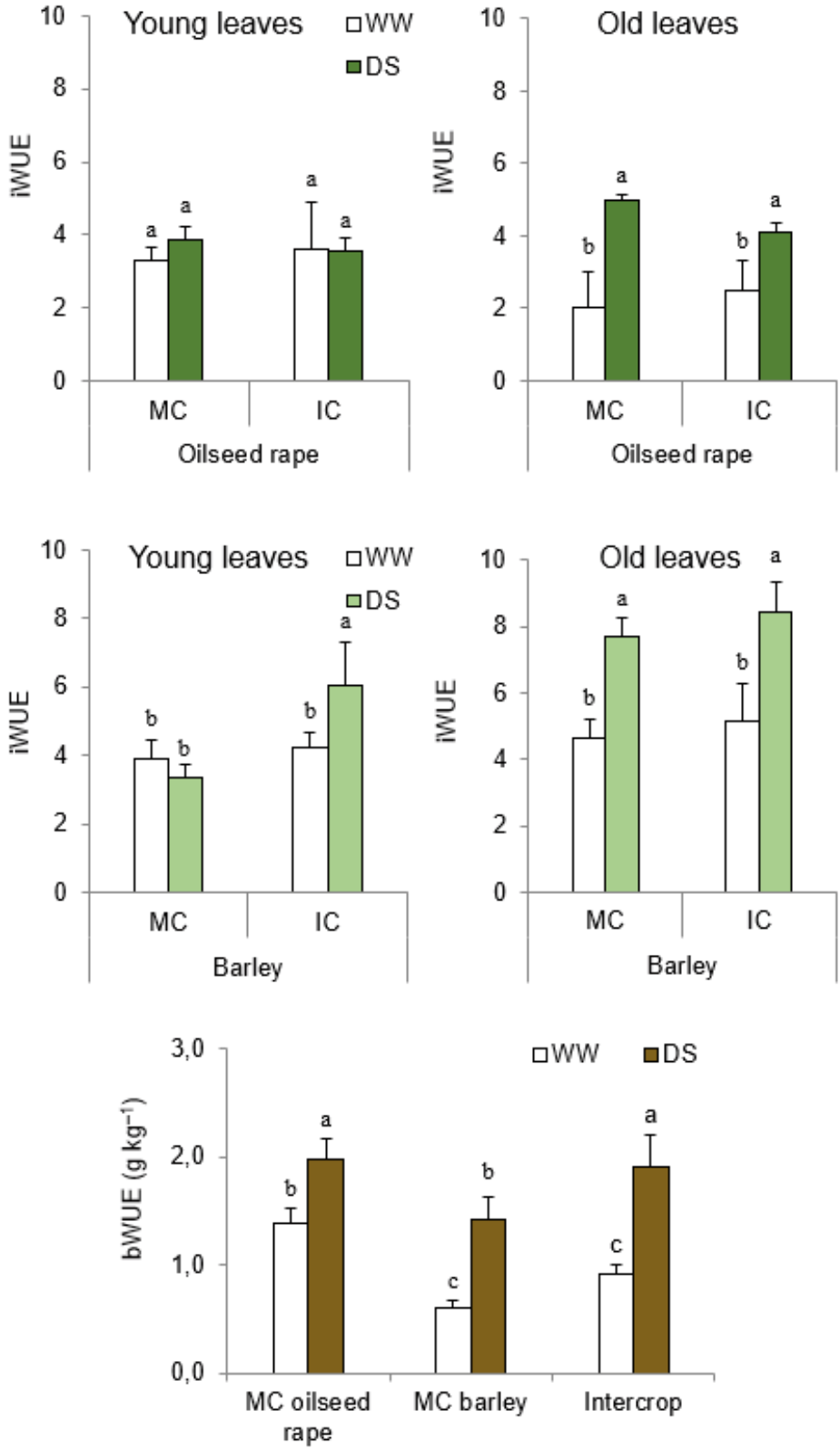


Figure 3: Instant water use efficiency (iWUE) in the young and old leaves of oilseed rape and barley and biomass water use efficiency (bWUE) in the monocrop (MC) or intercrop (IC) pots after eight weeks cultivation under well-watered (WW) and drought stress (DS) conditions. Bars within each culture mode indicated by the different letters are significantly different ($p < 0.05$)

Daily water consumption gradually increased during the two months experiment in both MC and IC pots under WW conditions (Fig. 4). Difference between MC and IC pots were obvious from 30 days after intercrop onward, and at the end of experiment, daily water consumption in the IC pots was considerably higher than that in MC pots (Fig. 4). Under DS conditions, the water consumption sharply decreased subsequent to omitting watering and remained lower throughout the experiment. Daily water consumption was consistently higher

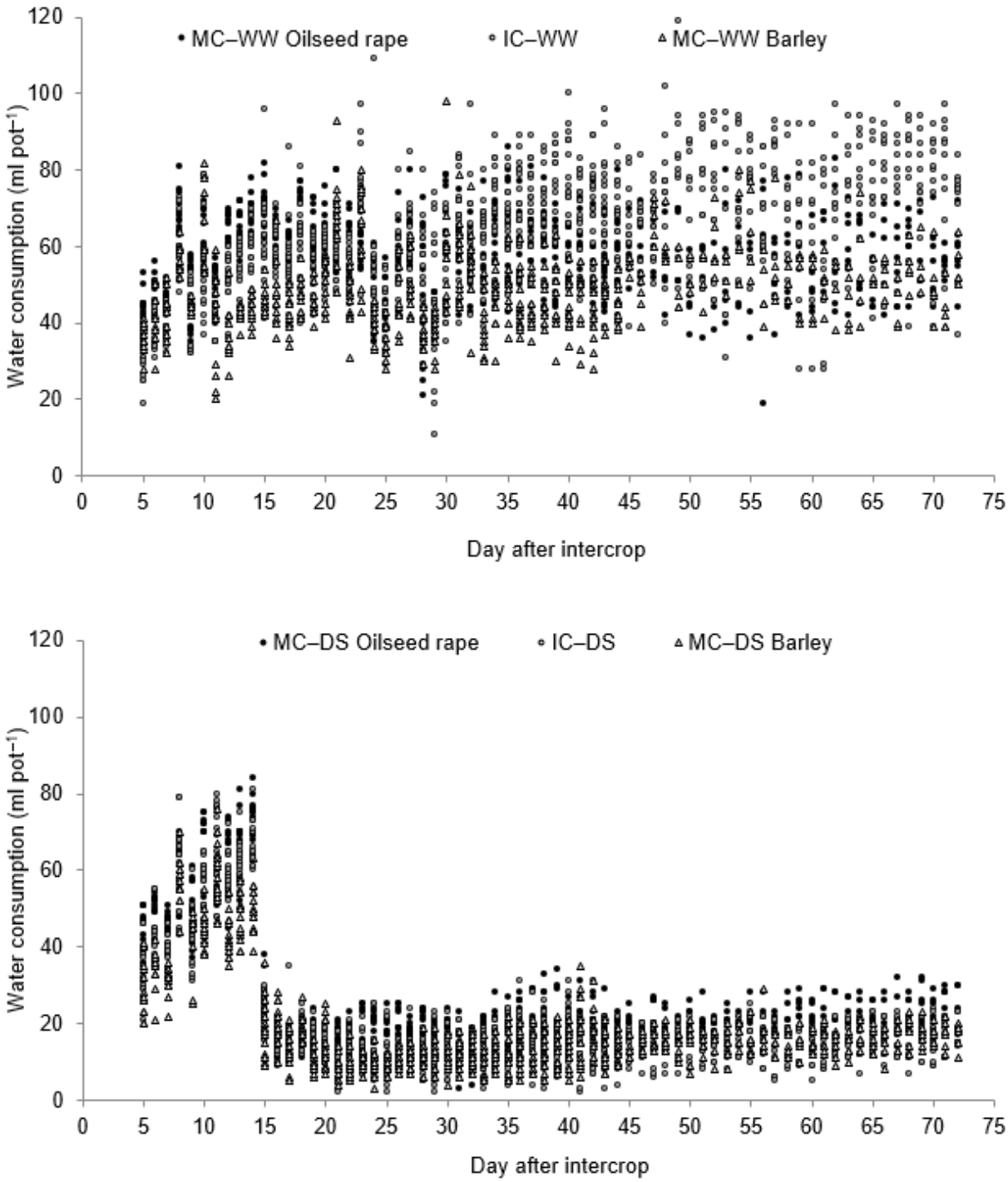


Figure 4: Daily water consumption of the monocrop (MC) pots of oilseed rape and barley and of the intercrop (IC) pots under well-watered (WW) (above) or drought stress (DS) (below) conditions for eight weeks

in the MC oilseed rape pots compared with IC and MC barley pots (Fig. 4).

3.6 EFFECT OF DS AND IC ON THE OSMOTIC HOMEOSTASIS OF LEAVES AND ROOTS

Leaf osmotic potential decreased under DS conditions in both species. Effect of DS on the root osmotic potential, however, was significant only in oilseed rape (Table 2). Leaf concentration of organic osmolytes increased under DS conditions in oilseed rape. Significant effect of DS, however, was observed for proline in the young leaves and for soluble sugars in the old leaves while free amino acids contributed equally to the osmolytes concentration in the old and young leaves of this species (Table 2). In barley leaves, soluble sugars did not respond to the treatments. The effect of DS on the proline concentration was not significant but the free amino acids increased in the young leaves of this species in response to DS. In the roots, proline and soluble sugars accumulated in both species while free amino acids did not responds to DS in none of species (Table 2).

One of the most common stress tolerance strategies in plants is the overproduction of different types of compatible organic solutes including soluble sugars, free amino acids, proline and glycinebetaine (Singh et al., 2015). These osmolytes protect plants through contribution to osmotic adjustment, detoxification of ROS, and the stabilization of membranes, native structures of enzymes and proteins (Verbruggen and Hermans, 2008). Oilseed rape responded more to the DS than barley regarding the accumulation of osmolytes in the leaves. This may allow this species to have higher RWC despite higher transpiration that help also to produce biomass under DS conditions. Of particular importance was the proline accumulation particularly in the young leaves of oilseed rape that was much higher than that in barley. Proline accumulation is caused by a combination of increased biosynthesis and slow oxidation in mitochondria (Parida et al., 2008) and play important roles including stabilization of macromolecules, ROS scavenging, a sink for excess reductant

Table 2: Osmotic potential and concentrations of proline, free amino acids and soluble sugars in the young and old leaves and roots of oilseed rape and barley cultivated either as monocrop (MC) or intercrop (IC) under well-watered or drought stress conditions for eight weeks. Data of each column indicated by the different letters are significantly different ($p<0.05$)

Oilseed rape		Young leaf	Old leaf	Roots	Young leaf	Old leaf	Roots
		Osmotic potential (-MPa)			Proline (μmol g^{-1} FM)		
Well-watered	MC	0.564 ± 0.030 ^{b}	0.469 ± 0.079 ^{b}	0.101 ± 0.013 ^{b}	0.72 ± 0.18 ^{c}	0.25 ± 0.06 ^{b}	0.40 ± 0.06 ^{b}
	IC	0.524 ± 0.015 ^{b}	0.511 ± 0.003 ^{b}	0.118 ± 0.009 ^{b}	0.76 ± 0.09 ^{c}	0.35 ± 0.04 ^{b}	0.27 ± 0.06 ^{b}
Drought stress	MC	0.904 ± 0.039 ^{a}	0.832 ± 0.003 ^{a}	0.196 ± 0.011 ^{a}	3.10 ± 0.69 ^{b}	0.67 ± 0.22 ^{b}	1.50 ± 0.16 ^{a}
	IC	0.962 ± 0.032 ^{a}	0.909 ± 0.049 ^{a}	0.224 ± 0.031 ^{a}	5.47 ± 0.61 ^{a}	1.14 ± 0.35 ^{a}	1.63 ± 0.63 ^{a}
		Free amino acids (μmol g^{-1} FW)			Soluble sugars (mg g^{-1} FM)		
Well-watered	MC	5.22 ± 0.11 ^{c}	3.04 ± 0.78 ^{d}	4.09 ± 1.28 ^{b}	29.43 ± 3.37 ^{b}	25.91 ± 5.27 ^{b}	0.63 ± 0.07 ^{c}
	IC	7.48 ± 0.69 ^{b}	7.28 ± 1.14 ^{b}	4.78 ± 1.45 ^{b}	28.87 ± 0.99 ^{b}	24.88 ± 6.11 ^{b}	1.42 ± 0.08 ^{c}
Drought stress	MC	8.67 ± 1.69 ^{b}	5.23 ± 0.57 ^{c}	5.90 ± 0.97 ^{b}	35.91 ± 3.12 ^{ab}	48.79 ± 4.17 ^{a}	2.76 ± 0.28 ^{b}
	IC	17.0 ± 2.53 ^{a}	11.4 ± 1.09 ^{a}	9.09 ± 0.48 ^{a}	47.03 ± 11.7 ^{a}	49.89 ± 10.8 ^{a}	4.07 ± 0.78 ^{a}
Barley		Young leaf	Old leaf	Roots	Young leaf	Old leaf	Roots
		Osmotic potential (-MPa)			Proline (μmol g^{-1} FM)		
Well-watered	MC	0.588 ± 0.014 ^{b}	0.543 ± 0.048 ^{b}	0.122 ± 0.008 ^{b}	0.25 ± 0.04 ^{a}	0.15 ± 0.02 ^{b}	0.22 ± 0.03 ^{c}
	IC	0.521 ± 0.057 ^{b}	0.511 ± 0.033 ^{b}	0.131 ± 0.008 ^{b}	0.24 ± 0.07 ^{a}	0.16 ± 0.04 ^{b}	0.23 ± 0.05 ^{c}
Drought stress	MC	0.856 ± 0.177 ^{a}	0.898 ± 0.071 ^{a}	0.141 ± 0.005 ^{b}	0.36 ± 0.04 ^{a}	0.31 ± 0.07 ^{ab}	0.66 ± 0.00 ^{b}
	IC	0.993 ± 0.022 ^{a}	0.985 ± 0.032 ^{a}	0.258 ± 0.028 ^{a}	0.41 ± 0.15 ^{a}	0.39 ± 0.15 ^{a}	1.48 ± 0.14 ^{a}
		Free amino acids (μmol g^{-1} FW)			Soluble sugars (mg g^{-1} FM)		
Well-watered	MC	2.79 ± 0.80 ^{b}	4.07 ± 0.07 ^{a}	1.82 ± 0.37 ^{b}	7.22 ± 1.52 ^{a}	7.25 ± 2.25 ^{a}	0.72 ± 0.03 ^{c}
	IC	2.85 ± 0.58 ^{b}	4.46 ± 0.58 ^{a}	1.63 ±0 .21 ^{b}	7.16 ± 1.29 ^{a}	7.98 ± 2.96 ^{a}	0.94 ± 0.09 ^{c}
Drought stress	MC	5.49 ± 1.53 ^{a}	4.28 ± 0.45 ^{a}	2.03 ± 0.13 ^{b}	7.93 ± 1.88 ^{a}	7.32 ± 2.31 ^{a}	1.99 ± 0.27 ^{b}
	IC	4.94 ± 0.43 ^{a}	4.45 ± 0.40 ^{a}	2.86±0.24 ^{a}	7.58 ± 2.96 ^{a}	11.0 ± 2.51 ^{a}	3.94 ± 0.15 ^{a}

and a source for carbon and nitrogen for use after relief of water deficit (Verbruggen and Hermans, 2008; Szabados and Savoure, 2009).

Intercropping did not influence the leaf or root osmotic potential in oilseed rape but decreased it in the roots of barley grown under DS conditions (Table 2). In the oilseed rape plants grown under DS conditions, IC plants showed higher concentration of proline and free amino acids in the leaves and higher free amino acids and soluble sugars in the roots compared to the MC plants. In the WW oilseed rape plants, only the leaf concentration of free amino acids was altered by the IC treatment. In barley, leaves did not respond to the IC treatment either in the DS or WW plants, while the roots accumulated all three osmolytes under DS conditions and proline and soluble sugars under WW conditions (Table 2).

The mechanisms behind the influence of the cropping pattern on the osmolyte accumulation are obscure. Intensification of water deficit following an increased competition for water and a faster depletion from the substrate in the IC pots could not be the mechanism for higher osmolytes accumulation. Indeed, the severity of DS could not be affected by cropping pattern because of daily irrigation up to the desired FC in our experiment. In addition, the total water consumption was rather lower in the IC pots compared to the MC oilseed rape (Fig. 1). A modification in the metabolism of plants under the effects of belowground root interactions is not restricted to the influence on the concentrations of organic osmolytes observed in this work and seems to be rather common in intercropping systems. In a proteomics analysis in the millet/peanut intercrop system, the expression of several proteins that are mainly involved in carbon and nitrogen metabolism are upregulated by interspecific root interactions (Zou et al., 2019).

To evaluate the relevance of the different osmotic adjustment parameters in the plants responses to the applied treatments, data were subjected to PCA (Fig. 5). The result showed that the photosynthesis and transpiration rates and the RWC were clustered with biomass data and, thus, were likely the most important parameters determining the plants response to the applied treatments (Fig. 5). Contrastingly, the osmotic adjustment parameters were separately clustered from the biomass data in both species. This was unexpected because the osmolytes contribute undoubtedly to sustaining leaf turgor required for photosynthesis and growth. Nevertheless, results of this analysis may highlight the negative effect of osmolytes synthesis on plants biomass production due to its high carbon and energy costs. Collectively, these data may suggest that, different patterns of osmolytes accumulation could not explain the biomass response of plants to the IC or DS conditions in our experiment.

3.7 EFFECT OF INTERCROPPING INDEPENDENT FROM WATERING TREATMENT

An improvement in the shoot growth of oilseed rape but reduction of it in barley under IC conditions was observed irrespective the watering treatment in this work (Fig. 1). Response of dry matter production to the neighboring plants has been observed for several intercrop systems. Quite different effects have been found: improvement in both crops (Xue et al., 2016), increase of growth only in one of the crops (Zuo et al., 2003), reduction in both (Inal et al., 2007) or even without biomass response (Zuo et al., 2004). Here, higher biomass production in oilseed rape after 10 weeks intercrop may be partly related to the competition for nutrients with barley favoring growth of oilseed rape. Nevertheless, an improved shoot biomass in oilseed rape upon intercropping with barley has also been observed in the hydroponically grown plants supplied with adequate nutrients provided through repeated replacement of nutrient solution (Sadeghzadeh et al., 2021). This may suggest additional mechanisms for the benefit of oilseed rape from an intercropping system.

Similarly, reduction of biomass in barley under IC conditions cannot only be explained by competition for nutrients. Growth impairment in intercropped plants may be mediated by chemical factors released from the roots of neighboring plants including, but not restricted to, allelochemicals. In an oilseed rape/barley intercrop system, we have observed activation of defense pathways, including phenylpropanoid- and salicylic acid-mediated pathways in barley but not in oilseed rape (Hajiboland, unpublished data). Activation of defense that was also observed in other mixed cropping systems (Schmid et al., 2013; Fu et al., 2015), may divert carbon resources from the growth and is likely the mechanism for reduction of dry matter production in barley under IC conditions. Interspecific relations independent from nutrient acquisition capacity in intercropped systems has attracted much less attention and our knowledge about the underlying mechanisms of belowground interactions is largely limited compared to other types of biotic interactions (Subrahmaniam et al., 2013).

The measured physiological parameters subjected to PCA (Fig. 6) showed a distinct clustering of four treatment combinations in oilseed rape. In barley, in contrast, the physiological parameters relevant to the cropping pattern were not clustered separately under WW conditions. This confirmed again the prominent effect of intercropping in oilseed rape irrespective the watering treatment and suggested that, barley may benefit from IC only under DS conditions.

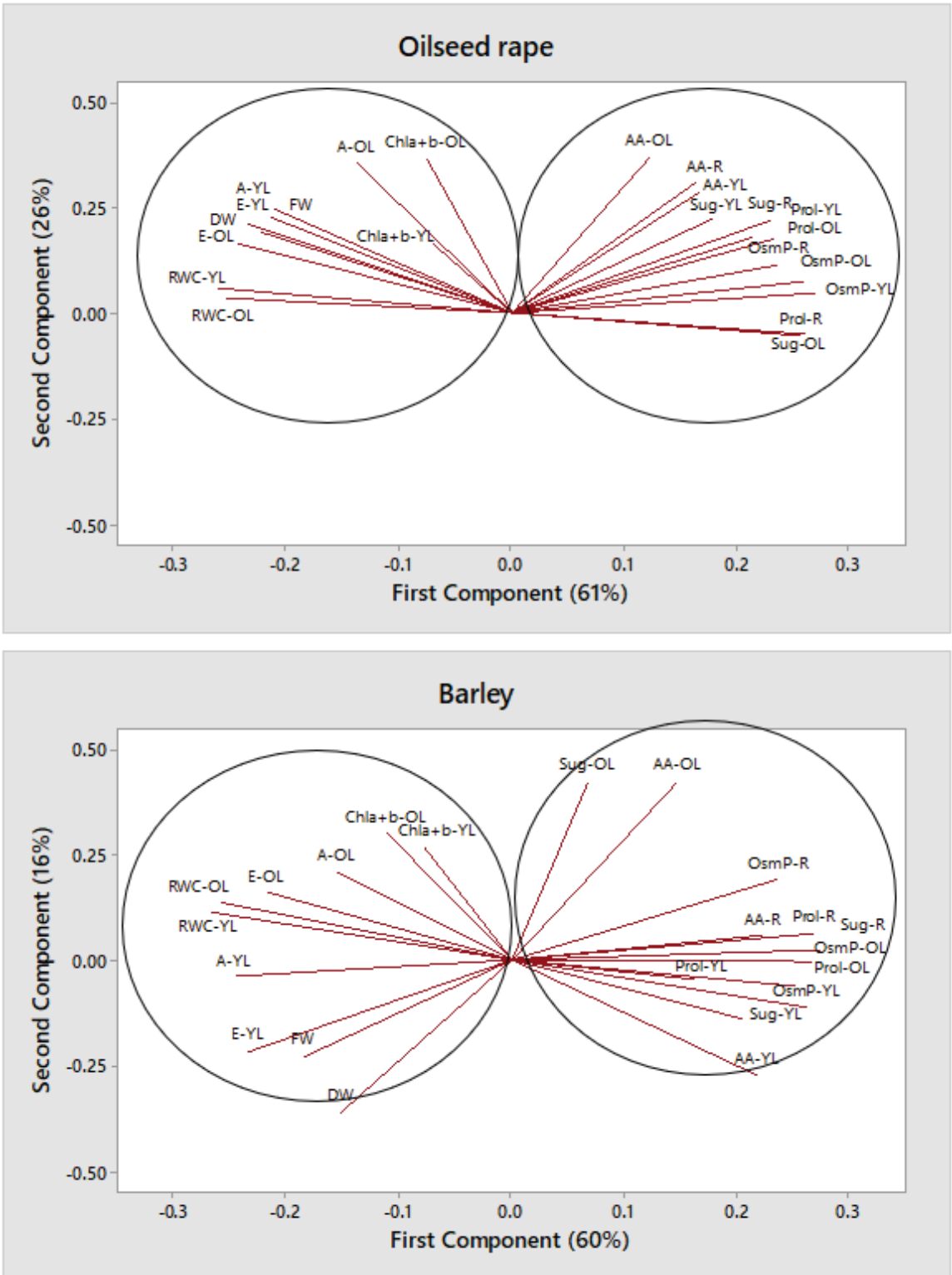


Figure 5: Principal component analysis of various physiological parameters in the young (YL) and old leaves (OL) and roots (R) of oilseed rape and barley cultivated either as monocrop or intercrop under well-watered or drought stress conditions for eight weeks. Abbreviations: Chl (chlorophyll), A: photosynthesis, E: transpiration, FM: fresh mass, DM: dry mass; RWC: relative eater content, AA: concentration of free amino acids, Sug: concentration of soluble sugars, Prol: concentration of proline, Osm: osmotic potential

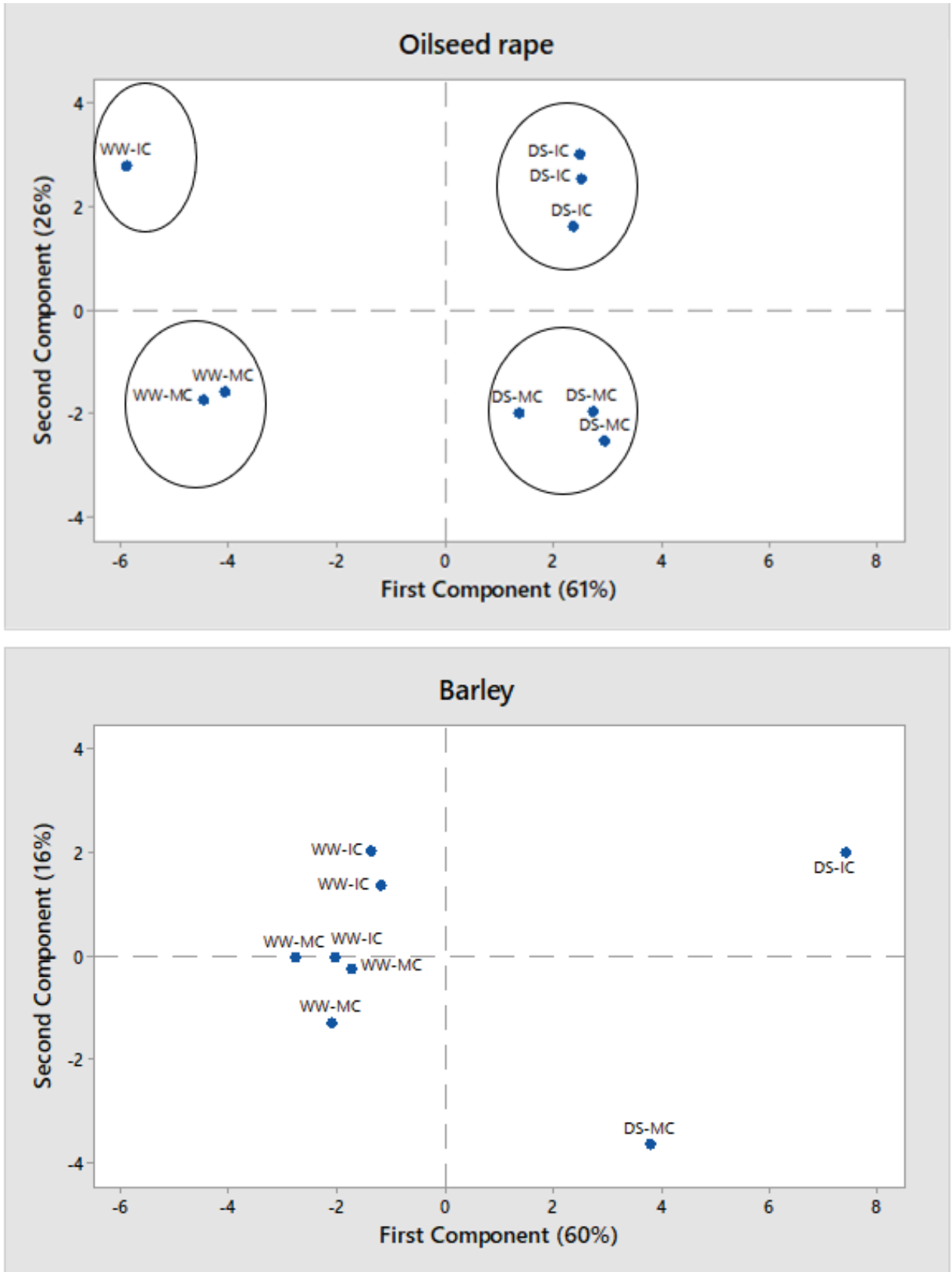


Figure 6: Principal component analysis of various physiological parameters in oilseed rape and barley cultivated either as monocrop (MC) or intercrop (IC) under well-watered (WW) or drought stress (DS) conditions for eight weeks

Fig. 7: Difference in the greenness of the old leaves in oilseed rape cultivated either as monocrop (above) or intercrop (below) with barley under drought stress conditions for eight weeks

3.8 DIFFERENCE BETWEEN THE YOUNG AND OLD LEAVES

The separate analysis of young and old leaves in this work showed differences between these leaves in the two species. The IC-mediated increase in the RWC was observed only in the young leaves of both species. There are evidences on the different response of leaves to drought stress depending on the leaf ontogenetic stage (Chastain et al., 2016). It has been stated that the leaves which develop after imposition of drought stress are more tolerant to water deficit than the old leaves; both in primary photochemistry and carbon reactions (Hajiboland et al., 2014; Chastain et al., 2016). In oilseed rape, further differences in the response to DS and IC between the young and old leaves were observed. The accumulation of proline under DS conditions was much higher in the young than in the old leaves (Table 2).

3.9 LEAF SENESCENCE AS AFFECTED BY INTERCROPPING

The old leaves of intercropped oilseed rape plants retained much better their green colour than the MC plants. This was particularly found under DS conditions (Fig. 7) and was also obvious from the Chl a + b data associated with a higher photosynthesis rate (Table 1). Drought-induced leaf senescence that is characterized by reduction of Chl and photosynthesis rate is an intricate process resulting in remobilization of nutrients to younger leaves thereby contributing to plant fitness (Jan et al., 2019). A direct role in the regulation of drought-induced leaf senescence has been demonstrated for cytokinins and ABA operating at opposite manner (Munné-Bosch and Alegre, 2004). Cytokinin levels that show a positive correlation with the photosynthetic rate and Chl content decrease under drought stress (Munné-Bosch and Alegre, 2004). The mechanism for the IC-mediated prevention of leaf senescence in oilseed rape plants was not addressed here, but could likely be related to an elevated level of cytokinin as was also observed in other belowground root interactions (Chen et al., 2018). Similar to our work on the improvement of Chl and photosynthesis in the oilseed rape, in the peanut/maize intercrop system, a proteomics study showed a three-fold increase in the expression of Rubisco small and large subunits, Rubisco activase and Chl a/b binding proteins compared to monocrop peanut young leaves (Xiong et al., 2013). Our data on the postponing of senescence in the old leaves of oilseed rape by intercropping will putatively increase the leaf area duration in this species and may contribute significantly to the higher biomass production under IC conditions in this species.

4 CONCLUSIONS

Cropping pattern considerably influenced the plants water and osmotic homeostasis under drought stress conditions. Elevated RWC, WUE and an improved osmotic adjustment in both species showed a conspicuous effect of belowground root interaction on plants response to water deficit conditions. Further benefits of IC were higher biomass production and leaf area duration in oilseed rape plants and higher root : shoot ratio in barley. Such plasticity in plant morphological and physiological traits is expected to increase plant performance, canopy photosynthesis and productivity and enhance water capture under intercropping conditions in the field. These results suggest intercropping as a suitable agricultural practice for oilseed rape and barley cultivated under water scarce or rainfed conditions. It is necessary, however, to examine the efficiency of different intercropping patterns for obtaining higher biomass and WUE under field conditions.

5 REFERENCES

Brooker, R. W., Bennett, A. E., Cong, W. F., Daniell, T. J., George, T. S., Hallett, P. D., Hawes, C., Iannetta, P. P., Jones, H. G., Karley, A. J., & Li, L. (2015). Improving intercropping: a synthesis of research in agronomy, plant physiology and ecology. *New Phytologist, 206*(1), 107–117. https://doi.org/10.1111/nph.13132

Chastain, D.R., Snider, J.L., Choinski, J.S., Collins, G.D., Perry, C.D., Whitaker, J., Grey, T.L., Sorensen, R. B., van Iersel, M., Byrd, S.A., & Porter, W. (2016). Leaf ontogeny strongly influences photosynthetic tolerance to drought and high temperature in *Gossypium hirsutum*. *Journal of Plant Physiology, 199*, 18–28. https://doi.org/10.1016/j.jplph.2016.05.003

Chaves, M. M., Flexas, J., & Pinheiro, C. (2009). Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Annals of Botany, 103*, 551–560. https://doi.org/10.1093/aob/mcn125

Chen, B. J., Hajiboland, R., Bahrami-Rad, S., Moradtalab, N., & Anten, N. P. (2019). Presence of belowground neighbors activates defense pathways at the expense of growth in tobacco plants. *Frontiers in Plant Science, 10*, 751. https://doi.org/10.3389/fpls.2019.00751

Cruz de Carvalho, M.H. (2008). Drought stress and reactive oxygen species: production, scavenging and signaling. *Plant Signaling & Behavior, 3*, 156–165. https://doi.org/10.4161/psb.3.3.5536

Daneshnia, F., Amini, A., & Chaichi, M. R. 2015. Surfactant effect on forage yield and water use efficiency for berseem

clover and basil in intercropping and limited irrigation treatments. *Agricultural Water Management, 160*, 57–63. https://doi.org/10.1016/j.agwat.2015.06.024

Gao, Y., Duan, A., Sun, J., Li, F., Liu, Z., Liu, H., & Liu, Z. (2009). Crop coefficient and water-use efficiency of winter wheat/spring maize strip intercropping. *Field Crops Research, 111*, 65–73. https://doi.org/10.1016/j.fcr.2008.10.007

Grema, A.K., & Hess, T.M. (1994). Water balance and water use of millet-cowpea intercrops in north east Nigeria. *Agricultural Water Management, 26*, 169–185. https://doi.org/10.1016/0378-3774(94)90056-6

Hajiboland, R., Norouzi, F., & Poschenrieder, C. (2014). Growth, physiological, biochemical and ionic responses of pistachio seedlings to mild and high salinity. *Trees*, *28*, 1065–1078. https://doi.org/10.1007/s00468-014-1018-x

Jan, S., Abbas, N., Ashraf, M., & Ahmad, P. (2019). Roles of potential plant hormones and transcription factors in controlling leaf senescence and drought tolerance. *Protoplasma, 256*, 313–329. https://doi.org/10.1007/s00709-018-1310-5

Koch, G., Rolland, G., Dauzat, M., Bédiée, A., Baldazzi, V., Bertin, N., Guédon, Y., & Granier, C. (2019). Leaf production and expansion: a generalized response to drought stresses from cells to whole leaf biomass– a case study in the tomato compound leaf. *Plants, 8*, 409. https://doi.org/10.3390/plants8100409

Kong, C. H., Zhang, S. Z., Li, Y. H., Xia, Z. C., Yang, X. F., Meiners, S. J., & Wang, P. (2018). Plant neighbor detection and allelochemical response are driven by root-secreted signaling chemicals. *Nature Communications, 9*, 3867. https://doi.org/10.1038/s41467-018-06429-1

Krasensky, J., & Jonak, C. (2012). Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *Journal of Experimental Botany, 63*, 1593–1608. https://doi.org/10.1093/jxb/err460

Kumar, S., Kaushal, N., Nayyar, H., & Gaur, P. (2012). Abscisic acid induces heat tolerance in chickpea (*Cicer arietinum* L.) seedlings by facilitated accumulation of osmoprotectants. *Acta Physiologiae Plantarum, 34*, 1651–1658. https://doi.org/10.1007/s11738-012-0959-1

Lemoine, R., Camera, S. L., Atanassova, R., Dédaldéchamp, F., Allario, T., Pourtau, N., Bonnemain, J. L., Laloi, M., Coutos-Thévenot, P., Maurousset, L., Faucher, M., Girousse, C., Lemonnier, P., Parrilla, J., & Durand, M. (2013). Source-to-sink transport of sugar and regulation by environmental factors. *Frontiers in Plant Science, 4*, 272. https://doi.org/10.3389/fpls.2013.00272

Lugojan, C., & Ciulca, S. (2011). Evaluation of relative water content in winter wheat. *Journal of Horticultural Science and Biotechnology, 15*, 173–177.

Martin-Guay, M. O., Paquette, A., Dupras, J., & Rivest, D. (2018). The new green revolution: sustainable intensification of agriculture by intercropping. *Science of the Total Environment, 615*, 767–772. https://doi.org/10.1016/j.scitotenv.2017.10.024

Mc Adam, S. A., Brodribb, T. J., & Ross, J. J. (2016). Shoot-derived abscisic acid promotes root growth. *Plant, Cell Environment, 39*, 652–659. https://doi.org/10.1111/pce.12669

Mommer, L., Kirkegaard, J., & van Ruijven, J. (2016). Root–root interactions: towards a rhizosphere framework. *Trends in Plant Science, 21*, 209–217. https://doi.org/10.1016/j.tplants.2016.01.009

Noctor, G., Mhamdi, A., & Foyer, C. H. (2014). The roles of reactive oxygen metabolism in drought: not so cut and dried. *Plant Physiology*, *164*, 1636–1648. https://doi.org/10.1104/pp.113.233478

Parida, A. K., Dagaonkar, V. S., Phalak, M.S., & Aurangabadkar, L. P. (2008). Differential responses of the enzymes involved in proline biosynthesis and degradation in drought tolerant and sensitive cotton genotypes during drought stress and recovery. *Acta Physiologiae Plantarum, 5*, 619–627. https://doi.org/10.1007/s11738-008-0157-3

Rees, D. J. (1986). The effects of population density and intercropping with cowpea on the water use and growth of sorghum in semi-arid conditions in Botswana. *Agricultural and Forest Meteorology, 37*, 293–308. https://doi.org/10.1016/0168-1923(86)90067-5

Sadeghzadeh, N., Hajiboland, R., Moradtalab, N., Poschenrieder, C. 2021. Growth enhancement of *Brassica napus* under both deficient and adequate iron supply by intercropping with *Hordeum vulgare*: a hydroponic study. *Plant Biosystems, 155*, 632–646. https://doi.org/10.1080/11263504.2020.1769215

Sánchez-Blanco, M. J., Alvarez, S., Ortuño, M. F., & Ruiz-Sánchez, M. C. (2014). Root system response to drought and salinity: root distribution and water transport. In: Morte, A. & Varma, A. (Eds.) *Root Engineering* (pp 325–352). Germany, Springer. https://doi.org/10.1007/978-3-642-54276-3_15

Semchenko, M., Saar, S., & Lepik, A. (2014). Plant root exudates mediate neighbour recognition and trigger complex behavioural changes. *New Phytologist, 204*, 631–637. https://doi.org/10.1111/nph.12930

Shackel, K. A., & Hall, A. E. (1984). Effect of intercropping on the water relations of sorghum and cowpea. *Field Crops Research, 8*, 381–387. https://doi.org/10.1016/0378-4290(84)90085-6

Singh, M., Kumar, J., Singh, S., Singh, V. P., & Prasad, S. M. (2015). Roles of osmoprotectants in improving salinity and drought tolerance in plants: a review. *Reviews in Environmental Science and Biotechnology, 14*, 407–426. https://doi.org/10.1007/s11157-015-9372-8

Singh, S., Narwal, S. S., & Chander, J. (1988). Effect of irrigation and cropping systems on consumptive use, water use efficiency and moisture extraction patterns of summer fodders. *International Journal of Tropical Agriculture, 6*, 76–82.

Skirycz, A., & Inze, D. (2010). More from less: plant growth under limited water. *Current Opinion in Biotechnology*, *21*, 197–203. https://doi.org/10.1016/j.copbio.2010.03.002

Szabados, L., & Savouré, A. (2009). Proline: a multifunctional amino acid. *Trends in Plant Science*, *15*, 89–97. https://doi.org/10.1016/j.tplants.2009.11.009

Tambussi, E. A., Bort, J., & Araus, J. L. (2007). Water use efficiency in C_3 cereals under Mediterranean conditions: a review of physiological aspects. *Annals of Applied Biology, 150*, 307–321. https://doi.org/10.1111/j.1744-7348.2007.00143.x

Tardieu, F., Simonneau, T., & Muller, B. (2018). The physiological basis of drought tolerance in crop plants: a scenario-dependent probabilistic approach. *Annual Review of Plant*

Biology, 69, 733–759. https://doi.org/10.1146/annurev-arplant-042817-040218

Tardieu, F., Granier, C., & Muller, B. (2011). Water deficit and growth. Coordinating processes without an orchestrator? *Current Opinion in Plant Biology, 14*, 283–289. https://doi.org/10.1016/j.pbi.2011.02.002

Verbruggen, N., & Hermans, C., (2008). Proline accumulation in plants: a review. *Amino Acids, 35*, 753–759. https://doi.org/10.1007/s00726-008-0061-6

Xiong, H., Shen, H., Zhang, L., Zhang, Y., Guo, X., Wang, P., Duan, P., Ji, C., Zhong, L., Zhang, F., & Zuo, Y. (2013). Comparative proteomic analysis for assessment of the ecological significance of maize and peanut intercropping. *Journal of Proteomics, 78*, 447–460. https://doi.org/10.1016/j.jprot.2012.10.013

Zuo, Y. (2013). Comparative proteomic analysis for assessment of the ecological significance of maize and peanut intercropping. *Journal of Proteomics, 78*, 447–460. https://doi.org/10.1016/j.jprot.2012.10.013

Xue, Y., Xia, H., Christie, P., Zhang, Z., Li, L., & Tang, C. (2016). Crop acquisition of phosphorus, iron and zinc from soil in cereal/legume intercropping systems: a critical review. *Annals of Botany, 117*, 363–377. https://doi.org/10.1093/aob/mcv182

Zhou, S., Duursma, R. A., Medlyn, B. E., Kelly, J. W. G., & Prentice, I. C. (2013). How should we model plant responses to drought? An analysis of stomatal and non-stomatal responses to water stress. *Agricultural and Forest Meteorology, 182/183*, 204–214. https://doi.org/10.1016/j.agrformet.2013.05.009

Zuo, Y., Li, X., Cao, Y., Zhang, F., & Christie, P. (2003). Iron nutrition of peanut enhanced by mixed cropping with maize: possible role of root morphology and rhizosphere microflora. *Journal of Plant Nutrition, 26*, 2093–2110. https://doi.org/10.1081/PLN-120024267

Zuo, Y., Liu, Y., Zhang, F., & Christie, P. (2004). A study on the improvement iron nutrition of peanut intercropping with maize on nitrogen fixation at early stages of growth of peanut on a calcareous soil. *Soil Science & Plant Nutrition, 50*, 1071–1078. https://doi.org/10.1080/00380768.2004.10408576

Yemm, E. W., & Willis, A. J. (1954). The estimation of carbohydrates extracts by anthrone. *Biochemistry Journal*, *57*, 508–514. https://doi.org/10.1042/bj0570508

Fu, X., Wu, X., Zhou, X., Liu, S., Shen, Y., & Wu, F. (2015). Companion cropping with potato onion enhances the disease resistance of tomato against *Verticillium dahliae*. *Frontiers in Plant Science, 6*, 726. https://doi.org/10.3389/fpls.2015.00726

doi:10.14720/aas.2022.118.3.2363 Original research article / izvirni znanstveni članek

Viability of seeds of two varieties of *Coffea arabica* L. using different pretreatments in the tetrazolium test

José Duván BUENDÍA CONTRERAS [1], Laura Yolima MORENO ROZO [2], Seir Antonio SALAZAR MERCADO [2, 3]

Received October 06, 2021; accepted August 04, 2022.
Delo je prispelo 6. oktobra 2021, sprejeto 4. avgusta 2022

Viability of seeds of two varieties of *Coffea arabica* L. using different pretreatments in the tetrazolium test

Abstract: This research attempted to determine the efficacy of the tetrazolium test in the evaluation of the seed viability of two varieties of *Coffea arabica* L. ('Castillo' and 'Cenicafé'). The fruits were obtained from crops located in the municipalities of Salazar de las Palmas and Arboledas (Norte de Santander - Colombia). The test was carried out with embryos manually extracted from the seeds using tweezers. Three pretreatments were established: distilled water, sodium hypochlorite (2.5 %), sucrose (10 %), and a control (no pretreatment). Embryos were placed in a cysteine solution (0.5 %) to prevent oxidation, then immersed in tetrazolium solutions with concentrations of 0.035 %, 0.075 %, and 0.1 % for a period of 6, 9, and 12 hours in darkness. The results of the viability test were validated with seed germination, using the wet paper towel method in darkness. The best viability percentages were found with the application of sodium hypochlorite (NaClO 2.5 %), with a high correlation with the germination percentage. The use of pretreatments improved the efficiency of the viability test and allowed the use of low concentrations of the reagent (0.035 %), giving the farmer a quick and less expensive alternative to determine germination capacity.

Key words: coffee; germination; optimization; pretreatments

Viabilnost semen dveh sort kavovca (*Coffea arabica* L.) z uporabo različnih predobravnavanj pred tetrazolijevim testom

Izvleček: V raziskavi smo poskušali določiti učinkovitost tetrazolijevega testa za ovrednotenje viabilnosti semen dveh sort kavovca, *Coffea arabica* L. ('Castillo' in 'Cenicafé'). Plodovi kavovca so bili pridobljeni z območij Salazar de las Palmas in Arboledas (Norte de Santander - Colombia). Test je bil izveden na embrijih, ki smo jih s pinceto izolirali iz semen. Uporabljena so bila tri predobravnavanja in sicer: distilirana voda, natrijev hipoklorit (2,5 %), saharoza (10 %) in kontrola (brez predobravnavanja). Embriji so bili najprej vstavljeni v raztopino cisteina (0,5 %) za preprečitev oksidacije in nato potopljeni v različne koncentracije raztopin tetrazolija (0,035 %; 0,075 % in 0,1 %) za 6, 9, za 12 ur v temi. Rezulti viabilnosti embrijev so bili ovrednoteni s kalitvijo semen v temi na vlažnem filtrirnem papirju. Največji odstotek viabilnih semen je bil ugotovljen pri uporabi natrijevega hipoklorita (NaClO 2,5 %), z največjo korelacijo z odstotkom kalitve. Uporaba predobravnavanj je izboljšala učinkovitost testa viabilnosti in je dopuščala uporabo majnih koncentracij reagenta (0,035 %), kar daje kmetom hitro in manj drago alternativo določanja kalivosti.

Ključne besede: kavovec; kalitev; optimizacija; predobravnavanja

1 Master of Biological Sciences, Universidad Francisco de Paula Santander, San José de Cúcuta, Colombia
2 Department of Biology, Universidad Francisco de Paula Santander, San José de Cúcuta, Colombia
3 Corresponding author, e-mail: seirantoniosm@ufps.edu.co

1 INTRODUCTION

Coffea arabica represents one of the most important crops in the world (RAMÍREZ et al., 2016; Selmar et al., 2008). Global coffee consumption has been increasing due to its pleasant aroma and flavor, as well as its beneficial health effects, leading to a large amount of by-product generation (Khochapong et al., 2021). Coffee is one of the most traded beverages in the world and in 2016/17 more than 9 million tons were consumed (da Silva et al., 2021). The main coffee producers are Brazil, Colombia, Vietnam, India, and Indonesia which represents relevant economic importance for these countries (da Silva et al., 2021). 12.5 million households worldwide receive income from coffee cultivation (Montagnon et al., 2021). Commercially cultivated coffee species are *Coffea arabica* L. and *C. canephora* Pierre ex A.Froehner (Mishra & Slater, 2012). Tropical African countries such as Ethiopia, Sudan, Kenya, Guinea, or Mozambique are usually pointed out as possible centers of origin, although the most accepted is Ethiopia (Rojo, 2014).

The coffee plant belongs to the Rubiaceae family and there are more than 70 species of the *Coffea* L. genus (da Silva et al., 2021), it is a tropical evergreen tree, growing at altitudes between 700 and 2000 m.a.s.l. (Tinoco & Peña, 2019). The green seeds of *Coffea arabica* are very rich in secondary metabolites. These metabolites have antioxidant properties that reduce the incidence of cancer and diabetes (Aissaoui et al., 2020). The coffee seed is a nut, oblong, flat-convex, of variable size (10-18 mm long and 6.5-9.5 mm wide) and mostly constituted by a horny endosperm where, at one of its ends and very superficially, lies an embryo of 3.5 to 4.5 mm long, with a conical radicle and cordate cotyledons (Arcila et al., 2007). An undesirable feature of coffee seeds is that they have slow and asynchronous germination, which prevents obtaining seedlings of desirable quality; this type of germination hinders rapid evaluations of viability and/or vigor due to the excessive time required to obtain results (Da Rosa et al., 2010).

The use of quick methods to know the viability is important, to accelerate decision-making regarding the management of seed lots, (Medeiros et al., 2015). For this reason, the tetrazolium salt test has assumed a prominent place for some crops, mainly due to the large amount of information that the test provides. *Tetrazolium* (TZ) can be used regardless of the degree of seed dormancy, becoming very important for species with this problem. It is also a very useful tool for seed producers, graders, and traders as it can help in decisions that need to be made quickly. (França-Neto and Krzyzanowski, 2019). The tetrazolium test is based on the activity of dehydrogenase enzymes that reduce the tetrazolium salt in living seed tissues by generating triphenyl formazan, a non-diffusible red-colored compound, which indicates respiratory activity and viability of cells and tissues; in contrast, dead tissues show no coloration (Salazar et al., 2018; Salazar et al., 2020a). It is important to indicate that the effectiveness of the tetrazolium test is mediated by the development of preconditioning procedures, which facilitate the entry of the tetrazolium solution into the seed (Hosomi et al., 2017; Salazar et al., 2019).

Decreasing the concentration of tetrazolium and verifying its efficacy allows the evaluator a reasonable and optimal use of this reagent, reducing the total cost of the test. According to the above, this work aims to determine the efficacy of the tetrazolium test in the evaluation of seed viability of two varieties of *Coffea arabica* using different pretreatments.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL

The ripe fruits of the Castillo coffee variety (*C. arabica*) were collected in village Alto del Angulo in the municipality of Salazar de las Palmas. The Cenicafé variety drupes were collected in plantations in the village of San Onofre in the municipality of Arboledas, both belonging to Norte de Santander (Colombia). The material was placed in plastic containers with lids, lined with newspaper to avoid deterioration, and transported to the Biology laboratories of the Faculty of Basic Sciences at the Universidad Francisco de Paula Santander where the research study was carried out.

2.2 PRETREATMENT AND VIABILITY OF SEEDS

Solutions of 2,3,5-triphenyl tetrazolium chloride were prepared with concentrations of 0.035 %, 0.075 % and 0.1 %. To avoid embryo oxidation, a 50 mg l^{-1} cysteine solution was prepared (Azofeifa, 2009). The solutions were kept in dark bottles and stored in the refrigerator at 5 °C (Salazar et al., 2020b; Salazar et al., 2020c). Three pretreatments were established: 6-hour immersion in 2.5 % sodium hypochlorite (NaClO), 10 % sucrose (C12H22O11), and water (H2O). The fourth group of seeds corresponded to the control group.

The fleshy layers (protective skin, pulp, parchment) and the integument were removed from the fruits to obtain the endosperm containing the embryos. The embryos were extracted manually, using a dissecting case and forceps. One hundred embryos were placed in each Petri dish immersed in 5 ml of tetrazolium solution. Each test

had 5 replicates for a total of 500 embryos for each exposure time established (6 h, 9 h, 12 h) in complete darkness. An intense reddish coloration of the embryo was determined as positive for the tetrazolium test.

2.3 GERMINATION TEST

The germination test was performed to corroborate the data obtained in the viability test. Five replicates of 100 seeds were used for 500 seeds (Salazar et al., 2020b). For the test, the seeds were removed from the endosperm, washed with distilled water, and placed to germinate for 30 days in total darkness on paper towels moistened with distilled water in previously disinfected plastic containers. Germinated seeds were considered to be those that presented a root growth at least 4 mm long or that presented geotropic curvature and the result was expressed as germination percentage.

2.4 STATISTICAL ANALYSIS

For the viability test and germination test, data were randomly distributed with 5 replicates and 100 seeds per replicate. The experimental design consisted of a completely randomized block analysis. Data were analyzed using Infostat statistical software by analysis of variance (ANOVA), followed by Tukey's multiple range HSD (Honest Significant Difference) test, to compare averages and determine significant differences at a level of $p \leq 0.05$.

3 RESULTS AND DISCUSSION

3.1 PRETREATMENT AND VIABILITY OF SEEDS

The tetrazolium test evaluates the physical and physiological conditions of the embryonic structure of each seed (França-Neto & Krzyzanowski, 2019). Moreover, viable seeds are easily identified by their carmine red coloration (Figure 1), due to the reductive reaction of the tetrazolium solution under the action of dehydrogenase enzymes in cellular respiration (Salazar et al., 2020a).

In the results shown in Table 1, it can be observed that the pretreatment with chlorine (2.5 % 6 h) was the one that obtained the highest viability in all concentrations and exposure times with tetrazolium, values that ranged between 92 % and 100 % viability in *C. arabica* 'Castillo'. However, no significant differences were found between the control and sucrose pretreatment at the following concentrations and exposure time with tetrazolium: 0.035 % 6, 9, 12 h; 0.075 % 9, 12 h; 0.1 % 6, 9, 12 h (Table 1). The lowest viability values were found in the pretreatment with distilled water in most of the concentrations and exposure time with tetrazolium, unlike the pretreatments with sucrose (0.035 % 12 h, 0.075 % 6 h, and 0.1 % 6 h) where the viability using the tetrazolium test were lower.

The findings in the Cenicafe variety showed few significant differences among pretreatments, concentrations, and exposure times with tetrazolium (Table 2). However, the chlorine treatment had a better effect on seed viability. The lowest viability value (88 %) was found in the sucrose pretreatment, at the tetrazolium concentration and exposure time of 0.035 % 9 h (Table 2). In addition, the preconditioning with distilled water identified statistically homogeneous means with the chlorine pretreatment data, unlike the concentration and exposure period of 0.075 % 12 h of tetrazolium.

According to Lamarca and Barbedo (2014), the higher the concentration and exposure period to tetrazolium, the greater the intensity of embryo coloration, which facilitates the analysis and evaluation of viable and non-viable seeds. According to Tola et al. (2019), exposure times of 5, 16, and 24 h and concentrations of 0.1 %, 0.5 %, and 1 % tetrazolium are suitable for viability analysis in coffee seeds. However, in this research, it was possible to obtain reliable viability levels thanks to the use of pre-treatments with chlorine (2.5 % 6 h), in concentrations of 0.035 %, 0.075 %, 0.1 %, and exposure times of 6, 9, 12 h of tetrazolium. These parameters are decisive in the tetrazolium test since they improve the efficiency of the viability test. Likewise, Salazar et al. (2020b) increased the efficiency of the tetrazolium test on *S. lycopersicum* L. seeds, using 1 % sodium hypochlorite and

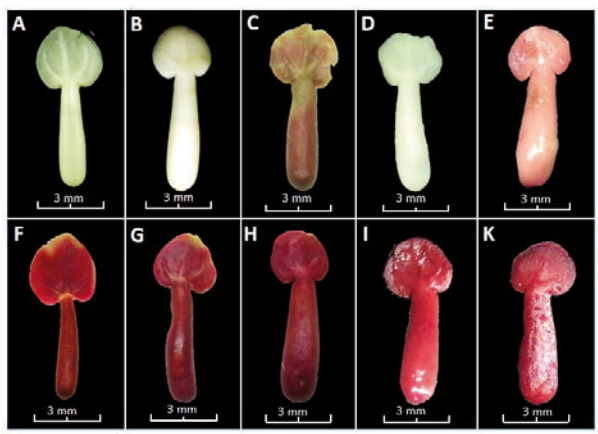


Figure 1: Viability of *Coffea arabica* seeds using the tetrazolium test. (A, B, C) non-viable seeds *C. arabica* Castilla' (D, E) non-viable seeds *C. arabica* 'Cenicafe'. (F, G, H): viable seed *C. arabica* 'Castilla' (I, K) viable seed *C. arabica* 'Cenicafe'

Table 1: Viability of *Coffea arabica* 'Castilla' seeds

	Tetrazolium concentration and exposure time								
Pretreatments	0.035 % 6 h	0.035 % 9 h	0.035 % 12 h	0.075 % 6 h	0.075 % 9 h	0.075 % 12 h	0.1 % 6 h	0.1 % 9 h	0.1 % 12 h
Control	90 ± 5.4 a	98 ± 4.4 a	96 ± 4.4 a	94 ± 8.9 a	98 ± 4.4 a	100 a	96 ± 5.4 ab	98 ± 4.4 a	100 a
H_2O 6h	72 ± 8.3 b	84 ± 8.9 b	96 ± 5 a	96 ± 5.4 a	88 ± 4.0 b	98 ± 4.4 a	92 ± 8.3 ab	98 ± 3.4 a	92 ± 5.4 b
Sucrose 10% 6h	92 ± 8.3 a	96 ± 54 a	94 ± 5.4 a	78 ± 7.8 b	98 ± 4.1 a	100 a	82 ± 7.8 a	98 ± 4.2 a	98 ± 1.9 a
Chlorine 2.5% 6h	92 ± 7.5 a	98 ± 4.4 a	96 ± 8.9 a	98 ± 4.4 a	98 ± 3.9 a	100 a	100 b	100 a	100 a

Different letters indicate significant differences ($p \leq 0.05$)
± Standard deviation

Table 2: Viability of *Coffea arabica* 'Cenicafe' seeds

	Tetrazolium concentration and exposure time								
Pretreatments	0.035 % 6 h	0.035 % 9 h	0.035 % 12 h	0.075 % 6 h	0.075 % 9 h	0.075 % 12 h	0.1 % 6 h	0.1 % 9 h	0.1 % 12 h
Control	89 ± 4.4 a	98 ± 4.4 a	98 ± 4.4 a	98 ± 4.4 a	96 ± 5.4 a	100 a	98 ± 4.4 a	98 ± 4.4 a	98 ± 3.5 a
H_2O 6h	96 ± 5.4 a	98 ± 4.2 a	94 ± 8.9 a	96 ± 5.4 a	98 ± 4.4 a	92 ± 8.3 b	98 ± 4.4 a	98 ± 4.4 a	98 ± 4.1 a
Sucrose 10% 6h	96 ± 5.4 a	88 ± 4.1 b	98 ± 4.4 a	98 ± 4.4 a	98 ± 4.4 a	100 a	92 ± 8.3 a	92 ± 8.4 a	98 ± 3 a
Chlorine 2.5% 6h	96 ± 5.2 a	98 ± 4.2 a	98 ± 4.2 a	98 ± 4.4 a	98 ± 4.4 a	100 a	98 ± 5.4 a	98 ± 4.4 a	100 ± 5 a

Different letters indicate significant differences ($p \leq 0.05$)
± Standard deviation

tetrazolium concentrations of 0.25% and 0.15% in 24h. However, in a study by Clemente et al. (2011) and Clemente et al. (2012), subjecting sodium hypochlorite (5 %, 6 %) for 6 h negatively impairs tetrazolium test results in *C. arabica* seeds with a moisture content below 25 %. Strobel et al. (2016) state that chlorine doses can generate embryo damage, leading to decreased seed viability. In addition, Salazar and Maldona, (2020) express, that chlorine generates toxic effects on cells at low doses, because it is a strong oxidant compound (Jiang et al., 2017).

In several studies sucrose solution (10 %) has had a positive effect on seed viability because sucrose is linked to the activation of metabolic processes related to dehydrogenase enzymes in cellular respiration, helping to maintain an osmotic balance in the seed, which prevents damage to the embryo (Mercado and Jaimes, 2022; Salazar and Botello, 2020; Salazar et al., 2019; Hosomi et al., 2017; Hosomi et al., 2012). Moreover, distilled water treatments resulted in low viability in the Castillo variety at several periods and tetrazolium concentrations. These results differ with Mercado et al. (2020), where pretreatment with distilled water on *Epidendrum microtum* Lindl seeds had 100% viability. According to Clemente et al. (2012), an imbibition time of 48 hours facilitates embryo extraction and does not affect the results of the tetrazolium test. According to Carvalho et al. (2014), hydration favors the absorption of tetrazolium and provides the activation of enzymatic metabolism.

3.2 COMPARISON OF GERMINATION AND VIABILITY

The viability test should be validated with the germination test, indicating the consistency of the findings concerning the behavior of the physiological quality of the seeds (Tola et al., 2019). In the Castillo variety, the average germination percentage was 95 %, comparing with the viability results, the same trend was maintained, where no statistically significant differences were found with the hypochlorite treatments. Similarly, in the cenicafe variety, the germination percentage was 96 %, which is positively correlated with the viability test. This reflects the efficacy of the pretreatments in improving seed viability. According to Salazar et al. (2020c), the germination test indicates whether the seeds did not germinate because they were dormant or because they showed embryo deterioration, being used to corroborate the vi-

ability results generated by the tetrazolium test. However, in most cases the viability test generates higher percentages concerning the germination test, agreeing with the studies conducted by Tola et al. (2019) on *Coffea arabica* seeds.

According to the results reported by Fantazzini et al. (2020), the tetrazolium test is not suitable for seeds with germination percentages lower than 60 %. Likewise, these authors report that for seeds with a germination value greater than 60 %, the results of the tetrazolium test were similar to those of the germination test on *C. arabica* seeds. Likewise, Figueiredo et al. (2017) point out, that several investigations have shown the discrepancy between the results of the tetrazolium and germination test in coffee seeds, specifically in those of lower quality.

Germination of *C. arabica* seeds occurs slowly and inconsistently. Therefore, the tetrazolium test is presented as a quick alternative to evaluate viability, provided that seed preparation and imbibition are established (Clemente 2012). In this research, germination percentages were higher than 93 % in the Castillo and Cenicafe varieties and indicated a close relationship with viability percentages with tetrazolium. According to the above, the use of pretreatments can improve the efficacy of the tetrazolium test on *C. arabica* seeds. In this case, the seeds exposed to sodium hypochlorite (2.5 % 6 h) generated high viability percentages, which allows reducing the concentrations and exposure periods of tetrazolium to 0.035 % and 6 h, respectively, which leads to a reduction in the cost of the reagent triphenyl tetrazolium chloride to determine the viability of *C. arabica* seeds.

4 CONCLUSIONS

Rapid identification of viability in a seed group is important because it allows decisions to be made about it. Coffee seed gives germination results in 25 to 30 days, but this can be extended up to 40 days. The use of tetrazolium determines effectively and with real results the viability of the seeds, considering the prolonged germination time. For the Castillo and Cenicafé varieties, the best viability percentages were found with the application of sodium hypochlorite (NaClO 2.5 %), with a high correlation with the germination percentage. The use of pretreatments improves the efficiency of the viability test, allowing the use of low concentrations, up to 0.035 %, giving the farmer a more economical alternative to the expenses generated by the use of the reagent.

5 REFERENCES

Arcila P, J.; Farfan V., F.; Moreno B., A.M.; Salazar G., L.F.; Hincapié G., E. (2007). *Sistemas de producción de café en Colombia*. Chinchiná, Centro Nacional de Investigaciones en Café (CENICAFE) 309 p. Disponible en https://www.cenicafe.org/es/publications/sistemas_de_produccion.pdf

Arvy, M. P.; Gallouin, F.; Ubillos, M. Á. M. y Montalbán, J. M. (2007). *Especias, aromatizantes y condimentos*. Mundi-Prensa, p. 77. Disponible en https://books.google.com.gt/books/about/Especias_aromatizantes_y_condimentos.html?id=yR9R2SBJlQ0C

Aissaoui, M., Rahmoun, N. M., Barek, S., Bensouici, C., & El Haci, I. A. (2020). Structural characterization of phenolic content, antioxidant and antibacterial activities of *Coffea arabica* green seeds. *Vegetos, 33*(3), 466-474. https://doi.org/10.1007/s42535-020-00130-9

Azofeifa-Delgado, L. (2008). Problemas de oxidación y oscurecimiento de explantes cultivados in vitro. *Agronomía Mesoamericana*, *20*(1), 153. https://doi.org/10.15517/am.v20i1.4990

Bello Ramírez, L. A., Daza Hernández, D. C., Forero Sarmiento, J. E., Linares Bautista, M. Ángel, & Lesmes Vesga, R. A. (2017). Evaluación de tratamientos pre-germinativos en semillas de café (*Coffea arabica* L.) variedad Castillo. *Revista Sennova: Revista Del Sistema De Ciencia, Tecnología E Innovación*, *2*(1), 136–161.

Carvalho, T., Ribeiro, C., Castro, O. & Panobianco, M. (2014). Adaptation of the tetrazolium test method for estimating the viability of sorghum seeds. *Journal of Seed Science*, *36,* 246-252. https://doi.org/10.1590/2317-1545v32n2713

Clemente, A. D. C. S., Carvalho, M. L. M. D., Guimarães, R. M., & Zeviani, W. M. (2011). Preparo das sementes de café para avaliação da viabilidade pelo teste de tetrazólio. *Revista Brasileira de Sementes*, *33*, 38-44. https://doi.org/10.1590/S0101-31222011000100004

Clemente, A. D. C. S., Carvalho, M. L. M. D., & Guimarães, R. M. (2012). Suitability of the tetrazolium test methodology for recently harvested and stored coffee seeds. *Ciência e Agrotecnologia 36*, 415-423. https://doi.org/10.1590/S1413-70542012000400005

Da Rosa, S. D. V. F., McDonald, M. B., Veiga, A. D., Vilela, F. D. L., & Ferreira, I. A. (2010). Staging coffee seedling growth: a rationale for shortening the coffee seed germination test. *Seed Science and Technology*, *38*(2), 421-431. https://doi.org/10.15258/sst.2010.38.2.15

Da Silva, C. Q., da Silva Fernandes, A., Teixeira, G. F., França, R. J., da Costa Marques, M. R., Felzenszwalb, I., .. & Ferraz, E. R. A. (2021). Risk assessment of coffees of different qualities and degrees of roasting. *Food Research International*, *141*, 110089. https://doi.org/10.1016/j.foodres.2020.110089

França-Neto, J. D. B., & Krzyzanowski, F. C. (2019). Tetrazolium: an important test for physiological seed quality evalu-

ation. *Journal of Seed Science*, *41*(3), 359-366. https://doi.org/10.1590/2317-1545v41n3223104

Fantazzini, T. B., Franco da Rosa, S. D. V., Carvalho, G. R., Liska, G. R., de Carvalho, M. L. M., Coelho, S. V. B., ... & Ribeiro, F. A. S. (2020). Correlation between historical data of the germination test and of the tetrazolium test in coffee seeds by GAMLSS. *Seed Science and Technology*, *48*(2), 179-188. https://doi.org/10.15258/sst.2020.48.2.05

Figueiredo, M.A., Coelho, S.V.B., Rosa, S.D.V.F., Vilela, A.L. and Silva, L.C. (2017). Exploratory studies for cryopreservation of *Coffea arabica* L. seeds. *Journal of Seed Science*, *39*, 150-158. http://doi.org/10.1590/2317-1545v39n2171123

Hosomi ST, de Souza TB, Custódio CC, Neto NBM (2017) Refining the tetrazolium test for evaluation of *Cattleya labiata* and *C. tigrina* seeds viability. *Australian Journal of Crop Science*, *11*(10), 1320-1326. https://doi.org/10.21475/ajcs.17.11.10.pne606

Hosomi, S.T., Cust_odio, C.C., Seaton, P.T., Marks, T.R., Barbosa, N.M.N. (2012). Improved assessment of viability and germination of *Cattleya* (Orchidaceae) seeds following storage. *In Vitro Cellular & Developmental Biology Plant, 48*, 127–136. https://doi.org/10.1007/s11627-011-9404-1

Jiang, H., Chen, M. & Lee, Y. (2017). In vitro germination and low-temperature seed storage of *Cypripedium lentiginosum* P. J. Cribb & S.C. Chen, a rare and endangered lady's slipper orchid. *Scientia Horticulturae, 225*, 471-479. https://doi.org/10.1016/j.scienta.2017.07.040

Khochapong, W., Ketnawa, S., Ogawa, Y., & Punbusayakul, N. (2021). Effect of in vitro digestion on bioactive compounds, antioxidant and antimicrobial activities of coffee (*Coffea arabica* L.) pulp aqueous extract. *Food Chemistry*, *348*, 129094. https://doi.org/10.1016/j.foodchem.2021.129094

Lamarca, E. V., & Barbedo, C. J. (2014). Methodology of the tetrazolium test for assessing the viability of seeds of *Eugenia brasiliensis* Lam., *Eugenia uniora* L. and *Eugenia pyriformis* Cambess. *Journal of Seed Science*, *36*(4), 427-434. http://doi.org/10.1590/2317-1545v36n41029

Medeiros, A. P. S. R., Mendoça, A. F. J., Barros, S. T., Nogueira, N. W., & Oliveira, R. M. F. (2015). Teste de tetrazólio para avaliação da qualidade siológica de sementes de *Vigna unguiculata* (L.) Walp. *Revista Ciência Agronômica*, *46*(3), 638-644. http://doi.org/10.5935/1806-6690.20150048

Mercado, S., Caleño, J., and Rozo, L. (2020). Improvement of the methodology of the tetrazolium test using different pretreatments in seeds of the genus *Epidendrum* (Orchidaceae). *Journal of Seed Science*, *42*, e202042013. https://doi.org/10.1590/2317-1545v42231028

Mercado, S. A. S., & Jaimes, Y. M. O. (2022). Implementation of organic components to the culture medium to improve the in vitro propagation of *Cattleya warscewiczii* and *Cattleya gaskelliana*. *South African Journal of Botany*, *148*, 352-359. https://doi.org/10.1016/j.sajb.2022.05.002

Mishra M.K., Slater A. (2012). *Recent advances in the genetic transformation of coffee*. Biotechnical Research Institute. Article ID 580857. https://doi.org/10.1155/2012/580857

Montagnon, C., Mahyoub, A., Solano, W., & Sheibani, F. (2021). Unveiling a unique genetic diversity of cultivated *Coffea arabica* L. in its main domestication center: *Yemen. Genetic Resources and Crop Evolution*, 1-12. https://doi.org/10.1007/s10722-021-01139-y

Rojo J.; Pérez-Urria Carril, E. (2014) Café I (G. Coffea). *REDUCA Biología*, *7* (2), 113-132. ISSN 1989-3620 https://eprints.ucm.es/id/eprint/27835/1/1757-2066-1-PB.pdf

Salazar, S., Botello, H., y Quintero, J. (2019). Efecto de pretatamientos en la prueba de tetrazolio en semillas de *Epidendrum barbaricum* Hágsater & Dodson. *Acta Agronómica, 68*(4), 306-311. Doi: https://doi.org/10.15446/acag.v68n4.79619

Salazar, S. Quintero, J. & Bustos, V. (2020a). Implementación de la prueba de tetrazolio en las semillas de *Raphanus sativus* L. *Revista Facultad De Ciencias Básicas*, *15*(2), 7-15. https://doi.org/10.18359/rfcb.3831

Salazar, S., Botello, H., y Quintero, J. (2020b). Optimización de la prueba de tetrazolio para evaluar la viabilidad en semillas de *Solanum lycopersicum* L. *Ciencia & Tecnología Agropecuaria*, *21*(3). https://doi.org/10.21930/rcta.vol21_num3_art:1344

Salazar, S., Quintero, Rojas, J. (2020c). Optimization of the tetrazolium test in three species of orchids of the Andean forest. *Australian Journal of Crop Science, 14*(5), 822-830. https://doi.org/10.21475/ajcs.20.14.05.p2276

Salazar, S. Maldonado, H. (2020). Evaluation of the cytotoxic potential of sodium hypochlorite using meristematic root cells of *Lens culinaris* Med. *Science of the Total Environment, 701*, 134992. https://doi.org/10.1016/j.scitotenv.2019.134992.

Salazar, S y Botello, E. (2020). Effect of the medium composition on the asymbiotic germination and in vitro development of the x*Laeliocattleya* hort. ex Rolfe, 1887 hybrid. *South African Journal of Botany*. *135*, 80-60. https://doi.org/10.1016/j.sajb.2020.08.011

Selmar, D., Bytof, G., & Knopp, S. E. (2008). The storage of green coffee (*Coffea arabica*): decrease of viability and changes of potential aroma precursors. *Annals of Botany*, *101*(1), 31–38. https://doi.org/10.1093/aob/mcm277

Strobel T, Koch F, Geison RA, Szareski V, Carvalho I, Nardino M, Souza V, Tiago Z, Queiroz Z, Amaral F, Aumonde TZ. (2016). Physical and physiological quality of soybean seeds harvested under different trial systems after storage periods. *Australian Journal of Basic and Applied Science, 10,* 124-130.

Tinoco, H. A., & Peña, F. M. (2019). Mechanical and geometrical characterization of fruits *Coffea arabica* L. var. Colombia to simulate the ripening process by finite element analysis. *Engineering in Agriculture, Environment and Food*, *12*(3), 367-377. https://doi.org/10.1016/j.eaef.2019.04.004

Tola, C. C., Ninacanchi, J. C. H., & Choque, D. C. (2019). Determinación de la concentración de tetrazolio y tiempo de tinción adecuado para el análisis de viabilidad en semillas de café. *Apthapi*, *5*(3), 1671-1682

doi:10.14720/aas.2022.118.3.2194 Original research article / izvirni znanstveni članek

Evaluation of forage maize yield and soil organic matter content under green manure cultivation

Elias ARAZMJOO [1, 2], Alireza MOGHRI FERIZ [3], MohammadAli BEHDANI [4], Sohrab MAHMOODI [4]

Received April 29, 2021; accepted August 08, 2022.
Delo je prispelo 29. Aprila 2021, sprejeto 8. avgusta 2022

Evaluation of forage maize yield and soil organic matter content under green manure cultivation

Abstract: To investigate the effect of different green manures from Gramineae and Brassicaceae families on yield, some agronomic traits of forage maize, overgrowth with weeds and soil organic matter, an experiment was conducted based on a randomized complete block design with three replications for three consecutive years (2017-2020) at the Agricultural and Natural Resources Research and Education Centre of Southern Khorasan. Experimental treatments included control (without application of green manure) and application of green manures from the cultivation of barley, triticale, canola, arugula with their optimum and twice optimum densities. The results showed that barley and triticale at twice optimum density with 865.7 and 802.9 g m^{-2}, respectively, had a higher green manure dry mass at the time of returning to the soil. Just before maize cultivation, soil organic matter with an average of 0.73 % was higher in barley green manure at twice optimum density compared to other treatments. Based on the results, the highest maize forage yield with 45.7 and 44.9 t ha^{-1} were achieved after treatment with barley green manure in twice optimum and optimum density (22.8 and 20.7 percent more than control treatment) and after that triticale in both densities, and canola and arugula at twice optimum density had the highest yield.

Key words: barley; triticale; canola; arugula; forage maize; production; weeds

Ovrednotenje pridelka silažne koruze in vsebnosti organske snovi v tleh v razmerah zelenega podora

Izvleček: Za ovrednotenje učinka različnega zelenega podora iz družin trav in križnic na pridelek in nekatere komponente pridelka silažne koruze, poraslosti s pleveli in vsebnosti organske snovi v tleh je bil izveden popolni naključni bločni poskus s tremi ponovitvami v treh zaporednih rastnih sezonah (2017-2020) na posestvu Agricultural and Natural Resources Research and Education Centre of Southern Khorasan. Obravnavanja v poskusu so obsegala kontrolo (brez uporabe zelenega podora) in uporabo zelenega podora z ječmenom, trtikalo, oljno ogrščico in rukvico, z optimalno in dvakrat optimalno gostoto setve. Rezultati so pokazali, da je imelo zeleno gnojenje z ječmenom in tritikalo pri dvakratniku optimalne setve, 865,7 in 802,9 g m^{-2}, večjo suho maso zelenega podora. Pred začetkom setve koruze je bila suha masa organske snovi v tleh, poprečno 0,73 g, večja pri zelenem gnojenju z ječmenom pri dvakratni optimalni gostoti kot pri drugih obravnavanjih. Največji pridelek silažne koruze, 45,7 in 44,9 t ha^{-1}, je bil dosežen pri zelenem gnojenju z ječmenom pri dvakratniku optimalne gostote setve in optimalni gostoti setve (22,8 in 20,7 odstotkov več kot pri kontrolnem obravnavanju) in potem pri tritikali pri obeh gostotah setve ter pri dvakratniku optimalne gostote setve pri oljni ogrščici in rukvici.

Ključne besede: ječmen; tritikala; oljna ogrščica; rukvica; silažna koruza; pridelek; pleveli

1 Horticulture Crops Research Department, South Khorasan Agricultural and Natural Resources Research and Education Centre, AREEO, Birjand, Iran
2 Corresponding author, e-mail: e.arazmjo@areeo.ac.ir
3 Soil and Water Research Department, South Khorasan Agricultural and Natural Resources, Research and Education Centre, AREEO, Birjand, Iran
4 Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Birjand, Birjand, Iran

1 INTRODUCTION

Maize (*Zea mays* L.) is the third most important cereal grain in the world, after wheat and rice, providing nutrients for humans and animals and serving as a basic raw material for the production of starch, oil, protein, alcoholic beverages, food sweeteners, and fuel (Bouis and Welch, 2010).

With the increasing population and increasing human need for chicken and eggs, the area under maize cultivation is increasing. According to the World Food Program, the annual production of maize is about 1.06 billion tons, and the largest producers in the world, the United States and China, together account for 58 % of this amount (FAO, 2020). In 2020, the area under maize cultivation in Iran was 200,000 hectares with a production of 1,400,000 tons, while its domestic consumption in the country was equal to 8,900,000 tons in this year and the gap between annual production and demand was provided with an import of 7,500,000 tons (USDA, 2020). One of the important limiting factors for the development of animal husbandry and the production of livestock materials is the provision of fodder to feed the country's livestock. In such a way that the import of fodder and fodder grains constitute considerable several imported items of the country. In this regard, the importance of forage production is increasingly felt.

The use of chemical fertilizers to produce crops around the world is also increasing (Abril et al., 2007), the continued use of which poses serious risks to the environment and human health (Graham and Vanca, 2000). In Iran, the indiscriminate use of chemical fertilizers, especially nitrogen fertilizers, and the lack of application of organic fertilizers in recent years has been the cause of a significant reduction in the amount of organic matter in agricultural soils (Malakouti, 1999). Soils with more than 3 % organic matter are needed to make suitable soils for plant growth (Pramanik et al., 2004). Additionally, the use of chemical fertilizers does not have a beneficial effect on physical soil properties. Adverse effects of fertilizers and pesticides on the environment have led to more attention and the use of methods without the use of chemicals, and the issue of sustainability in agriculture to be considered. One practical way to meet this goal is to use green manures that can reduce the use of chemical fertilizers.

The application of green manures is one of the management methods of choice in many agricultural productions systems because these fertilizers can reduce soil erosion and improve the physical properties of the soil, increase organic matter and soil fertility, increase nutrient circulation and reduce global warming potentials and finally increase the system stability (Dinnes et al., 2002). Plants used as green manure increase soil water storage in arid lands by increasing water infiltration, reducing evaporation, and improving soil structure. Return of green manures to the soil as a result of microbiological processes increase soil organic matter and release nutrients in plants for plants (Talgre et al., 2009).

In the study of Abdi et al. (2012) the plants of Gramineae (sorghum, millet, and oat), Brassicaceae (arugula), and Leguminosae (white clover, red clover, bersim clover, sainfoin, and vetch) were used as green manure, and the evaluation of soil nutrient changes, and nitrogen mineralization were studied and the highest amount of organic carbon was obtained by returning sorghum forage residues to the soil (1.59 %). The amount of total soil nitrogen in all tested plants increased during different sampling times. The highest amounts of total nitrogen (0.23 %) were obtained by white clover in five months after the return of the remains.

Clement et al. (1995) examined different types of green manures and found that the ratio of lignin and polyphenols to nitrogen and tannins to nitrogen controlled the amount of nitrogen released. On the other hand, the decomposition of green manure and the release of its nutrients depends on soil physical (moisture, temperature, texture, minerals, and pH), chemical (carbon/nitrogen ratio, soil nutrient content), and biological properties (the rate of soil biological activity) (Myers et al., 1994), among which the ratio of carbon/nitrogen has a greater impact on the mineralization of organic matter than other factors. Regarding the effect of red clover, common alfalfa, vetch, and oats as green manure on bioavailable nitrogen, it was observed that the amount of soil nitrogen increased significantly under common alfalfa use and the wheat grain protein content in the next crop was the highest (Maikstenien and Arlauskiene, 2004). It has been reported that in dry winters, nitrate accumulates in topsoil and the cover plant controls nitrate leaching during the early stages of growth, and that vetch is less efficient in leaching control in contrast to barley but increased soil nitrogen storage (Gabriel et al., 2012). Additionally, the combined application of vetch winter cover plant and a small amount of fertilizer can significantly improve the sustainability of low-input maize-based conservation agriculture (Dubeab et al., 2013).

Green manure in Iran is used only in some areas and to a very limited extent. Animal manures are also not stored and used properly. Besides, the high cost of livestock manure and the lack of common use of them have caused organic fertilizers to play a negligible role in increasing fertility and improving Iran soils. This can cause serious problems in agricultural planning and operations, especially in large-scale agriculture. Thus, the purpose of this study was to investigate the effect of Gramineae (bar-

ley and triticale) and Brassicaceae (canola and arugula) as green manure in two different densities on the maize yield, weeds growth and percentage of soil organic matter during the periods after adding green manure residues to the soil and finally introducing the desired plant or plants as green manure in the studied conditions.

2 MATERIALS AND METHODS

A field experiment was conducted between 2017 and 2020 at the Agricultural and Natural Resources Research and Education Centre of South Khorasan Province, Birjand, Iran (32° 52′N, 58° 59′E). Before cultivation, a pre-planting composite sample of the soil was taken from a depth of 0-30 cm for determination of particle size distribution, pH, EC and soil organic matter. The results of soil analysis during the years of the experiment are presented in Table 1 and meteorological information during the years of the experiment is also presented in Figure 1.

There were nine treatments in the trial, laid out in a randomized complete block design in three replications. Plants used as green manure included barley (*Hordeum vulgaris* L.), triticosecale (*Triticosecale* spp.), canola (*Brassica napus* L.), and arugula (*Eruca sativa* L.). Experimental treatments included control (none application of green manure) and application of green manures from the cultivation of barley (with optimum density), barley (with twice optimum density), triticale (with optimum density), triticale (with twice optimum density), canola (with optimum density), canola (with twice optimum density), arugula (with optimum density), arugula (with twice optimum density).

Cultivation of barley with two densities of 400 and 800, triticale with two densities of 400 and 800, canola with two densities of 70 and 140, and arugula with two densities of 80 and 160 plants per square meter and based on their 1000-grain mass on five 60 cm rows with a length of five meters was done in November of 2017, 2018 and 2019. The distance between the plots was one meter and between the replication was two meters. After planting, irrigation was done and a total of two irrigations were carried out in autumn and one irrigation in spring. In April, the green manures were cut into small pieces using a disk and returned to the soil with plowing.

Considering that weed control by green manure as well as the contribution of green manure in increasing soil organic matter is directly related to the dry mass produced, the biomass of each green manure at the time of return to the soil was evaluated. To estimate the dry mass of green manures, sampling was performed before returning them to the soil. For this purpose, one square meter was randomly taken from each plot and the samples were dried in an oven at 70 °C for 48 hours and then weighed. After returning the green manure the land was left uncultivated and then maize was planted in early summer.

Maize cultivar SC647 was cultivated in the first half of July in 2018, 2019, and 2020 in furrows with a distance of 75 cm and a length of five meters, and a density of 20 plants per square meter. Before planting maize, samples were taken from each plot to measure the amount of soil organic matter. Fertilizers used include 300 kg ha^{-1} urea (50 kg ha^{-1} before planting, 150 kg ha^{-1} at 6-7 leaves stage, and 100 kg ha^{-1} before the emergence of male inflorescence), 100 kg ha^{-1} triple superphosphate (before sowing) and 100 kg ha^{-1} of potassium sulfate (before sowing) were used during the maize growing period based on the dimensions of the plots. During the maize growing season, irrigation was carried out in the first months with an interval of eight days and in the last month with an interval of 10 days, and a total of nine irrigations was carried out. During the growing season, traits such as number of days to emergence of tassels, number of days to emergence of silk, number of rows per ear, number of grain per row, ear length, ear diameter, plant height, and forage yield of maize, as well as weed biomass and weed density, were measured or weighted and recorded. To measure ear length, ear diameter, and plant height by observing the margin effect, five plants were randomly selected and the average of five plants for each of these traits was recorded. The number of rows per ear, the number of grain per row, and the number of grain per ear were counted and recorded in five ears after harvest. A sampling of weeds in maize cultivation was done in 4-6 leaf stage in a quadrate of one square meter in each plot and after determining the density of weeds, these samples were dried at a temperature of 70 degrees in an oven for 48 hours and then weighed. To measure the maize forage yield, the area of one square meter was harvested and the fresh mass of the forage was weighed by observing the margin effect.

After ensuring the homogeneity of variance of experimental error with Bartlett test, the data were analyzed using SAS statistical software based on a randomized complete block design and means comparison based on Duncan test at 5 % probability level.

3 RESULTS AND DISCUSSION

The results of combined analysis of variance for three years of the experiment showed that year had a significant effect at the level of 1 % on the dry mass of green manure, the number of grain per row and weeds dry mass and a significant effect at the level of 5 % on ear

Table 1: The results of soil physiochemical properties

Year	Sand (%)	Silt (%)	Clay (%)	Soil texture	pH	EC (dS.m^{-1})	Organic matter (%)
2017	38	42	20	Loam	7.9	4.79	0.36
2018	40.5	36	23.5	Loam	7.82	4.27	0.49
2019	46.5	36	17.5	Loam	7.85	5.67	0.44

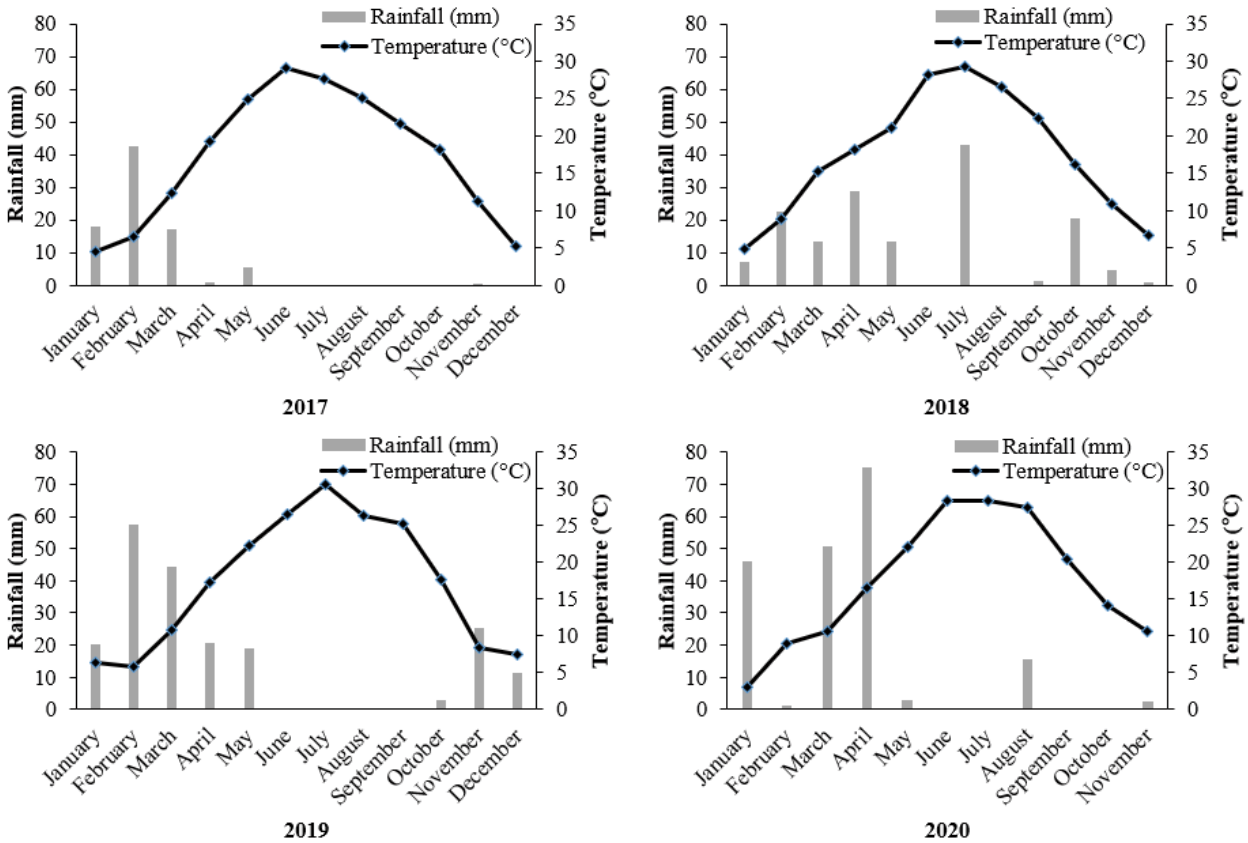


Figure 1: Mean temperature and rainfall during 2017-2020 (Climate data: Iran, Birjand - Tutiempo.net)

length (Table 2). Also, except for the number of days to emergence, the number of days to the emergence of silk and the diameter of the ear, the year had a significant effect on the other studied traits, whereas the interaction of year in green manure was significant only on the dry mass of green manure at the time of return to soil (Table 2).

The results of mean comparing for the year effect showed that the highest dry mass of green manure with an average of 564.4 g m^{-2} and the highest weeds dry mass with an average of 175.6 g m^{-2} were observed in the second year of the experiment (Table 3). The highest number of grain per row with 36.7 grains and an ear diameter of 48.8 mm was also found in the third year whereas the difference in ear length between the third and the second year was not significant (Table 3). According to the meteorological statistics presented in Figure 1, one of the reasons for the better growth of green manures and their higher dry mass when returning to the soil in the spring in the second and third years of the experiment, was more precipitation during the autumn, winter, and spring during these years compared to the first year when the amount of precipitation was lower.

The results of mean comparison of the traits showed that at the time of return to the soil, barley and triticale green manure at twice optimum density treatment with 865.7 and 802.9 g m^{-2}, respectively, had higher dry mass compared with other treatments (Table 3). After control or none green manure cultivation, canola, and arugula treatments had the lowest dry mass at the optimum density at the time of return to the soil, so that their dry mass was about 55 percent lower compared to barley green

manure at twice optimum density (Table 3). Means comparison for the interaction effect of year in green manure also showed that barley green manure treatment with twice optimum density in the second year with an average of 937 g m^{-2} had the highest dry mass among other treatments, but not statistically different with barley green manure treatments with twice optimum density in the third year and triticale with twice optimum density in the second and third year. In contrast, canola, and arugula green manures with averages of 291.1 and 314.7 g m^{-2} in the first year had the lowest dry mass among other treatments, respectively (Table 4). Naturally, the more residues returned to the soil, the more organic will be added to the soil. Abdi et al. (2012) also reported in their study that compared to the green manures of sorghum, millet, oats, arugula, and several types of clover, sorghum produced the highest fresh and dry mass of shoots and consequently it has also led to the production of higher organic carbon in the soil.

In terms of the number of grain rows per ear, barley green manure had the highest rate at the optimum density with an average of 16.6 rows and there was no statistically significant difference between this treatment with barley and arugula treatments with twice optimum density, whereas control treatment with 14.1 rows had the lowest amount (Table 3).

There was no significant difference in the number of grain per row between barley, triticale, canola, and arugula green manures, but these treatments were significantly superior to the control (Table 3).

The number of grains per ear had the lowest values in control and canola with optimum density treatments, while barley with optimum density and then barley and arugula with double optimum density was in the superior statistical group in this regard (Table 3). Maize ear length was higher in barley green manure treatments at both twice and optimum densities and also arugula at twice optimum density (Table 3).

Barley green manure in twice optimum density with an average of 212.5 cm had the highest plant height among other treatments and resulted in a 16.5 % increase in maize plant height compared to the control treatment and its difference with barley treatments with optimal density, triticale and canola with twice optimum density and arugula with both densities and twice the density were not statistically significant (Table 3). It seems that these treatments provided more access to nutrients to the plant than other treatments and an increased in ear length and plant height are observed. Evaluation of legume winter cover crops in maize cultivation also showed a 37 % increase in maize plant height (Miguez and Bollero, 2005). Increasing the ear length in the application of green manure is consistent with the results of Ghasemi et al. (2016).

According to the results, the lowest amount of maize forage with 37.2 t ha^{-1} was related to the control treatment or no green manure cultivation, and barley green manure at twice optimum density with 45.7 t ha^{-1} had the highest maize forage which had no significant difference with barley in optimum density treatment with 44.9 t ha^{-1}. Also, after them, triticale green manure in both density and canola and arugula at twice optimum density had higher forage yield and there was no statistically significant difference between them (Table 3). Barley at optimum and twice optimum density leads to an increase of 20.7, 22.8 %, triticale at optimum and twice optimum density leads to an increase of 14.5 and 14.8 %, canola at optimum and twice optimum density leads to an increase of 10.2 and 14.8 % and finally arugula at optimum and twice optimum density led to an increase of 9.7 and 18.3 % in the yield of maize forage compared to the control treatment (Table 3). The increase in maize forage yield as a result of the return of the mentioned green fertilizers can be justified by increasing the soil organic matter and the availability of nutrients for the next crop as well as improving the biological and physical properties of the soil. In addition to improving soil structure and nutrient accumulation in soil surface layers (Cherr et al., 2006), green manure has been reported to be the most important source for bacterial activity, and bacteria are more efficient in these conditions (Orhana et al., 2006). The use of organic fertilizers by increasing soil organic matter strengthens the properties of aggregates, microbial activity, soil quality, crop fertility, and storage capacity of nutrients such as nitrogen, phosphorus, potassium, zinc, and iron in the soil (Wei and Liu, 2005).

The predominant weeds in the maize field included *Amaranthus retroflexus* L., *Portulaca oleracea* L., *Convolvulus arvensis* L., and *Alhaji camelorum* L. The results of the experiment showed the superiority of barley and triticale green manures in controlling weeds in the maize field. Namely, the barley treatment at twice optimum density with 54.2 g m^{-2} and triticale in twice optimum density with 83.8 g m^{-2} had the lowest weeds dry mass, respectively (Table 3). Barley and triticale at twice optimum density resulted in a reduction of 85.3 % and 77.2 % in weeds dry mass compared to the control (Table 3). The highest weeds density and weeds dry mass of 155.9 plants m^{-2} and 368.8 g m^{-2} were obtained from control treatment or no cultivation of green manure, followed by canola and arugula at the optimum density. Plants with high biomass and more shading can control weeds well. One of the reasons for the decrease in the weeds density and their dry mass in green manure treatments compared to fallow has been reported to be the sharp reduction of

Table 2: The results of analysis of variance for investigated traits

		Mean squares												
Source of Variation	df.	Green manure dry mass	No. of days to emergence of tassels	No. of days to emergence of silks	No. row per ear	No. grain per row	No. grain per ear	Ear length	Ear diameter	Plant height	Forage yield	Weeds dry mass	Weeds density	Soil organic matter
Year	2	179642**	23.19 ns	43.90 ns	37.16 ns	155.85**	22694 ns	105.92*	376.9 ns	2218.5 ns	246.2 ns	11219.9**	391.66 ns	0.138 ns
Block (Year)	6	3719.4	4.88	11.07	0.74	2.92	2557.69	9.11	35.79	668.71	0.68	420.82	400.91	0.002
Green manure	8	583358**	4.73 ns	10.06 ns	4.55**	21.62**	17751.2**	28.81**	28.87 ns	1280.6**	58.64**	86129.5**	10243.9**	0.052**
Green manure*Year	16	5970.1*	0.92 ns	2.46 ns	0.22 ns	0.41 ns	491.7 ns	1.87 ns	0.69 ns	42.82 ns	7.05 ns	544.29 ns	275.65 ns	0.0006 ns
Residual error	48	2951.5	25.08	25.86	0.73	4.85	2883.08	4.76	27.02	150.87	6.41	533.59	206.70	0.002
Coefficient of variation	-	10.78	8.03	7.25	5.62	6.39	10.53	7.94	11.34	5.86	5.97	14.20	21.39	7.93

* and ** are significantly different at α = 0.05 and α = 0.01, respectively and ns is non-significant

Table 3: The results of mean comparison for effects of year and green manure on investigated traits

Year	Green manure dry mass (g m^{-2})	No. of days to emergence of tassels	No. of days to emergence of silks	No. row per ear	No. grain per row	No. grain per ear	Ear length (cm)	Ear diameter (cm)	Plant height (cm)	Forage yield (t ha^{-1})	Weeds dry mass (g m^{-2})	Weeds density (plant m^{-2})	Soil organic matter (%)
2017-2018	411.3 b	61.4 a	68.7 a	15.6 a	31.9 c	498.7 ab	25.3 b	41.6 b	204.9 a	38.9 b	139.1 b	65.6 a	0.51 b
2018-2019	564.4 a	63.3 a	71.2 a	14.0 b	34.8 b	487.8 b	27.8 ab	47.1 ab	220.2 a	44.5 a	175.6 a	64.5 a	0.64 a
2019-2020	536.7 a	62.3 a	70.6 a	16.2 a	36.7 a	542.5 a	29.3 a	48.8 a	204.0 a	43.8 a	173.1 a	71.5 a	0.63 a
Green Manure													
Control	0.0 f	62.9 a	71.4 a	14.1 c	31.0 b	424.3 c	25.0 c	42.7 a	185.7 c	37.2 c	368.8 a	155.9 a	0.47 e
Barley optimum density	599.7 b	62.6 a	69.9 a	16.6 a	36.5 a	584.6 a	29.9 a	46.3 a	216.3 a	44.9 a	90.5 d	58.0 b	0.63 bc
Barley twice optimum density	865.7 a	63.3 a	71.7 a	15.6 ab	35.2 a	530.5 ab	30.0 a	49.1 a	221.5 a	45.7 a	54.2 e	57.0 b	0.73 a
Triticale optimum density	552.5 bc	62.8 a	70.7 a	14.9 bc	34.2 a	494.5 bc	26.6 bc	45.8 a	195.3 bc	42.6 ab	101.3 d	55.6 b	0.59 cd
Triticale twice optimum density	802.9 a	61.3 a	68.6 a	14.9 bc	35.2 a	508.9 b	27.5 abc	47.3 a	216.7 a	42.7 ab	83.8 d	52.7 b	0.68 ab
Canola optimum density	379.6 e	61.3 a	68.9 a	14.9 bc	33.6 ab	482.9 bc	26.1 bc	45.7 a	206.3 ab	41.0 b	228.5 b	57.8 b	0.54 d
Canola twice optimum density	467.6 d	62.8 a	70.7 a	15.1 bc	34.8 a	508.1 b	26.7 bc	46.0 a	215.5 a	42.7 ab	174.7 c	48.2 b	0.58 cd
Arugula optimum density	378.2 e	62.4 a	70.1 a	15.2 bc	34.4 a	505.2 b	26.3 bc	44.4 a	211.8 a	40.8 b	212.2 b	68.1 b	0.54 d
Arugula twice optimum density	491.0 cd	61.7 a	69.7 a	15.9 ab	35.5 a	548.1 ab	29.1 ab	45.1 a	218.4 a	44.0 ab	149.7 c	51.5 b	0.59 cd

Means followed by similar letters in each column are not significantly different at p = 5 % based on Duncan test.
Barley optimum and twice optimum densities: 400 and 800 plants m^{-2}, respectively
Triticale optimum and twice optimum densities: 400 and 800 plants m^{-2}, respectively
Canola optimum and twice optimum densities: 70 and 140 plants m^{-2}, respectively

Table 4: The results of mean comparison for interaction effect on the dry mass of investigated green manures (g m^{-2})

Green Manure	2017-18	2018-19	2019-20
Control	0 ^{k}	0 ^{k}	0 ^{k}
Barley optimum density	482.4 ^{egf}	678.3 ^{bc}	638.4 ^{c}
Barley twice optimum density	738.6 ^{b}	937.0 ^{a}	921.6 ^{a}
Triticale optimum density	430.4 ^{efgh}	638.9 ^{c}	588.1 ^{cd}
Triticale twice optimum density	632.4 ^{c}	921.7 ^{a}	854.7 ^{a}
Canola optimum density	291.1 ^{j}	443.6 ^{efgh}	404.1 ^{ghi}
Canola twice optimum density	375.9 ^{hij}	529.2 ^{de}	497.8 ^{defg}
Arugula optimum density	314.7 ^{ij}	417.2 ^{fgh}	402.6 ^{ghi}
Arugula twice optimum density	436.4 ^{efgh}	513.9 ^{def}	522.7 ^{de}

Means followed by similar letters in each column are not significantly different at $p = 5$ % based on Duncan test.
Barley optimum and twice optimum densities: 400 and 800 plants m^{-2}, respectively
Triticale optimum and twice optimum densities: 400 and 800 plants m^{-2}, respectively
Canola optimum and twice optimum densities: 70 and 140 plants m^{-2}, respectively
Arugula optimum and twice optimum densities: 80 and 160 plants m^{-2}, respectively

light reaching the lower parts of the plant canopy in these treatments, reducing the weeds photosynthetic activity of and thus reducing their density (Bilalis et al., 2009). Residues mixed with the soil of green plants with allelopathic effects (Ohno et al., 2000), stimulation of soil pathogens (Conklin et al., 2002), impact on nutrients access (Gallandt et al., 1999), increase crop growth, and improving its competitiveness with weeds (Boquet et al., 2004) can reduce weed density and growth. A report states that non-legume species such as canola and rye are suitable if the main purpose of using cover crops is to control weeds (Campiglia et al., 2009).

The amount of soil organic matter in barley green manure treatment with twice optimum density with an average of 0.73 was higher than other treatments. Since the highest biomass produced among green manures was related to this treatment, the higher amount of soil organic matter in this treatment can be attributed to this factor. After this treatment, triticale with twice optimum density and barley with optimum density was in the second and third ranks in terms of soil organic matter (Table 3). The high percentage of organic carbon in these treatments is probably due to the larger volume of soil-derived residues in these treatments. In the study of Ghaffari et al. (2013), rye, barley and triticale treatments with three times planting density and rye with normal density increased 26, 25, 21, and 25 % of soil organic carbon content, respectively, compared to the control treatment. In some studies, an increase in soil organic carbon content due to the application of green manure compared to the conventional low-input system (without fertilizer) has been reported (Clark et al., 1998). It has been reported that the return of green manure plants to the soil increases carbon, organic matter, total nitrogen, and soil fertility, which occurs as a result of microbiological processes and causes the release of nutrients for plants (Talgre et al., 2009).

4 CONCLUSIONS

Based on the results, the highest maize forage yield with 45.7 and 44.9 t ha^{-1} was obtained from barley green manure at twice optimum density and its optimum density, followed by triticale at both density and canola and arugula at twice optimum density. Due to severe organic matter deficiency in many soils of South Khorasan province, cultivation of green manure plants before maize cultivation, depending on the type of green manure selected and its density can increase maize forage yield by 9.6 to 20.7 % compared to not cultivating them. Reducing weeds in the soil will be another advantage of growing green manure before maize cultivation.

5 REFERENCES

Abdi, S., Tajbakhsh, M., Abdollahi Mandulakani, B. & Rasouli Sadaghiani, M. (2012). Effect of green manure on the soil organic matter and nitrogen. *Journal of Agricultural Knowledge*, *5*(7), 41-52.

Abril, A., Baleani, D., Casado-Murillo, N. & Noe, L. (2007). Effect of wheat crop fertilization on nitrogen dynamics and balance in the Humid Pampas, Argentina. *Agriculture, Ecosystems & Environment*, *119*, 171-176. https://doi.org/10.1016/j.agee.2006.07.005

Bilalis, D., Karkanis, A. & Efthimiadou, A. (2009). Effects of two legume crops, for organic green manure, on weed flora, under Mediterranean conditions: competitive ability of five winter season weed species. *African Journal of Agricultural Research*, *4*(12), 1431-1441.

Boquet, D. J., Hutchinson, R. L. & Breitenbeck, G. A. (2004). Long-term tillage, cover crop, and nitrogen rate effects on cotton: plant growth and yield components. *Agron-*

omy Journal, *96*, 1443.1452. https://doi.org/10.2134/agronj2004.1443

Bouis, H.E. & Welch, R.M. (2010). Biofortification - A sustainable agricultural strategy for reducing micronutrient malnutrition in the global south. *Crop Science*, *50*(1), 20-32. https://doi.org/10.2135/cropsci2009.09.0531

Campiglia, E., Paolini, R., Colla, G. & Mancinelli, R. (2009). The effects of cover cropping on yield and weed control of potato in a transitional system. *Field Crops Research*, *112*, 16-23. https://doi.org/10.1016/j.fcr.2009.01.010

Cherr, C. M., Scholberg, J. M. S. & McSorley, R. (2006). Green manure as nitrogen source for sweet corn in a warm–temperate environment. *Agronomy Journal*, *98(*5), 1173-1180. https://doi.org/10.2134/agronj2005.0036

Clark, M. S., Horwath, W. R., Sherman, C. & Scow, K. M. (1998). Change in soil chemical properties resulting from organic and low-input farming practices. *Agronomy Journal*, *90*, 662-671. https://doi.org/10.2134/agronj1998.00021962009000050016x

Clement, A., Ladha, J.K. & Chalifour, F.P. (1995). Crop residue effects on nitrogen mineralization, microbial biomass, and rice yield in submerged soils. *Soil Science Society of America Journal*, *59*, 1595–1603. https://doi.org/10.2136/sssaj1995.03615995005900060013x

Conklin, A. E., Erich, MS. & Liebman, M. (2002). Effects of red clover (*Trifolium pratense*) green manure and compost soil amendments on wild mustard (*Brassica kaber*) growth and incidence of disease. *Plant and Soil*, *238*, 245.25. https://doi.org/10.1023/A:1014448612066

Dinnes, D.L., Karlen, D.L., Jaynes, D.B., Kaspar, T.C., Hatfield, J.L., Colvin, T.S. & Cambardella, C.A. (2002). Nitrogen management strategies to reduce nitrate leaching in tile-drained Midwestern soils. *Agronomy Journal*, *94*, 153-171. https://doi.org/10.2134/agronj2002.1530

Dubeab, E., Chiduzaa, C. & Muchaonyerwac, P. (2013). Conservation agriculture effects on plant nutrients and maize grain yield after four years of maize–winter cover crop rotations. *South African Journal of Plant and Soil*, *30*(4), 227-232. https://doi.org/10.1080/02571862.2013.867458

FAO statistics. (2020). Retrieved from: http://www.fao.org/

Gabriel, J. L., Munoz-Carpena, R. & Quemada, M. (2012). The role of cover crops in irrigated systems: Water balance, nitrate leaching, and soil mineral nitrogen accumulation. *Agriculture, Ecosystems and Environment*, *155*, 50-61. https://doi.org/10.1016/j.agee.2012.03.021

Gallandt, E. R., Liebman, M. & Huggins, D. R. (1999). Improving soil quality: implications for weed management. *Journal of Crop Production*, *2*, 95-121. https://doi.org/10.1300/J144v02n01_06

Ghaffari, M., Ahmadvand, G., Ardakani, M.R., Mosadeghi, M.R. & Ghaffari, M. (2013). Effect of winter cereals as pre plant cover crops on weeds control, improving soil fertility, yield and yield components of potato. *Journal of Plant Production, 36*(4), 29-42.

Ghasemi, A., Ghanbari, A., Fakheri, B.A. & Fanaie, H.R. (2016). Effect of different fertilizer resources on yield and yield components of grain maize (*Zea mays* L.) influenced by tillage managements. *Journal of Agroecology, 7*(4), 499-512.

Graham, Ph. & Vanca, C.P. (2000). Nitrogen fixation in perspective: an overview of research and extension needs. *Field Crops Research*, *65*, 93-106. https://doi.org/10.1016/S0378-4290(99)00080-5

Maiksteniene, S. & A. Arlauskiene. (2004). Effect of preceding crops and green manure on the fertility of clay loam soil. *Agronomy Research*, *2*(1), 87-97.

Malakouti, M. (1999). *Sustainable agriculture and increasing yield by optimizing fertilizer use in Iran*. 1 st Edition. Agricultural Education Publishing, Karaj.

Miguez, F.E. & Bollero, G.A. (2005). Review of corn yield response under winter cover cropping systems using meta-analytic methods. *Crop Science Society of America*, *45*(6), 2318-2329. https://doi.org/10.2135/cropsci2005.0014

Myers, R.J.K., Palm, C.A., Cuevas, E. & Gunatilleke, I.U.N. (1994). The synchronization of nutrient mineralization and plant nutrient demand. In: P.L. Woomer. & M.J. Swift, (Eds.), *The Biological Management of Tropical Soil Fertility*. Wiley-Sayce Publication, Chichester, UK, pp. 81–116.

Ohno, T., Doolan, K. & Zibilske, L.M. (2000). Phytotoxic effects of red clover amended soils on wild mustard seedling growth. *Agriculture Ecosystems & Environment*, 78, 187-192. https://doi.org/10.1016/S0167-8809(99)00120-6

Orhana, E., Esitkena, A., Ercislia, S., Turanb, M. & Sahinc, F. (2006). Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents in organically growing raspberry. *Scientia Horticulturae*, *111*(1), 38-43. https://doi.org/10.1016/j.scienta.2006.09.002

Pramanik, MYA., Sarkar, MAR., Islam, MA. & Samad, MA. (2004). Effect of green manures and different levels of nitrogen on the yield and yield components of transplant Aman rice. *Journal of Agronomy*, *3*(2), 122-125. https://doi.org/10.3923/ja.2004.122.125

Talgre, L., Lauringson, E., Roostalu, H. & Astover, A. (2009). The effects of green manures on yields and yield quality of spring wheat. *Agronomy Research*, *7*(1), 125-132.

USDA statistics. (2020). Retrieved fromhttps://www.usda.gov/

Wei, Y. & Liu, Y. (2005). Effects of sewage sludge of compost application on crops and cropland in a 3-years field study. *Chemosphere*, *59*, 1257-65. https://doi.org/10.1016/j.chemosphere.2004.11.052

doi:10.14720/aas.2022.118.3.2485 Original research article / izvirni znanstveni članek

Fruit collapse incidence and quality of pineapple as affected by biopesticides based on *Pseudomonas fluorescens* and *Trichoderma harzianum*

Diego Mauricio CANO-REINOSO [1], Loekas SOESANTO [1, 2], KHARISUN [1], Condro WIBOWO [3]

Received December 28, 2021; accepted August 10, 2022.
Delo je prispelo 28. decembra 2021, sprejeto 10. avgusta 2022

Fruit collapse incidence and quality of pineapple as affected by biopesticides based on *Pseudomonas fluorescens* and *Trichoderma harzianum*

Abstract: In this study the effect of *Pseudomonas fluorescens* and *Trichoderma harzianum* based biopesticides on fruit collapse disease incidence and pineapple quality was investigated. The experiment was implemented in a split-plot design with two factors, one involving two inoculation methods (spray and inject), and a second factor involving four treatments, A (control: no biopesticides used), B (Bio P32 from 13 weeks before harvest), C (Bio T10 from 13 weeks before harvest) and D (Bio P32 + Bio T10 from 13 weeks before harvest). The inoculated pathogen was *Dickeya zeae*. The incidence of fruit collapse, total soluble solids, total acidity, sucrose, ascorbic acid, mineral content, and electrolyte leakage were determined. The inject method caused more fruit collapse incidence than the spray method. Treatments C and D provided the best results having a low incidence of fruit collapse (spray: 5 and 1.7 %, inject: 20 % in both cases), high antioxidant capacity (regarding ascorbic acid), high mineral nutrient content (in terms of Ca and Mg), and low electrolyte leakage content (< 70 % in average), with a healthier cell wall characteristic. Meanwhile, treatments A and B were less efficient in these aspects and promoted the incidence of fruit collapse, especially when the inject method was used, as this was more harmful regarding the fruit physiology. In conclusion, the biopesticides employed can reduce the incidence of fruit collapse and positively affect the fruit quality.

Key words: biopesticide; *Dickeya zeae*; fruit quality; disease incidence; *Pseudomonas fluorescens*; *Trichoderma harzianum*

Vpliv uporabe biopesticidov na osnovi bakterije *Pseudomonas fluorescens* in glive *Trichoderma harzianum* na propad in kakovost plodov ananasa

Izvleček: V raziskavi je bil preučevan učinek uporabe biopesticidov na osnovi vrst *Pseudomonas fluorescens* in *Trichoderma harzianum* na propad in kakovost plodov ananasa. Poskus je bil izveden kot faktorski poskus z deljenkami, kjer je prvi dejavnik obsegal dva načina vnosa patogena (pršenje in injeciranje), drugi pa naslednja štiri obravnavana: A (kontrola: brez uporabe biopesticidov), B (uporaba Bio P32 13 tednov pred pobiranjem), C (uporaba Bio T10 13 tednov pred pobiranjem) in D (uporaba Bio P32 + Bio T10 13 tednov pred pobiranjem). Inokuliran patogen je bila bakterija *Dickeya zeae*. Po obravnavanjih so bili določeni naslednji parametri: pojav propada plodov, vsebnost topnih snovi v plodovih in njihova celukopna kislost, vsebnost saharoze, askorbinske kisline in mineralov ter puščanje elktrolitov iz plodov. Injeciranje patogena je povzročilo večji propad plodov kot pršenje. Obravnavanji C in D sta dali najbojše rezultate z najmanjšim propadanjem plodov (pri pršenju 5 in 1,7 %, pri injeciranju 20 % v obeh primerih), veliko vsebnostjo antioksidantov (vsebnost askorbinske kisline), mineralov (kot vsebnost Ca in Mg), manjšo vsebnost elektrolitov v iztoku (v poprečju manj kot 70 %) in bolj zdrave celične stene. Obravnavanji A in B sta bili glede na prej naštete parametre manj učinkoviti in sta pospešili propadanje plodov, posebej še pri injeciranju patogena, kar je bilo tudi bolj škodljivo glede na fiziološke lastnosti plodov. Zaključimo lahko, da uporaba biopesticidov zmanjša propadanje plodov in pozitivno vpliva na njihovo kakovost.

Ključne besede: biopesticidi; *Dickeya zeae*; kakovost plodov; pojav bolezni; *Pseudomonas fluorescens*; *Trichoderma harzianum*

1 Department of Agrotechnology, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Indonesia
2 Corresponding author, e-mail: lukassusanto26@gmail.com
3 Department of Food Science and Technology, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Indonesia

1 INTRODUCTION

Pineapple diseases are common problem affecting fruit quality, with infections usually beginning in the field and before harvest (Rohrbach and Johnson, 2003; Sipes and Pires de Matos, 2018). Fruit collapse is caused by the bacterium *Dickeya zeae* (formerly *Erwinia chrysanthemi* (Peckham et al., 2010; Sueno et al., 2014), which is characterized by exudation of sap and gas in the form of bubbles, an olive-green skin color and cavities within the skeletal fibers that show up in the flesh of the fruit (Aeny et al., 2020; Cano-Reinoso et al., 2021). *D. zeae* can infect the plant via infection vectors coming from the field, such as already infected plants, ants, beetles, and flies that attack during flower induction, or directly affecting the developed fruit when high temperatures weeks before harvest increase transpiration and allow the bacterium to penetrate directly through the stomata of the skin (Pires de Matos, 2019; Cano-reinoso et al., 2021). For this reason, fruit collapse symptoms usually occur just before harvest or during postharvest handling, as *D. zeae* can remain latent for a long time (Rohrbach and Johnson, 2003; Pires de Matos, 2019).

Recently, low acidity pineapple hybrids have been reported to be more susceptible to this disease (Soteriou et al., 2014; Cano-Reinoso et al., 2021). Currently, these hybrids are the most commonly exported by the industry as fresh fruit because they are attractive to consumers (Chen et al., 2009; Kleemann, 2016). Therefore, a solution must be found to address this problem. In addition, the solution should preserve the quality of the fruit and protect the environment, as the use of chemical pesticides is expected to be reduced worldwide in the near future. In this context, the use of biopesticides presents itself as an alternative, as they are environmentally friendly and easier to apply. These can interact with the plant and fruit during development through the plant stomata, lenticels, and natural cracks, and can also be applied after harvest (Soesanto et al., 2011, 2018).

Bio P32 is a biopesticide derived from *Pseudomonas fluorescens*, a strain of *P. fluorescens* isolated from the rhizosphere of wheat. Bio T10 is another biopesticide base on *Trichoderma harzianum*, a soil-borne fungus used for biological control of plant pathogens (Soesanto et al., 2011, 2018). These biopesticides have been widely used on various crops, both preharvest and postharvest, to reduce the incidence of bacterial diseases and improve crop characteristics, such as dragon fruit (Hamarawati et al., 2017), eggplant (Soesanto et al., 2011), and cucumber (Soesanto et al., 2020). However, since few studies has been reported on the effect of these products on fruit collapse and pineapple quality, this experiment aims to evaluate the effect of *Pseudomonas fluorescens* and *Trichoderma harzianum* based biopesticides on fruit collapse disease incidence and pineapple quality. This will involve a comparison of different inoculation methods that represent how *D.zeae* infect the plant during flowering or in the weeks just prior to harvest, and investigating different variables that could characterize the optimal fruit traits for future consumption in a low acid hybrid.

2 MATERIAL AND METHODS

2.1 EXPERIMENT DESIGN AND TREATMENTS

The research was set in pineapple fields located in Lampung, Sumatra island of Indonesia, between February and June of 2020. A pineapple low acid hybrid (MD2) was used for this experiment. MD2 is known for its exceptional sweetness, consistency and uniformed size at harvest; currently is one of the most exported fresh cultivars, with a price tree times higher than any acid hybrid (Bin Thalip et al., 2015). The fruits were harvested in 148 days after flowering, considered an optimal time in MD2 to obtain the best physico-chemical characteristics for a future consumption (Bin Thalip et al., 2015; Ding and Syazwani, 2016).

The soil was previously fertilized with 200 kg ha^{-1} of di-ammonium phosphate, 1000 kg ha^{-1} K_2SO_4, and 200 kg ha^{-1} kieserite crystal. Several foliar applications were carried out after the planting using 700 kg ha^{-1} urea, 700 kg ha^{-1} $(NH_4)_2SO_4$, 1000 kg ha^{-1} K_2SO_4, 170 kg ha^{-1} $MgSO_4$, 60 kg ha^{-1} $FeSO_4$, 60 kg ha^{-1} $ZnSO_4$, in intervals of 30 days during three months; besides, after flowering borax was sprayed on the plant in doses of 30 kg ha^{-1}. The pedological and mineral characteristic of the soil where the experiment was implemented are presented in Table 1. Furthermore, during the experiment, a weather station (LSI Lastem; equipped with a CR6 data logger from Campbell Scientific; Italy) measured an average relative humidity (RH) of 89.34 %, an ambient temperature of 26.8 °C, solar radiation of 16.83 w m^{-1}, and a monthly average rainfall of 133.77 mm.

The experiment was arranged in a split-plot design, with two factors. One factor concerning two methods of inoculation of *D. zeae* bacterium and a second factor about four treatments implemented. Each treatment had three replications with 44 fruits. Field rows where the treatments were administrated consisted of 0.4 m width and 3.75 m length. Pineapple plants were arranged in two lines of 22 plants in the rows with a separation of 0.25 m. Observations were carried out once every two weeks, from six weeks before harvest. The arrangement of the experiment factors used with their respective characteristics are presented in Table 2.

Table 1: Pedological and mineral nutrients characteristics of the soil in the experiment

Texture	
Clay (%)	18.56
Loam (%)	13.01
Sand (%)	68.43
Chemical composition	
pH (H_2O)	6.8
C (%)	0.7
N (mg kg^{-1})	800
P (mg kg^{-1})	43.75
K (mg kg^{-1})	319.8
Ca (mg kg^{-1})	638
Mg (mg kg^{-1})	235.2
Na (mg kg^{-1})	4.6

*The N, P, K, Ca, Mg and Na represent the available mineral nutrients content in the soil

Table 2: The organization of the experiment design employed in the research

Factor one (Inoculation method)
1. Spraying before the open-heart stage.
2. Injection into the fruit flesh from six weeks before harvest.
Factor two (Treatments)
A. Control (No biopesticides used)
B. Bio P32 from 13 weeks before harvest
C. Bio T10 from 13 weeks before harvest
D. Bio P32 + Bio T10 from 13 weeks before harvest

The control had only inoculated the bacterium for each of the methods used (sprayed or injected, respectively). For both inoculation methods, juice of previously infected fruits extracted from the flesh (including *D. zeae*) was employed. For the spray method, doses of 20 ml juice/plant-fruit were employed using a hand sprayer. These doses were selected after field trails before the beginning of this experiment demonstrated that with their employment the fruits exposed symptoms of fruit collapse just after flowering. Also, those trials proved that sprays applications during flowering were more effective to cause fruit collapse than injections. The plants were sprayed at night, in two and one week before flowering and one week thereafter (13, 12 and 11 weeks before harvest). The sprayings moment tried to represent the typical field infection during flower induction, where a latent bacterium in the environment enters the plant through the nectarthodes (Wang et al., 2011; Sipes and Pires de Matos, 2018).

On the other hand, for the inject method were administrated doses of 0.2 ml juice/fruit with a syringe. These doses were implemented after previous field trials exposed that with these doses a fruit can present fruit collapse symptoms during advance stage of development, close to harvest. Also, these doses were employed by Barral et al. (2017). They demonstrated that injections with these doses in pineapple are enough to inoculate a disease before harvest. Moreover, these trials demonstrated that for an advance fruit development, *D. zeae* inoculations with injections were more effective than sprayings. The sprayed inoculations on the plant were administrated in six, four, and two weeks before harvest. For this method, four eyes of the pineapple shell were inoculated by pushing a syringe through them. Two eyes were inoculated on the upper part and two on the lower part of the shell, similar to the technic described in Barral et al. (2017). The inoculation time selected for the inject method intend to replicate another typical moment of infection by *D. zeae*, in this case close to harvest, entering through the shell stomata, as described in Sipes and Pires de Matos (2018).

Concerning the biopesticides applications, from 13 to 10 weeks before harvest, those were employed weekly. Later after ten weeks, those were applied one time every two weeks until harvest. Bio P32 [in 1 l of product solution: 10 % of snail meat, 2 g of fermented Shrimp, and 10 ml of *P. fluorescens* - (10^{12} cell ml^{-1}) - strain 32] and Bio T10 [in 1 l of product solution: 10 g of rice flour and white sugar, and 10 ml of *T. harzianum* (10^8 conidia ml^{-1}) - strain 10] were used in doses 20 ml/per plant-fruit (v/v: 20 ml l^{-1}) during night time. Furthermore, where the fruits had an advance maturation, the biopesticides were not only sprayed in the leaves, also directly into the shell and crown, understanding that the stomata and lenticels available in those structures could permit their absorption and assimilation, as recommend by Soesanto et al. (2011) and (2020).

2.2 DETECTION OF THE TOTAL SOLUBLE SOLIDS (TSS), TOTAL ACIDITY (TA) AND FRUIT COLLAPSE INCIDENCE

The TSS and TA were calculated following the procedures described in Shamsudin et al. (2020), in a composition of four fruits per replication of each treatment arranged. TSS was measured by implementing a handheld refractometer (MASTER-53 α; Atago: Japan), while the TA was detected by titration to pH 8.1 with 0.1 M NaOH using phenolphthalein as an indicator and re-

vealed as a percentage of citric acid. The incidence of fruit collapse was measured by detecting and collecting the percentage of fruits presenting the disease symptoms described in Cano-Reinoso et al. (2021).

2.3 ASCORBIC ACID (ASA) AND SUCROSE CONTENT DETERMINATION

The AsA and sucrose content of the fruits was measured by the method reported in Siti Roha et al. (2013), using a High-Performance Liquid Chromatography (model L-2000 instrument; Hitachi: USA) with a Refractive Index detector model L-2490. A juice extracted from the fruit flesh adjacent to the core was used. The samples were obtained from a composition of four fruits per replication in each of the treatments arranged. Standard solutions of AsA and sucrose were dissolved in distilled water and filtered through a Millipore 0.45 µm membrane filter. The AsA and sucrose content were quantified, comparing the peak area by a chromatographic procedure.

2.4 MINERAL NUTRIENTS DETERMINATION

The calcium and magnesium content of the fruits was calculated using atomic absorption spectrometry (AAS 932 Plus; GBC scientific equipment: USA), employing a composition of four fruits per replication in each of the field treatments. The method applied was the one described in Benton-Jones (2001). Juice samples were put in a digestion tube with 5 ml of 65 % nitric acid and left overnight. Later, the samples were heated with a block digester at 125 °C for one hour. After that, 3 ml of 30 % hydrogen peroxide (H_2O_2) were added and reheated for one hour; thereafter, HNO_3 was used (1 ml residue) and 5 ml of nitric acid with distillate water (1:10) were added and shaken. Finally, the samples were move to a 25/50 ml flask quantitatively and pitched with distillate water, with the goal of creating an extract ready to determine the calcium and magnesium content. As the water content of the samples were previously detected, the results are expressed in a dry basis content.

2.5 DETECTION OF THE ELECTROLYTE LEAKAGE (EL)

Following the EL calculation in pineapple fruit reported in Chen and Paull (2001), the EL of the fruit flesh was obtained from the composition of four fruits per replication of the treatments implemented. Plugs were taken with a cork borer applying a longitudinal cut and then slides into a disk of 2 mm of thickness. Around 6 g of the disk were washed three times to remove any lysed material from the cell. For two hours, the disks were shaken and incubated in 60 ml of 0.3 M mannitol solution. Later on, the conductivity of the previous solution was obtained with a radiometer. After that, the samples were boiled for two hours to release all the electrolytes, and the conductivity was determined. The EL is shown as the percentage of the total conductivity.

2.6 SCANNING ELECTRON MICROSCOPE (SEM) EVALUATION

SEM analysis was performed using a similar method reported in Hu et al. (2012). A piece of tissue adjacent to the core ($5 \times 5 \times 2$ mm^3) was split from the middle of the flesh with a tweezer. Before scanning, the slices were dehydrated in a series of ethanol solutions and dried at a critical point of liquid CO_2 using a desiccator. The samples were mounted onto aluminum specimen stubs employing conductive silver glue and sputter-coated with gold. SEM was executed with a scanning electron microscope (ZEISS/EVO MA 10: German) equipped with an energy dispersive spectroscopy (EDS) at 15.00 kV.

2.7 STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS Version 22.0 software (SPSS Inc.; Chicago, IL: USA). All data were analyzed by a two-way ANOVA. Mean significant differences at $p < 0.05$ were determined by Duncan's multiple range tests and Kruskal-Wallis test (in case of the fruit collapse incidence data).

3 RESULTS AND DISCUSSION

3.1 TOTAL SOLUBLE SOLIDS (TSS), TOTAL ACIDITY (TA), AND SUCROSE CONTENT IN THE FRUIT

The TSS presented significant differences in the interaction results. The treatment D obtained the highest value (14.87 %), when the spray method was employed; however, the same treatment in the case of the inject method delivered the lowest outcome (12.33 %). Lower TSS content was associated with a higher fruit collapse incidence (Table 3). Previous studies reported that the value of the TSS for commercial consumption of pineapple low acid hybrids should be at least close to 12 % (Lu et al., 2014; Bartholomew and Sanewski, 2018; Ca-

no-Reinoso et al., 2022a); this requirement was assessed in the treatment results of both inoculation methods; also, this circumstance could have been promoted by the treatments used as previous authors have reported a positive effect on the TSS content by the administration of biopesticides based on of *P. fluorescens* and *T. harzianum* (Jiang et al., 2019; Carillo et al., 2020). Besides, it has been demonstrated that pathogens interfere with the metabolism of the host by increasing their sugar uptake, especially at the phloem level, decreasing the final TSS content in sink organs like the fruit (Morkunas and Ratajczak, 2014; Naseem et al., 2017). This fact explains why the TSS treatment results of the inject method were lower than the spray one, due to the most critical case of infection in this method causing fruit collapse.

In the case of the TA, there were no significant differences delivered in the interaction outcomes; However, The TA values were higher in the inject method while lower in the spray method (0.69 % and 0.51 %, respectively) (Table 3). This more superior TA content was linked to a higher fruit collapse incidence. In pineapple, TA mainly is a measuring of the citric acid level of the fruit (Saradhuldhat and Paull, 2007; Paull and Chen, 2018). In MD2, the total TA value range between 0.4–0.7 % (Lu et al., 2014; Paull and Chen, 2018). Values inside this range were represented in the interaction results at harvest. However, the higher content of TA in the inject method could have been provoked by a more superior citric acid accumulation. Nevertheless, further studies should be done on this matter.

Concerning the sucrose content, in the interaction results there were significant differences evidenced. The treatment D with inject method had the most reduced outcome (4.31 %); on the contrary, the highest result was observed in the same treatment but when the spray method was employed (9.85 %) (Table 3). A higher content of sucrose was noticeably associated with a more reduced incidence of fruit collapse. The most crucial sugar in pineapple is sucrose. Previous research reported that in low acid hybrids the sucrose should be between 7–9 % at harvest (Nadzirah et al., 2013; Lu et al., 2014). Values among that range were reflected in this research outcomes. It has been proved that cell wall invertase (CWI) is one of the enzymes highly correlated with the sucrose accumulation in pineapple (Saradhuldhat and Paull, 2007; Paull and Chen, 2018). Recently evidence indicated that pathogens generated the induction of CWI activity, producing more hexose as sugars to support their metabolic activities, interfering the normal sugar accumulation in the fruit (Yamada et al., 2016; Naseem et al, 2017). These previous facts inferred that *D.zeae* influencing the CWI activities affected the sucrose accumulation, especially with the inject method, causing a more superior fruit collapse incidence. However, despite reports explaining the increase of sucrose under biopesticides applications of *P. fluorescens* and *T. harzianum* in several fruits (Jiang et al., 2019; Carillo et al., 2020); this phenomenon was not fully evidenced in the inject method, as this was more harmful to the fruit, nullifying this positive characteristic, especially in treatment D. More studies could be elaborated to determine the relation of the biopesticides used in this experiment with the inoculation methods influencing sugar enzymes activities.

Table 3: Effects of the interaction between the treatments and the inoculation methods implemented on pineapple quality and fruit collapse incidence

Treatments*Inoculation methods	TSS (%)	TA (%)	Sucrose (%)	AsA (mg kg^{-1})	Fruit collapse Incidence (%)
A*Spray	13.87 ± 0.18 ab	0.52 ± 0.02 a	9.59 ± 0.21 ab	188.61 ± 28.09 a	3.33 bc
B*Spray	13.93 ± 0.18 ab	0.50 ± 0.02 a	9.22 ± 0.25 abc	91.35 ± 69.37 b	0.00 c
C*Spray	14.13 ± 0.24 ab	0.53 ± 0.01 a	9.11 ± 0.11 abc	53.07 ± 1.39 b	5.00 bc
D*Spray	14.87 ± 0.35 a	0.50 ± 0.01 a	9.85 ± 0.06 a	78.96 ± 15.37 ab	1.67 c
A*Inject	12.80 ± 0.61 ab	0.71 ± 0.13 a	7.93 ± 0.25 c	98.99 ± 5.75 ab	20.0 ab
B*Inject	12.80 ± 1.44 ab	0.65 ± 0.12 a	8.43 ± 1.05 bc	233.42 ± 72.41 a	23.3 a
C*Inject	12.33 ± 0.71 b	0.70 ± 0.16 a	8.77 ± 0.19 abc	132.10 ± 1.05 ab	20.0 ab
D*Inject	12.33 ± 0.27 b	0.70 ± 0.12 a	4.31 ± 0.06 d	181.75 ± 21.63 a	20.0 ab

**Each value represents a mean ± standard error. Mean values in each column followed by the same lower-case letters are not statistically different by Duncan's multiple range test and Kruskal-Wallis test (for the fruit collapse incidence and severity data) ($p < 0.05$)

***A (Control: No biopesticide used), B (Bio P32 from 13 weeks before harvest), C (Bio T10 from 13 weeks before harvest), D (Bio P32 + Bio T10 from 13 weeks before harvest). TSS (Total Soluble Solids), TA (Total Acidity), AsA (Ascorbic Acid)

3.2 ASCORBIC ACID (ASA) CONTENT IN THE FRUIT

Observations of the AsA results exposed significant differences in the interaction outcomes. The highest value was obtained in treatment B with the inject method (233 mg kg^{-1}), and the lowest outcome in treatment C with the spray one (53.07 mg kg^{-1}) (Table 3). Overall, higher val-

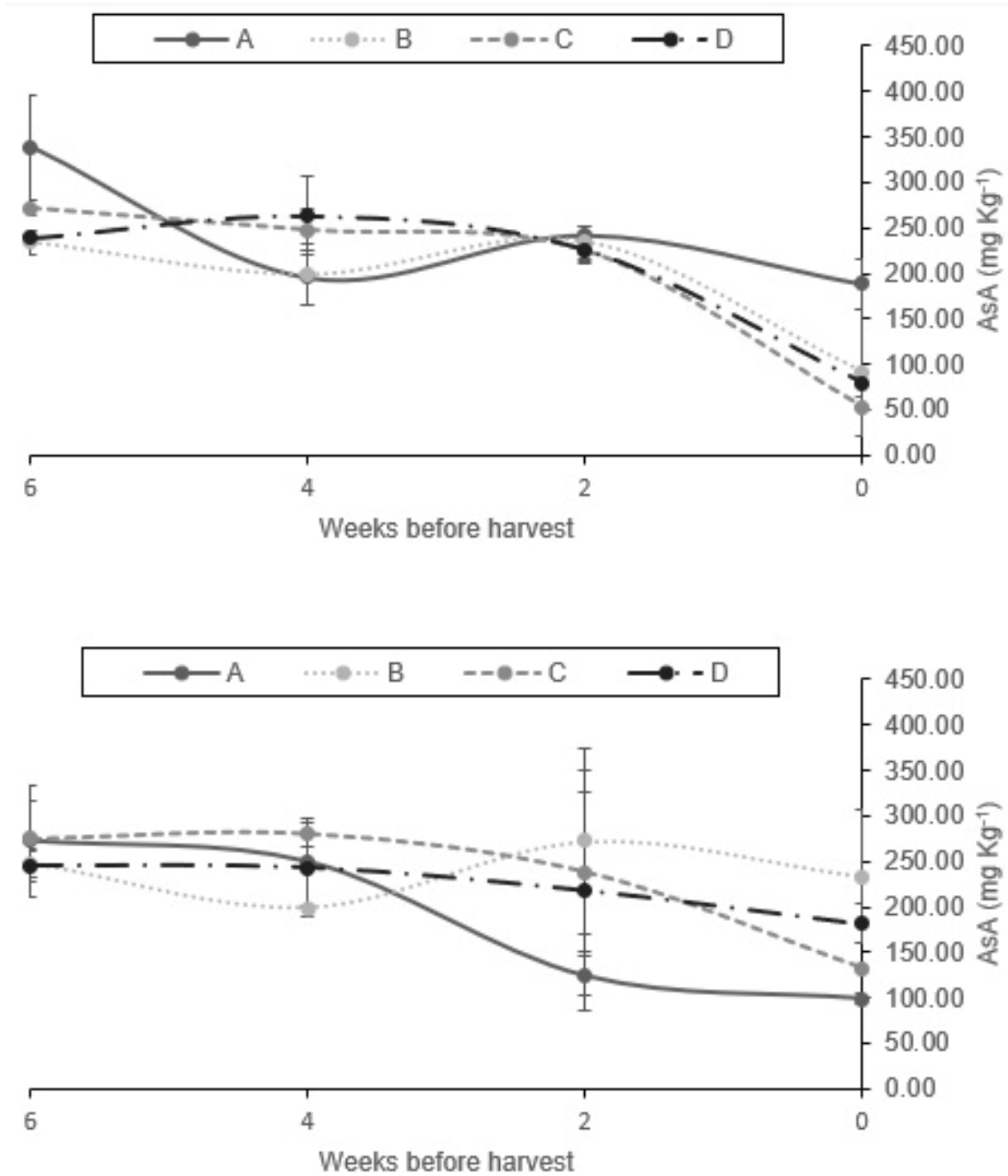


Figure 1: Trend of the ascorbic acid (AsA) content during the experiment for the treatments applied in both inoculation methods. A (Control: No biopesticide used), B (Bio P32 from 13 weeks before harvest), C (Bio T10 from 13 weeks before harvest), D (Bio P32 + Bio T10 from 13 weeks before harvest). Values are the mean of three replicates, and error bars represent the standard error

ues of AsA were linked to a more superior incidence of fruit collapse. The trend of the AsA content trough the experiment is presented in Figure (1). This figure shows that in four weeks before harvest there is a remarkable change in the trend of AsA in both inoculation methods, particularly in the inject one, which could have caused a physiological impact generating the final content at harvest.

The AsA in MD2 pineapple usually range between 300–600 mg kg^{-1} at harvest (Lu et al., 2014; Paull and Chen, 2018; Cano-Reinoso et al., 2022a). Besides, previous researches reported a positive correlation between the AsA content in pineapple and its antioxidant activity. The AsA values obtained in this research were lower than the range formerly determined; however, this could be ascribed to the plant's environmental conditions through the experiment time. It is possible to identify that from four weeks before harvest, when the organic acids accumulations start to happen, the AsA content never reach values close to 300 mg kg^{-1} (Figure 2). Seemingly, the irradiation, rainfall and resulting temperature could have affected the AsA accumulation in the fruit, as described in Ferreira et al. (2016) and Paull and Chen (2018).

As has been proved to encourage the activities of several scavenger enzymes like catalase (CAT), peroxidase (POD) and ascorbic peroxidase (APX) (Akram et al., 2017; Noichinda et al., 2017). Pathogen infections cause an increase in the reactive oxygen species (ROS); this circumstance creates a rise of AsA and subsequent scavenging activities to cope with these ROS generation in fruits (Lu et al., 2014; Noichinda et al., 2017). Furthermore, *T. harzianum* and *P. fluorescens* in different liquid culture applications have proved to enhance the antioxidant capacity, scavenger enzyme activities, and resistance mechanisms like hypersensitive responses (HR), in fruits and vegetables (Garcia-Seco et al., 2015; Sood et al., 2020).

The past information demonstrated why the treatments having biopesticides applications in the inject method increase substantially their AsA content. Besides, due to its more harmful impact, this method could have promoted a higher activity of scavenger enzymes, AsA accumulation, and HR to mitigated the fruit injure; a phenomenon that could have occurred also in treatment A without biopesticides used. However, the exhibition of this situation was not enough to reduce the damage created by the pathogen infection, which is evidenced in the higher fruit collapse incidence associated with the more superior AsA content, also in the inject method. Moreover, the insufficient AsA content detected weeks before harvest could have made more difficult to generate an optimal physiological respond of the fruit on these circumstances.

3.3 MINERAL NUTRIENTS CONTENT AND ELECTROLYTE LEAKAGE (EL)

Mineral nutrients interaction outcomes exposed significant differences at harvest. Nonetheless, the observation of the results exposed that the method of inoculation impacted these variables. For the calcium, the most elevated value was detected in treatment C with the inject method (2522.27 mg kg^{-1}); meanwhile, the lowest one was observed in the same treatment but using the spray method (1575.63 mg kg^{-1}). In the case of magnesium, the most elevated result was determined in treatment C using the inject method (2526.31 mg kg^{-1}) and the most reduced in treatment D with the spray one (1837.48 mg kg^{-1}) (Table 4). For the result of both mineral nutrients, the higher content was associated with a more superior incidence of the fruit collapse.

Calcium has been proved to rise the resistance of fruits and vegetables to pathogens attacks by increasing the cellular responses to biotic signals and reducing the cell wall breakdown (Madani et al., 2016; De Freitas and Resender Nassur, 2017). Concerning magnesium, this is a component of the middle lamella and also has been reported to activate calcium-dependent protein kinases (CPDKs) (Waraich et al., 2011; Huber and Jones, 2013); proteins that translated the Ca^{2+} signature into specific phosphorylation events generating signaling responses as part of plant defense mechanisms (Gao et al., 2014; De Freitas and Resender Nassur, 2017; Cano-Reinoso et al., 2022b). Evidently, due to the more severe infection generated by the inject method, the fruit as a protection mechanism could have promoted the increase in the uptake of calcium and magnesium to maintain the cell wall structure, encouraging more molecular ions assimilation and enzyme activities (Ca^{+2}, CDPKs, respectively). Besides, *T. harzianum* and *P. fluorescens* have been associated with a more remarkable assimilation of mineral nutrients content in terms of N, P, K, Ca and Mg in plants and fruits (Pérez-Rodriguez et al., 2020; Sood et al., 2020). These facts make clearer that the biopesticides may have an influence on the plant and fruit defense mechanism when a certain high degree of affectation is reached, in this case, triggering the respective calcium and magnesium increase. However, like the situation observed in the AsA results, these effects were not enough to decrease the incidence of fruit collapse in the inject method.

The interaction results for the EL content at harvest presented significant differences. The most elevated value was observed in treatment D using spray method of inoculation (72.10 %), while the most reduced value was obtained in treatment A with the inject method (54.19 %) (Table 4). A trend of the EL content trough the experi-

Table 4: Effects of the interaction between the treatments and the inoculation methods implemented on pineapple mineral nutrients content, and the electrolyte leakage (EL)

Treatments*Inoculation methods	Ca (mg kg^{-1})	Mg (mg kg^{-1})	EL (%)
A*Spray	1853.55 ± 106.34 bc	2077.48 ± 106.54 abc	65.27 ± 5.48 abcd
B*Spray	1736.12 ± 107.81 c	1993.41 ± 102.55 bc	67.99 ± 6.61 abc
C*Spray	1575.63 ± 90.16 c	1875.93 ± 94.90 c	70.81 ± 1.39 ab
D*Spray	1780.47 ± 29.35 c	1837.48 ± 81.18 c	72.10 ± 3.92 a
A*Inject	2335.41 ± 306.44 ab	2335.25 ± 237.64 ab	54.19 ± 1.70 d
B*Inject	2474.59 ± 233.43 a	2442.74 ± 195.10 ab	58.92 ± 2.59 bcd
C*Inject	2522.27 ± 188.09 a	2526.31 ± 96.49 ab	57.47 ± 2.63 cd
D*Inject	2399.15 ± 52.38 a	2451.99 ± 42.28 ab	57.69 ± 2.70 cd

** Each value represents a mean ± standard error. Mean values in each column followed by the same lower-case letters are not statistically different by Duncan's multiple range test ($p < 0.05$)
***A (Control: No biopesticide used), B (Bio P32 from 13 weeks before harvest), C (Bio T10 from 13 weeks before harvest), D (Bio P32 + Bio T10 from 13 weeks before harvest). EL (Electrolyte Leakage)

ment is presented in Figure (2). In this figure it is possible to observe that the EL had a noticeable increase in both methods of inoculation between four and two weeks before harvest, more remarkable in the treatment B of the inject method (around 80 %), which had a EL content much higher than those of the spray method. This outcome can provide a broader understanding concerning the relation of EL with the fruit collapse incidence at harvest, especially for treatment B.

The EL reflects a loss of integrity in cell membranes, common during a pathogen infection (Demidchik et al., 2014). In pineapple fruit, the EL speeds up from six weeks before harvest in concomitance with the sucrose accumulation (Paull and Chen, 2003, 2018). This research exposed that treatment using the spray method had higher EL, which should be correlated with a more superior incidence of fruit collapse; however, this only happened in the treatments employing the inject method. The differences in EL percentage between the two methods were more related to the experiment design and unique status of the sample analyzed. In the Figure (2) it is possible to observe that fruits of the inject method two weeks before harvest had an EL percentage almost like those of the spray method at harvest, especially in treatment B. This situation means that at this time, the fruits gathered from the inject method were predominantly affected by fruit collapse, while at harvest, the number of fruits with disease symptoms were highly reduced. Therefore, it is possible to infer that the EL in the inject method can be correlated to a more superior fruit collapse incidence, analyzing the results from two weeks before harvest. Furthermore, the higher EL percentages of the spray method can be more associated with the normal process of sugar accumulation in pineapple than a physiological response to the stress induced by the bacterium attack; because of that, the lower fruit collapse incidence. Concerning *T. harzianum* and *P. fluorescens*, there is still insufficient information of their influence on the EL in plants and fruits; however, their recognized beneficial impact on calcium uptake could suggest that these biopesticides would help to decrease the percentage of EL under a disease infection. High calcium accumulation has been related to a leakage reduce when a plant is subjected to an abiotic or biotic factor (Demidchik et al., 2014; De Freitas and Resender Nassur, 2017). More experiments could be done on this aspect.

3.4 SEM ANALYSIS

SEM analysis was conducted at harvest time in the treatments A, B, C, and D of the inject inoculation method (Figure 3). The sample of the treatments A and B showed characteristics of a low cell wall integrity, identified by arrows with lack of significant thickness, and an undulated shape not attached to the vascular bundles of the cell. On the contrary, in treatments C and D it was possible to observe symptoms of membrane well-function, with arrows presenting more significant thickness and turgor.

During infections bacteria can cause an increase in the activities of pectolytic and polygalacturonases (PG) enzymes, which are known for their the cell wall degrading properties (Hocking et al., 2016; De Freitas and Resender Nassur, 2017). The activities of these enzymes can be mitigated by minerals like calcium, which binds to the cell wall and increases its strength, making the cell wall matrix less accessible to them (De Freitas and Resender Nassur, 2017). This information suggested the treatments C and D of the inject method caused a lower

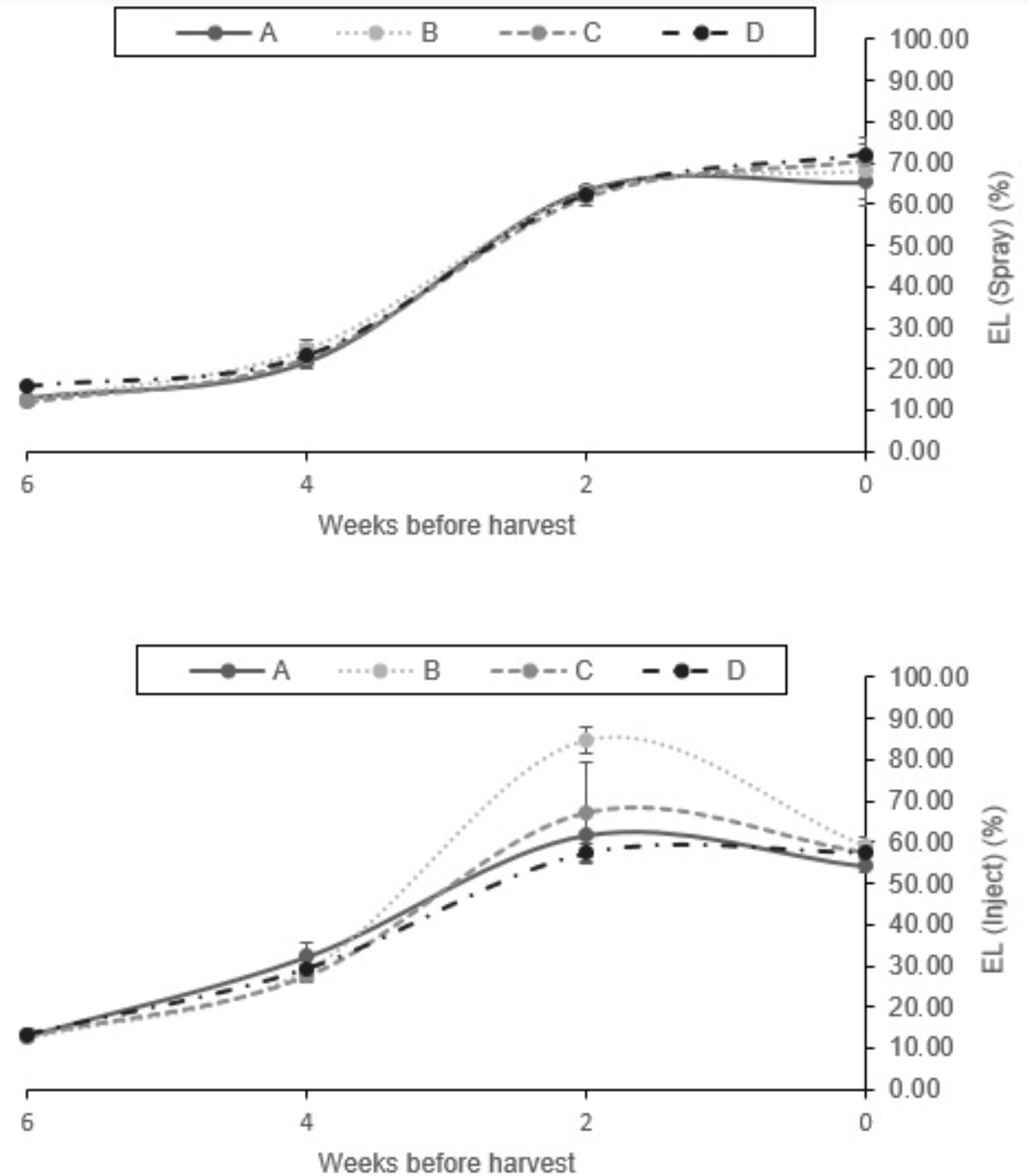


Figure 2: Trend of the Electrolyte leakage (EL) content during the experiment for the treatments applied in both inoculation methods. A (Control: No biopesticide used), B (Bio P32 from 13 weeks before harvest), C (Bio T10 from 13 weeks before harvest), D (Bio P32 + Bio T10 from 13 weeks before harvest). Values are the mean of three replicates, and error bars represent the standard error

activity of these enzymes, generating a healthier cell wall status, opposite to treatments A and B, as exposed in the SEM analysis. Despite of the high concentration content of calcium in the treatments A and B, their cell wall primary layer displayed unhealthy characteristics. This situation could be attributed to the lower assimilation

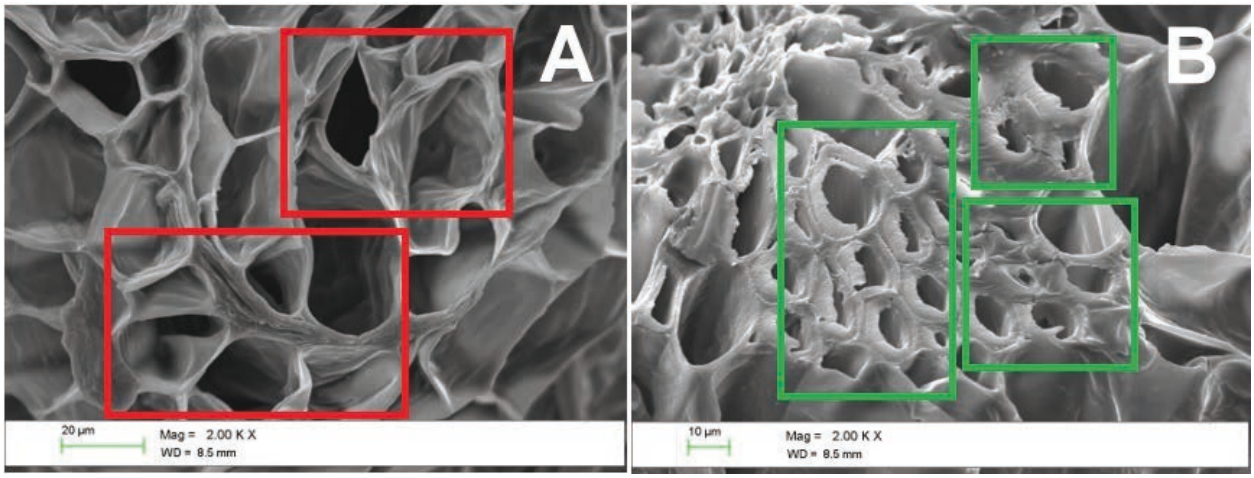


Figure 3: Effects of the treatments A, B, C, and D in the cell walls of the inject method of inoculation detected by SEM (20 and 10 µm size, respectively; with 2000 x of magnification). The smaller thickness and undulated arrows of the cell wall (red square) and more significant thickness and non-undulated arrows (green square) were examined. Treatments, A (Control: No biopesticide used), B (Bio P32 from 13 weeks before harvest), C (Bio T10 from 13 weeks before harvest), D (Bio P32 + Bio T10 from 13 weeks before harvest)

of calcium ions (Ca^{2+}) into the cell wall matrix produced by the harmful impact of the inject method when treatments A and B were implemented. Besides, the calcium ions of these treatments could have also been employed in other calcium-influenced-process like sugar production and fruit respiration (Hocking et al., 2016; Meeteren and Aliniaeifard, 2016); decreasing its sensing activity into the cell wall.

3.5 FRUIT COLLAPSE INCIDENCE

There were significant differences evidenced in the interaction outcomes of the fruit collapse incidence. The treatment B in the spray method obtained the lowest incidence (0 %), while the same treatment but in the in the inject method had the highest one (23.7 %) (Table 4). Moreover, the inject method delivered in average a higher incidence than the spray one for all the treatments (20.93 and 2.50 %, respectively). This evidence finally proves that the inject method was more effective in causing symptoms of this disease. On the other hand, the observation of the significant differences and the mean values of the interaction outcomes can provide the insight that C and D can be considered as the best options to control fruit collapse disease; meanwhile, A and B could be taken as less effective in this aspect. This affirmation can be supported by the examination of the influences of these treatments on the quality variables studied (specially the mineral nutrients content, AsA content, and EL), the cell wall status by the SEM analysis previously described, and their relation with the fruit collapse incidence in both inoculation methods. C and D despite not exposing always the highest outcomes, those delivered mostly optimal results in the previous parameters mentioned, primordially a healthy cell wall, which could help to predict that under a more harmful conditions of infection than the implemented in this experiment, these treatments could satisfactorily mitigate the fruit collapse occurrence. On the contrary, A and B, although displayed high outcomes, regarding antioxidants and resistance parameters, like AsA and Ca content, especially in the case of B, their high EL from weeks prior to harvest, together with their unhealthy cell wall status, suggested that under elevated infections these treatments could not provide enough protection to the fruit.

Furthermore, due to the characteristics of both inoculation methods used in this research, where the juice had to be extracted from previously infected fruits, it was complicated to determine in every juice concentration the number of colonies forming unit (CFU) existed. Previous laboratory trials before the beginning of this experiment demonstrated that the minimal number of CFU required to inoculate *D. zeae* in pineapple should be around 10^7–10^9 CFU ml^{-1}, which was in agreement with former experiments described by Sueno et al. (2014) and Aeny et al. (2020)HI, on a pineapple cultivar (Ananas comosus 'PRI 73-114'. This information could help to support the explanation about why the spray method was less effective in showing fruit collapse symptoms. Because of the characteristic of this method, the number of CFU ml^{-1} required to cause a *D. zeae* infection could be higher than the inject method. Moreover, because of the number of colonies necessary to produce an infection in

the inject method, the doses of biopesticides employed (20 ml/plant-fruit), together with the concentration number of cell ml^{-1} and conidia ml^{-1} in those products (*P. fluorescens* and *T. harzianum*, respectively), could not be enough to mitigate the impact of fruit collapse. For future experiments, the doses and the concentration number of cells and conidia per ml of *P. fluorescens* and *T. harzianum* should be increased in the case that this experiment wants to be replicated in pineapple. On top of that, those future trials could also implement a chemical pesticide treatment as positive control. These future arrangements could help to stablish the differences between the biopesticides administrated in this research and any conventional pesticide, essentially concerning pineapple quality and fruit collapse occurrence. As the employment of chemical agents were outside of the scope of this experiment, this should be a point to be observed eventually.

4 CONCLUSIONS

The biopesticides based on *Pseudomonas fluorescens* and *Trichoderma harzianum* affected the fruit collapse disease incidence and pineapple quality. Treatment C (Bio T10 from 13 weeks before harvest), and D (Bio P32 + Bio T10 from 13 weeks before harvest) delivered the best results having an ideal AsA, EL, mineral nutrients content, healthier cell wall characteristics, and a low fruit collapse incidence, essentially after analyzing their outcomes in both inoculation methods. Meanwhile, treatments A (Control: No biopesticide used), and B (Bio P32 from 13 weeks before harvest) were less effective in these aspects. Finally, the inject inoculation method caused more fruit collapse incidence than the spray one. The number of CFU of *D. zeae* were considered as the reasons why the inject method was more severe affecting the fruit physiology and effective in generating the higher incidence.

5 ACKNOWLEDGEMENTS

Sincere thanks want to be expressed by the authors of this article to PT Great Giant Pineapple in Lampung Indonesia, their research department, and the laboratory staff for the vital and logistic support with all the activities carried out during this experiment.

6 REFERENCES

Aeny, T. N., Suharjo, R., Ginting, C., Hapsoro, D. W. I., & Niswati, A. (2020). Characterization and host range assessment of *Dickeya zeae* associated with pineapple soft rot disease in east Lampung, Indonesia. *Biodiversitas*, *21*(2), 587–595. https://doi.org/10.13057/biodiv/d210221

Akram, N. A., Shafiq, F., & Ashraf, M. (2017). Ascorbic acid-a potential oxidant scavenger and its role in plant development and abiotic stress tolerance. *Frontiers in Plant Science*, *8*, 1–5. https://doi.org/10.3389/fpls.2017.00613

Barral, B., Chillet, M., Minier, J., Léchaudel, M., & Schorr-Galindo, S. (2017). Evaluating the response to *Fusarium ananatum* inoculation and antifungal activity of phenolic acids in pineapple. *Fungal Biology*, *121*(12), 1045–1053. https://doi.org/10.1016/j.funbio.2017.09.002

Bartholomew, D. P., & Sanewski, G. M. (2018). Inflorescence and Fruit Development and Yield. In G. M. Sanewski, D. P. Bartholomew, & R. E. Paull (Eds.), *The pineapple: botany, production and uses* (pp. 223–268). London, UK: CABI Publishing. https://doi.org/10.1079/9781786393302.0233

Benton-Jones, J. J. (2001). *Laboratory guide for conducting soil tests and plant analysis*. New York, USA: CRC Press. https://doi.org/10.1201/9781420025293

Bin Thalip, Amar Ahmadi, Tong P.S., & Casey Ng. (2015). The MD2 "Super Sweet" pineapple (Ananas comosus). *Utar Agriculture Science Journal*, *1*(4), 14–17.

Cano-Reinoso, D. M., Soesanto, L., Kharisun, & Wibowo, C. (2021). Review: Fruit collapse and heart rot disease in pineapple: Pathogen characterization , ultrastructure infections of plant and cell mechanism resistance. *Biodiversitas*, *22*(5), 2477–2488. https://doi.org/10.13057/biodiv/d220504

Cano-Reinoso, D. M., Soesanto, L. Kharisun, & Wibowo, C. (2022a). Effect of pre-and postharvest treatments with salicylic acid on physicochemical properties of pineapple cv. MD2. *Chiang Mai University Journal of Natural Sciences*, *21*(3), e2022039. https://doi.org/10.12982/CMUJNS.2022.039

Cano-Reinoso, D. M., Kharisun, K., Soesanto, L., & Wibowo, C. (2022b). Effect of calcium and silicon fertilization after flowering on pineapple mineral status and flesh translucency. *Plant Physiology Reports*, *27*(1), 96–108. https://doi.org/10.1007/s40502-022-00651-2

Carillo, P., Woo, S. L., Comite, E., El-nakhel, C., Rouphael, Y., Fusco, G. M., Borzacchiello, A., Lanzuise, S., & Vinale, F. (2020). Application of *Trichoderma harzianum*, 6-pentyl-α-pyrone and plant biopolymer formulations modulate plant metabolism and fruit quality of plum tomatoes. *Plants*, *9*(6), 1–15. https://doi.org/10.3390/plants9060771

Chen, C. C., & Paull, R. E. (2001). Fruit temperature and crown removal on the occurrence of pineapple fruit translucency. *Scientia Horticulturae*, *88*(2), 85–95. https://doi.org/10.1016/S0304-4238(00)00201-6

Chen, N. J., & Paull, R. E. (2017). Production and postharvest handling of low acid hybrid pineapple. *Acta Horticulturae*, *1166*, 25–34. https://doi.org/10.17660/ActaHortic.2017.1166.4

Chen, N. J., Paull, R. E., Chen, C. C., & Saradhuldhat, P. (2009). Pineapple production for quality and postharvest handling. *Acta Horticulturae*, *822*, 253–260. https://doi.org/10.17660/ActaHortic.2009.822.31

De Freitas, S. T., & Resender Nassur, R. C. M. (2017). Calcium

treatments. In S. Pareek (Ed.), *Novel postharvest treatments of fresh produce* (pp. 52–68). Boca Raton, USA: CRC Press. https://doi.org/10.1201/9781315370149-3

Demidchik, V., Straltsova, D., Medvedev, S. S., Pozhvanov, G. A., Sokolik, A., & Yurin, V. (2014). Stress-induced electrolyte leakage: The role of K+-permeable channels and involvement in programmed cell death and metabolic adjustment. *Journal of Experimental Botany*, *65*(5), 1259–1270. https://doi.org/10.1093/jxb/eru004

Ding, P., & Syazwani, S. (2016). Physicochemical quality, antioxidant compounds and activity of MD-2 pineapple fruit at five ripening stages. *International Food Research Journal*, *23*(2), 549–555.

Ferreira, E. A., Siqueira, H. E., Boas, E. V. V., Hermes, V. S., & Rios, A. de O. (2016). Compostos bioativos e atividade antioxidante de frutos de cultivares de abacaxizeiros. *Revista Brasileira de Fruticultura*, *38*(3), 1–7. https://doi.org/10.1590/0100-29452016146

Gao, X., Cox, K. L., & He, P. (2014). Functions of calcium-dependent protein kinases in plant innate immunity. *Plants*, *3*(1), 160–176. https://doi.org/10.3390/plants3010160

Garcia-Seco, D., Zhang, Y., Gutierrez-Mañero, F. J., Martin, C., & Ramos-Solano, B. (2015). Application of Pseudomonas fluorescens to blackberry under field conditions improves fruit quality by modifying flavonoid metabolism. *PLoS ONE*, *10*(11), 1–23. https://doi.org/10.1371/journal.pone.0142639

Hamarawati, E., Mugiastuti, E., Manan, A., Loekito, S., & Soesanto, L. (2017). Applications of *Pseudomnas fluorescens* P60 in Controlling Basal Stem Rot (*Sclerotium rolfsii* Sacc.) on Dragon Fruit Seedlings. *Asian Journal of Plant Pathology*, *12*(1), 1–6. https://doi.org/10.3923/ajppaj.2018.1.6

Hocking, B., Tyerman, S. D., Burton, R. A., & Gilliham, M. (2016). Fruit calcium: Transport and physiology. *Frontiers in Plant Science*, *7*, 1–17. https://doi.org/10.3389/fpls.2016.00569

Hu, H., Li, X., Dong, C., & Chen, W. (2012). Effects of wax treatment on the physiology and cellular structure of harvested pineapple during cold storage. *Journal of Agricultural and Food Chemistry*, *60*(26), 6613–6619. https://doi.org/10.1021/jf204962z

Jiang, M. Y., Wang, Z. R., Chen, K. W., Kan, J. Q., Wang, K. T., Zalán, Z. S., Hegyi, F., Takács, K., & Du, M. Y. (2019). Inhibition of postharvest gray mould decay and induction of disease resistance by *Pseudomonas fluorescens* in grapes. *Acta Alimentaria*, *48*(3), 288–296. https://doi.org/10.1556/066.2019.48.3.2

Kleemann, L. (2016). Organic Pineapple Farming in Ghana - A Good Choice for Smallholders? *The Journal of Developing Areas*, *50*(3), 109–130. https://doi.org/10.1353/jda.2016.0096

Lu, X. H., Sun, D. Q., Wu, Q. S., Liu, S. H., & Sun, G. M. (2014). Physico-chemical properties, antioxidant activity and mineral contents of pineapple genotypes grown in China. *Molecules*, *19*(6), 8518–8532. https://doi.org/10.3390/molecules19068518

Madani, B., Mirshekari, A., Sofo, A., & Tengku Muda Mohamed, M. (2016). Preharvest calcium applications improve postharvest quality of papaya fruits (*Carica papaya* L. cv. Eksotika II). *Journal of Plant Nutrition*, *39*(10), 1483–1492. https://doi.org/10.1080/01904167.2016.1143500

Martínez, J. I., Gómez-Garrido, M., Gómez-López, M. D., Faz, Á., Martínez-Martínez, S., & Acosta, J. A. (2019). *Pseudomonas fluorescens* affects nutrient dynamics in plant-soil system for melon production. *Chilean Journal of Agricultural Research*, *79*(2), 223–233. https://doi.org/10.4067/S0718-58392019000200223

Meeteren, U. V., & Aliniaeifard, S. (2016). Stomata and postharvest physiology. In S. Pareek (Ed.), *Postharvest ripening physiology of crops* (pp. 157–216). Boca Raton, USA: CRC Press.

Morkunas, I., & Ratajczak, L. (2014). The role of sugar signaling in plant defense responses against fungal pathogens. *Acta Physiologiae Plantarum*, *36*(7), 1607–1619. https://doi.org/10.1007/s11738-014-1559-z

Nadzirah, K. Z., Zainal, S., Noriham, A., Siti Roha, A. M., & Nadya, H. (2013). Physico- chemical properties of pineapple variety N36 harvested and stored at different maturity stages. *International Food Research Journal*, *20*(1), 225–231.

Naseem, M., Kunz, M., & Dandekar, T. (2017). Plant–Pathogen Maneuvering over Apoplastic Sugars. *Trends in Plant Science*, *22*(9), 740–743. https://doi.org/10.1016/j.tplants.2017.07.001

Noichinda, S., Bodhipadma, K., & Wongs-Aree, C. (2017). Antioxidant Potential and Their changes during postharvest handling of tropical fruits. In S. Pareek (Ed.), *Novel Postharvest Treatments of Fresh produce* (pp. 633–662). Boca Raton, USA: CRC Press. https://doi.org/10.1201/9781315370149-20

Paull, R. ., & Chen, C. C. (2003). Postharvest Physiology, Handling and Storage of Pineapple. In D. P. Bartholomew, R. E. Paull, & K. G. Rohrbach (Eds.), *The pineapple: botany, production and uses* (pp. 253–279). London, UK: CABI Publishing. https://doi.org/10.1079/9780851995038.0253

Paull, R. E., & Chen, C. C. (2018). Postharvest Physiology, Handling and Storage of Pineapple. In G. M. Sanewski, D. P. Bartholomew, & R. E. Paull (Eds.), *The pineapple: botany, production and uses* (pp. 295–323). London, UK: CABI Publishing. https://doi.org/10.1079/9781786393302.0295

Peckham, G. D., Kaneshiro, W. S., Luu, V., Berestecky, J. M., & Alvarez, A. M. (2010). Specificity of monoclonal antibodies to strains of *Dickeya* sp. that cause bacterial heart rot of pineapple. *Hybridoma*, *29*(5), 383–389. https://doi.org/10.1089/hyb.2010.0034

Pérez-Rodriguez, M. M., Pontin, M., Lipinski, V., Bottini, R., Piccoli, P., & Cohen, A. C. (2020). *Pseudomonas fluorescens* and *Azospirillum brasilense* Increase yield and fruit quality of tomato under field conditions. *Journal of Soil Science and Plant Nutrition*, *20*(4), 1614–1624. https://doi.org/10.1007/s42729-020-00233-x

Pires de Matos, A. (2019). Main pests affecting pineapple plantations and their impact on crop development. *Acta Horticulturae*, *1239*, 137–145. https://doi.org/10.17660/ActaHortic.2019.1239.17

Rohrbach, K. G., & Johnson, M. W. (2003). Pests, diseases and weeds. In D. P. Bartholomew, R. E. Paull, & K. G. Rohr-

bach (Eds.), *The pineapple: botany, production and uses* (pp. 203–251). London, UK: CABI Publishing. https://doi.org/10.1079/9780851995038.0203

Saradhuldhat, P., & Paull, R. E. (2007). Pineapple organic acid metabolism and accumulation during fruit development. *Scientia Horticulturae*, *112*(3), 297–303. https://doi.org/10.1016/j.scienta.2006.12.031

Shamsudin, R., Zulkifli, N. A., & Kamarul Zaman, A. A. (2020). Quality attributes of fresh pineapple-mango juice blend during storage. *International Food Research Journal*, *27*(1), 141–149.

Sipes, B., & Pires de Matos, A. (2018). Pests, diseases and weeds. In G. M. Sanewski, D. P. Bartholomew, & R. E. Paull (Eds.), *The pineapple: botany, production and uses* (pp. 269–294). London, UK: CABI Publishing. https://doi.org/10.1079/9781786393302.0269

Siti Roha, A. M., Zainal, S., Noriham, A., & Nadzirah, K. Z. (2013). Determination of sugar content in pineapple waste variety N36. *International Food Research Journal*, *20*(4), 1941–1943.

Soesanto, L., Mugiastuti, E., & Rahayuniati, R. F. (2011). Biochemical characteristic of *Pseudomonas fluorescens* P60. *Journal of Biotechnology and Biodiversity*, *2*, 19–26.

Soesanto, Loekas, Mugiastuti, E., & Khoeruriza. (2019). Granular formulation test of *Pseudomonas fluorescens* P60 for controling bacterial wilt (*Ralstonia solanacearum*) of tomato in planta. *Agrivita*, *41*(3), 513–523. https://doi.org/10.17503/agrivita.v41i3.2318

Soesanto, Loekas, Mugiastuti, E., Rahayuniati, R. F., Manan, A., Dewi, R. S., Java, C., & Java, C. (2018). Compatibility test of four *Trichoderma* spp. isolates on several synthetic pesticides. *AGRIVITA, Journal of Agricultural Science*, *40*(3), 481–489. https://doi.org/10.17503/agrivita.v40i3.1126

Soesanto, Loekas, Solikhah, A. N., Mugiastuti, E., & Suharti, W. S. (2020). Application of *Trichoderma harzianum* T10 liquid formula based on soybean flour against cucumber seedlings damping-off (*Pythium* sp.). *Akta Agrosia*, *23*(1), 11–18.

Sood, M., Kapoor, D., Kumar, V., & Sheteiwy, M. S. (2020). *Trichoderma* :The "Secrets" of a Multitalented. *Plants*, *9*, 762. https://doi.org/10.3390/plants9060762

Soteriou, G. A., Kyriacou, M. C., Siomos, A. S., & Gerasopoulos, D. (2014). Evolution of watermelon fruit physicochemical and phytochemical composition during ripening as affected by grafting. *Food Chemistry*, *165*, 282–289. https://doi.org/10.1016/j.foodchem.2014.04.120

Sueno, W. S. K., Marrero, G., de Silva, A. S., Sether, D. M., & Alvarez, A. M. (2014). Diversity of dickeya strains collected from pineapple plants and irrigation water in Hawaii. *Plant Disease*, *98*(6), 817–824. https://doi.org/10.1094/PDIS-03-13-0219-RE

Wang, Y., Fu, X. Z., Liu, J. H., & Hong, N. (2011). Differential structure and physiological response to canker challenge between 'Meiwa' kumquat and 'Newhall' navel orange with contrasting resistance. *Scientia Horticulturae*, *128*(2), 115–123. https://doi.org/10.1016/j.scienta.2011.01.010

Yamada, K., Saijo, Y., Nakagami, H., & Takano, Y. (2016). Regulation of sugar transporter activity for antibacterial defense in *Arabidopsis*. *Science*, *354*(6318), 1427–1430. https://doi.org/10.1126/science.aah5692

doi:10.14720/aas.2022.118.3.2454 Original research article / izvirni znanstveni članek

Enhance the phytoremediation efficiency of *Echinochloa colona* (L.) Link for Pb-contaminated soil by phosphorus solubilizing bacteria

Quang Trung DO [1, 2]

Received December 05, 2021; accepted August 22, 2022.
Delo je prispelo 5. decembra 2021, sprejeto 22. avgusta 2022

Enhance the phytoremediation efficiency of *Echinochloa colona* (L.) Link for Pb-contaminated soil by phosphorus solubilizing bacteria

Abstract: A promising solution for phytoremediation of metal-contaminated soils is to use plants in combination with phosphate-solubilizing bacteria (PSB). In this study, we subjected to isolate PSB from paddy soil and investigate their ability in improving the phytoremediation of lead (Pb^{2+}) by a weed plant (*Echinochloa colona* (L.) Link) as well as in promoting the growth of *E. colona* under Pb stress condition. Total 06 PSB (labeled from TB01 to TB06) were isolated and the TB04 showed the strongest phosphate-solubilizing activity with the highest values of phosphorus solubilization index (PSI = 7.13) obtained from $Ca_3(PO_4)_2$. Especially, the phosphorus solubilizing ability of the TB04 strain was not affected by the high Pb^{2+}concentration. The TB04 strain was identified as *Pseudomonas putida* Trevisan, 1889 (accession number FJ976601.1). Furthermore, *E. colona* inoculated with TB04 strain significantly increased the phytoremediation efficiency of Pb from Pb-contaminated soil and the growth was enhanced clearly. These results suggest that the TB04 strain could potentially use as an inoculant in combination with *E. colona* to construct novel constructed wetlands for phytoremediation of metal-contaminated soil.

Key words: lead immobilization; *Pseudomonas putida*; soil fertility; phytoremediation; metal-contaminated soil

Povečanje fitoremediacijske učinkovitosti vrste *Echinochloa colona* (L.) Link z bakterijami, ki sproščajo fosfor v tleh, onesnaženih s svincem

Izvleček: Obetajoča rešitev za fitoremediacijo s kovinami onesnaženih tal je uporaba rastlin v kombinaciji z bakterijami, ki sproščajo fosfor (PSB). V raziskavi so bili preučevani izolati teh bakterij iz riževih polj in njihova sposobnost izboljšanja fitoremediacije svinca (Pb^{2+}) s plevelno vrsto kostrebe (*Echinochloa colona* (L.) Link) kot tudi izboljšanje rasti te rastline v razmerah kovinskega stresa zaradi onesnaženja s svincem. Celokupno je bilo izoliranih 6 izolatov PSB (označenih kot TB01 do TB06), pri čemer je imel izolat TB04 največjo sposobnost sproščanja fosforja (z indeksom PSI = 7,13) iz kalcijevega fosfata ($Ca_3(PO_4)_2$). Na sposobnost sproščanja fosforja pri sevu TB04 niso vplivale velike koncentracije Pb^{2+}. Sev TB04 je bil identificiran kot vrsta bakterije *Pseudomonas putida* Trevisan, 1889 (številka akcesije FJ976601.1). Inokulacija kostrebe s sevom TB04 je značilno povečala njeno fitoremediacijsko učinkovitost za svinec v s svincem onesnaženih tleh, pri čemer je bila njena rast značilno pvečana. Rezultati nakazujejo, da bi sev TB04 lahko potencialno uporabili kot inokulant kostrebe kot nov način fitoremediacije s kovinami onesnaženih močvirnih tal.

Ključne besede: imobilizacija svinca; *Pseudomonas putida*; rodovitnost tal; fitoremediacija; s kovinami onesnažena tla

1 Central Institute for Natural Resources and Environmental Studies, Vietnam National University Hanoi, Ha Noi, Vietnam
2 Corresponding author, e-mail: trungcnsinh@gmail.com

1 INTRODUCTION

It is a fact that industrial development, agricultural practices, and human activities caused a quick increase in areas of soil contaminated with heavy metals (Xiao et al., 2021). Importantly, pollution with lead (Pb) was the most concern because it has no function in biology or physiology for the living cells, but was determined as a toxic chemical for living cells (Yahaghi et al., 2018; Aransiola et al., 2019). Especially, the metal chemicals were not biodegraded leading to their accumulation in the soil, which can increase the risk of these metals entering the food chain by uptake activity of crops (Noble et al., 2018; Xiao et al., 2021). Hence, the removal of metal pollutants from the soil is very important and necessary. Although there are several methods have been applied to remediate the metal pollution in soil, phytoremediation of heavy metals is a promising one that uses plants to uptake the metal pollutants from soil accumulating them in the above-ground part of the plant for disposal. Hence, phytoremediation is environmentally friendly, low-cost, and easy to set up (Noble et al., 2018; Xiao et al., 2021).

In agricultural practices, the application of PGPR, particularly phosphate solubilizing bacteria (PSB), to improve crop yield is becoming more and more frequent. Besides assisting plants in nutrient uptakes and disease protection, PSB also presented its ability to enhance plant growth in harsh conditions caused by contaminants in the soil such as metal pollutants (Noble et al., 2018; Adhikari et al., 2020). Therefore, the inoculation of PSB in the phytoremediation of metal pollutants from the soil is very potential. It was reported that a weed named *Echinochloa colona* (L.) Link that has a wide distribution in an agroecosystem and has played role in the uptake of heavy metals from metal-contaminated soil (Subhashini and Swamy, 2016; Noble et al., 2018). It demonstrated their efficiency in the phytoremediation of lead, nickel, zinc, cadmium, and chromium from contaminated soils (Subhashini and Swamy, 2016). In addition, Noble et al. (2018) reported that with the assistance of plantain peels the phytoremediation of Pd and Cd in soil by *E. colona* was significantly enhanced. Therefore, the application of *E. colona* for phytoremediation of metal-contaminated soils is very promising. However, it is a fact that phytoremediation presents some limitations such as being time-consuming, and the removal efficiency of metals depends strongly on the plants vegetated in that system.

Interestingly, the combination of plants and plant growth-promoting rhizobacteria (PGPR) could improve the phytoremediation efficiency (Noble et al., 2018; Xiao et al., 2021). However, the study using PSB to enhance the removal efficiency of metal pollutants from the soil by *E. colona* are scarce. Hence, this study's aims were (1) to isolate PSB from Thai Binh paddy soil and (2) to investigate their ability in improving the phytoremediation of lead (Pb^{2+}) by a weed plant (*Echinochloa colona*) as well as in promoting the growth of *E. colona* under Pb stress condition.

2 MATERIAL AND METHODS

2.1 ISOLATION OF PHOSPHORUS-SOLUBILIZING BACTERIA

Samples of soil were collected from different locations at agricultural fields in Thai Binh Province, Vietnam for isolation of PSB. About 2 g of each soil sample adhered to the rice roots was collected and carefully transferred into sterile tubes containing sterile deionized (DI) water (about 2 ml). Then, each test tube was vortexed thoroughly and let set for 5 minutes at room temperature. The 10-fold dilutions in the same buffer were applied. After that, it took 100 µl of diluted samples to plate onto Pikovskaya (PVK) media agar plates (Pikovskaya, 1948). The bacterial colonies with clear halos in the PVK agar plate indicated solubilizing activity of the phosphate. These were sub-cultured on PVK (Biobasic, Canada).

Similar methods were applied to screen for microorganisms that could solubilize aluminum phosphate ($AlPO_4$) and iron phosphate ($FePO_4$). In this experiment, the medium was modified from the PVK medium, in which the $Ca_3(PO_4)_2$ was altered by either 5 g l^{-1} of $AlPO_4$ or 5 g l^{-1} of $FePO_4$.

The PVK medium used in study include (g l^{-1}): glucose, 10; $(NH_4)_2SO_4$, 0.5; $MgSO_4.7H_2O$, 0.1; yeast extract, 0.5; KCl, 0.2; NaCl, 0.2; $FeSO_4.7H_2O$, 0.002; $MnSO_4.7H_2O$, 0.002; $Ca_3(PO_4)_2$, 5; pH 6.5 (for agar plate, 15 g of agar was added). The plate incubation was carried out at 30 °C for 7 days. All media and glassware used were sterilized in an autoclave before use.

2.2 MOLECULAR IDENTIFICATION OF TB04 STRAIN

The total DNA of strain TB04 was extracted using a Rapid Bacteria Genomic DNA Isolation Kit (Biobasic, Canada) as per the kit instructions. The PCR amplification of 16S rDNA was done with the extracted DNA by using the universal primers 27F and 1492R. The sequence of 16S rDNA sequences obtained was blasted on NCBI to identify the species. The sequences with high similarity were used for multiple cluster alignment and phylogenetic analysis on MEGA software (v.7.2).

2.3 DETERMINE PHOSPHATE SOLUBILIZING EFFICIENCY OF THE ISOLATES

Single colonies were cultured separately in liquid LB media at 30 °C for 24 h on the shaker (150 rpm). Then, bacterial cells of each strain were collected by applying a described procedure. The bacterial suspension of isolates (10^6 CFU ml^{-1}) was determined for their ability to solubilize different insoluble phosphorus compounds ($Ca_3(PO_4)_2$, sodium phytate, $FePO_4$, or $AlPO_4$) on either solid or liquid PVK medium. The condition for plate incubation was at 30 °C for seven days. The medium with no bacteria was used as the control.

After seven days of incubation, the determination of soluble P concentration in bacterial culture was done using the molybdenum blue method (Waterlot, 2018), and the phosphate solubilization index (PSI) of bacteria grown on plates was measured as the method described by Liu et al. (2015). The pH measurement of the bacterial culture was carried out by using the pH meter.

In addition, the isolated PSB were also characterized their P solubilizing efficacy in soil conditions by a method adapted from Wan et al. (2020). Different treatments have been done in triplicates: (T1) 100 g sterilized soil + 10 ml bacterial solution; (T2) 95 g sterilized soil + 10 ml bacterial solution + 5 g $Ca_3(PO_4)_2$, (T3) 95 g sterilized soil + 10 ml bacterial cultures + 5 g $Ca_3(PO_4)_2$ + 10 ml nutrient solution (PVK liquid medium removed $Ca_3(PO_4)_2$). Soil moisture in the experiments was adjusted to 80 % by sterile water and kept for 30 days at 25 °C. After that, the amount of available P (AP) in treated soils was determined by the molybdenum blue method (Waterlot, 2018).

2.4 INDOLE-3ACETIC ACID (IAA) PRODUCTION OF PSB

The isolates were also screened for IAA production by using LB medium supplemented 0.1 % L-tryptophan. The colorimetric method using ferric chloride-perchloric acid reagent ($FeCl_3$–$HClO_4$) as described by Luu et al. (2021) was applied to measure the amount of IAA produced.

2.5 PHOSPHORUS SOLUBILIZATION ABILITY OF TB04 ISOLATE UNDER LEAD STRESS

The isolates were then investigated for the solubilization of $Ca_3(PO_4)_2$ under Pb^{2+} stress. The Pb-contaminated soil was artificially produced by mixing the sterilized soil with $Pb(NO_3)_2$ (0, 200, 400, 800, 1600, or 2400 mg Pb/ kg soil) and $Ca_3(PO_4)_2$ (as the P source). Then, 100 ml of the culture of isolated PSB were added to the prepared soil and were kept for four months at room temperature. For control experiments, soil with only $Ca_3(PO_4)_2$. The moisture in all experiments was kept at 80 % by watering with sterile water every five days. After four months of incubation, the soil sample was collected, air-dried, ground, sieved through a 0.2-mm sieve, and subsequently extracted at room temperature for 30 min by a mixed solution of 0.025M HCl and 0.03 M NH_4F (1:10 soil:water ratios). The amount of the available P in the treated soil was determined by the molybdenum blue method (Waterlot, 2018).

2.6 EFFECT OF TB04 STRAIN INOCULATION TO THE DEVELOPMENT AND PB UPTAKE OF WEED PLANT (*Echinochloa colona*)

The pot experiments were prepared as the method described in Luu et al. (2021). Briefly, seeds of *E. colona* were sterilized on their surface by using ethanol 70 % for 30 s and sodium hypochlorite solution 2 % for 5 minutes. Then these seeds were washed three times with sterile water and dried on autoclaved filter papers. The TB04 strain with the highest efficiency of Pb uptake and IAA production was overnight grown, centrifugated, and washed with sterile water before being resuspended with sterile water to make a bacterial solution with OD = 1. The sterilized seeds were covered with selected PSB by soaking in the bacterial solution for 30 minutes before sowing. For the control, sterile water was used instead of the bacterial solution.

The treatment was done in triplicates by sowing ten bacterized seeds of *E. colona* per plastic pot filled with about 1 kg of lead-contaminated soil (600 mg kg^{-1} of $Pb(NO_3)_2$). After plant establishment, one plant per pot was done. The pots were kept in the nursery garden and soil moisture was held at 60 % of water holding capacity during the experiment by adding a specific amount of sterile water as the method described by Steadman et al. (2004). After one month, 100 ml of the bacterial culture (OD = 1) were added to the treated pot as biofertilizer while sterile water was used for the control.

The experiments were carried out in 3 months. The measured parameters for plant growth were plant height, shoot and root dry mass. The plant height was measured from the aboveground to the tip of the upper-most leaf of the plant. The root was cut from the plant and removed Pb ions bounding to its surface by washing with 1 mM $Ca(NO_3)_2.4H_2O$ and sterile water. The dry mass of root and shoot were determined after dried in an oven at 70 ˚C for 72 h. The Pb in the oven-dried shoot and root

was extracted by using a solution of HNO_3-HCl (70 %) and H_2O_2 (30 %) (Jones et al., 1990) and were measured by FAAS. All measurements were done in triplicates.

2.7 DATA ANALYSIS

All experiments were repeated three times the results were presented as mean values with ± SD. Tukey's honestly significant difference (HSD) method in SPSS (version 17) was applied to compare the means in all experiments.

3 RESULTS AND DISCUSSION

3.1 ISOLATION AND CHARACTERIZATION OF PHOSPHATE-SOLUBILIZING BACTERIA

Bacteria isolates that produced a transparent zone around colonies in the Pikovskaya (PVK) medium were determined as phosphate-solubilizing bacteria and were selected. There were six single colonies were observed and further transferred into new PVK plates for purification (Table 1).

All isolates showed different efficiency in solubilizing phosphorus after 7 days of incubation at 30 °C, which was illustrated by different values of PSI ranging from 1.53 to 7.13 (Table 1). A further characteristic of isolates indicated their ability in IAA production, in which the highest amount of IAA (7.86 mg l^{-1}) was observed for the TB04 strain.

Furthermore, the results also presented the different capabilities in solubilizing phosphorus compounds of all isolates from different phosphate sources. All isolated strains could solubilize multiple insoluble phosphorus compounds ($Ca_3(PO_4)_2$, $AlPO_4$, and $FePO_4$) but only TB03 and TB04 presented the phytate solubilization (Table 2). For inorganic P, the results indicated that $Ca_3(PO_4)_2$ was the most favorable compound for all strains demonstrated by the highest amount of soluble P (173.11-572.13 mg l^{-1}) released from this compound; and the TB04 also presented the highest efficiency. In addition, approximately 10-fold less of solubilization efficiency was observed for the remaining complexed phosphate sources including $AlPO_4$ (21.17 to 72.13 mg l^{-1}) and $FePO_4$ (10.51 to 29.73 mg l^{-1}) by most of the isolates (Table 2). Furthermore, only two strains, TB01 and TB04, showed the ability in solubilizing organic phytate supplemented with a modified PVK broth medium (1.53 and 3.61 mg l^{-1}, respectively). These results indicated that TB04 could solubilize multiple P sources and might be used to reverse insoluble phosphate to soluble form in agriculture.

Table 1: Characteristics of isolated phosphate-solubilizing bacteria (PSB)

PSB isolates	Phosphate solubilization index (Agar)	IAA production (mg l^{-1})
TB01	4.13 ± 0.11^{b}	1.87 ± 1.21^{c}
TB02	4.12 ± 0.12^{b}	2.02 ± 1.02^{c}
TB03	3.37 ± 0.31^{bc}	3.25 ± 1.01^{bc}
TB04	7.13 ± 0.15^{a}	7.86 ± 1.01^{a}
TB05	1.53 ± 0.23^{c}	4.52 ± 1.12^{b}
TB06	1.67 ± 0.12^{c}	1.91 ± 1.12^{c}

Data are means ± SE of three independent biological replicates. Data with the same letters in the same column are not significantly different from each other according to the honestly significant difference (HSD) test ($p < 0.05$)

It was a fact that the solubilization of $AlPO_4$ and $FePO_4$ was lower than the one of $(Ca_3(PO_4)_2$. It can be explained by two possible reasons. Firstly, it was reported that the interaction of aluminum (Al^{3+}) and iron (Fe^{3+}) with phosphate ion (PO_4^{3-}) is a reversible reaction. Hence, it could be that the acids produced by PSB during the solubilization might force the reversible reaction of aluminum (Al^{3+}) and iron (Fe^{3+}) with phosphate ion (PO_4^{3-}) to form insoluble complexes (Sánchez-Cruz, 2020) leading to an inefficient in solubilizing $FePO_4$ and $AlPO_4$. Secondly, it could be differences in affinity among cations and anions in the solution, in which the anions generated by PSB such as carboxylic and hydroxylic groups preferred calcium (Ca^{2+}) to aluminum (Al^{3+}) and iron (Fe^{3+}) and subsequently enhanced the phosphorus solubilization (Sánchez-Cruz, 2020). Moreover, the results indicated the pH reduction and production of a phytase of strains TB01 and TB04 played significant roles in solubilizing inorganic phosphate. These were demonstrated by some previous research (Kumar and Rai., 2015; Wan et al., 2020). All of these suggest the organic acids and/or phosphate solubilizing enzymes produced by PSBs play important roles in mineralizing phosphorus compounds (Walpola et al., 2013).

Moreover, the correlation analysis showed a low correlation between the values of PSI and the amount of soluble P released (r = 0.442), and between pH of supernatant and the amount of soluble P released (r = 0.501). These could be related to P solubilizing mechanisms, in which the PSB produced external metabolites such as hydrolytic enzymes, and/or organic acids that enhanced the solubilization of mineral phosphates and could reduce the pH of bacteria culture.

Some reports demonstrated a positive correlation between the pH of culture and the solubilized amount of

Table 2: Determination of phosphate solubilization ability in PVK broth medium with $Ca_3(PO_4)_2$, $AlPO_4$, $FePO_4$, and sodium phytate by isolated PSB

	PVK with $Ca_3(PO_4)_2$		PVK with $AlPO_4$		PVK with $FePO_4$		PVK with sodium phytate	
PSB isolates	Soluble P (mg l^{-1})	pH	Soluble P (mg l^{-1})	pH	Soluble P (mg l^{-1})	pH	Soluble P (mg l^{-1})	pH
TB01	251.15 ± 10.71^{b}	4.95 ± 0.21^{b}	54.31 ± 4.71^{b}	3.55 ± 0.12^{c}	10.51 ± 1.53^{d}	3.47 ± 0.23^{d}	ND	4.35 ± 0.21^{b}
TB02	176.15 ± 7.12^{c}	3.93 ± 0.11^{c}	72.13 ± 2.35^{a}	3.30 ± 0.17^{bc}	13.25 ± 1.31^{c}	3.64 ± 0.17^{c}	ND	3.71 ± 0.18^{c}
TB03	248.12 ± 12.72^{b}	3.78 ± 0.14^{c}	44.16 ± 2.31^{c}	3.25 ± 0.31^{bc}	29.73 ± 2.42^{a}	3.82 ± 0.16^{c}	1.53 ± 0.19^{b}	3.92 ± 0.13^{bc}
TB04	572.13 ± 12.41^{a}	4.22 ± 0.15^{c}	51.34 ± 3.17^{b}	3.27 ± 0.12^{bc}	23.15 ± 1.27^{ab}	3.53 ± 0.12^{cd}	3.61 ± 0.71^{a}	3.53 ± 0.51^{b}
TB05	182.13 ± 11.10^{c}	5.21 ± 0.13^{b}	21.17 ± 2.73^{e}	4.31 ± 0.15^{b}	20.53 ± 1.17^{b}	4.05 ± 0.13^{b}	ND	3.37 ± 0.33^{d}
TB06	173.11 ± 9.13^{c}	4.57 ± 0.21^{bc}	33.17 ± 2.23^{d}	3.78 ± 0.17^{bc}	19.56 ± 1.15^{b}	3.77 ± 0.32^{c}	ND	4.17 ± 0.27^{b}
Control media	ND	6.51 ± 0.11^{a}	ND	6.52 ± 0.13^{a}	ND	6.47 ± 0.15^{a}	ND	6.53 ± 0.31^{a}

Data are means ± SE of three independent biological replicates. Value with the same letter in the same row is not significantly different from each other according to the honestly significant difference (HSD) test ($p < 0.05$). ND: not detected

phosphorus complexes ($Ca_3(PO_4)_2$) (Marra et al., 2019). However, the results showed an uncorrelation between the soluble P release and pH reduction. This might be chelation between metal cations (Ca^{2+}, Al^{3+}, Fe^{3+}) and anion groups of produced organic acids (Stevenson, 2005) leading to pH decrease and subsequently the increase of soluble P. Therefore, it could be said that the solubilization of phosphorus compounds is simultaneously affected by pH decrease and acid production in the solution (Fankem et al., 2006).

3.2 MOLECULAR IDENTIFICATION OF STRAIN TBO4

The molecular identification of TB04 indicated that this strain was *Pseudomonas putida* (accession number FJ976601.1) (Figure 1). The sequence of 16S rDNA of TB04 was deposited in GenBank with an accession number OP141766. This strain showed a significant efficiency of $Ca_3(PO_4)_2$ solubilization compared to reported *Pseudomonas* sp. (such as *Pseudomonas fluorescens* (Flügge 1886) Migula, 1895 (184 mg l^{-1}) (Katiyar and Goel, 2003), *Pseudomonas putida* (247 mg l^{-1}) (Pandey et al., 2006). These differences can be explained due to the difference in isolated strains that were grown and developed under specific conditions.

3.3 EFFECT OF PSB AND TRICALCIUM PHOSPHATE IN UNCULTIVATED SOIL

Next steps, we investigated the phosphate solubilizing ability of the isolates in $Ca_3(PO_4)_2$ -rich soil conditions. As we expected, all isolated PSB could solubilize the $Ca_3(PO_4)_2$ incubated in soil (Figure 2).

After 30 days of incubation, the AP content in soil supplemented with TB04 was significantly higher in all experiments than the one in the control. Notably, the soil added with TB04 showed the highest amount of AP in the same soil treatments. Particularly, the AP amount in TB04-incubated soils, in T1 (soil + PSB), T2 (Soil + PSB + $Ca_3(PO_4)_2$), and T3 (Soil + PSB + $Ca_3(PO_4)_2$ + Nutrient) treatments were 0.55, 0.87, and 1.72 mg g^{-1}, respectively. Especially, the significantly higher values of AP in T3 treatment compared to those in T1 and T2 treatments ($p < 0.05$) were observed. This might be because of the results of the addition of sufficient nutrients for bacterial growth (Figure 2). As can be seen from Figure 2, the positive correlation between the AP amount in PSB-inoculated soil and added amount of $Ca_3(PO_4)_2$ in the presence of TB04 strain. These results were consistent with previous reports, which demonstrated the potential application of PSB in improving soil quality, particularly by increasing the amount of available P that directly influences the plant development and plant uptake and subsequently the yield (Himani and Reddy, 2011; Teng et al., 2019; Wan et al., 2020). These improvements might be due to the inoculated PSB in treatment solubilized the $Ca_3(PO_4)_2$ fertilizer to release soluble P that was partially used for the development of PSB, subsequently enhancing the phosphorus's efficiency. These explanations were demonstrated by studies that reported a positive correlation between the change in the amount of soil organic carbon and the change in bacterial development in soil (Nakhro and Dkhar, 2010; Wan et al., 2020). In addition, another contributor to the improvement of soluble P amount in treated soil might be the difference in hydrolytic enzymes (such as phosphatase, and phytase)

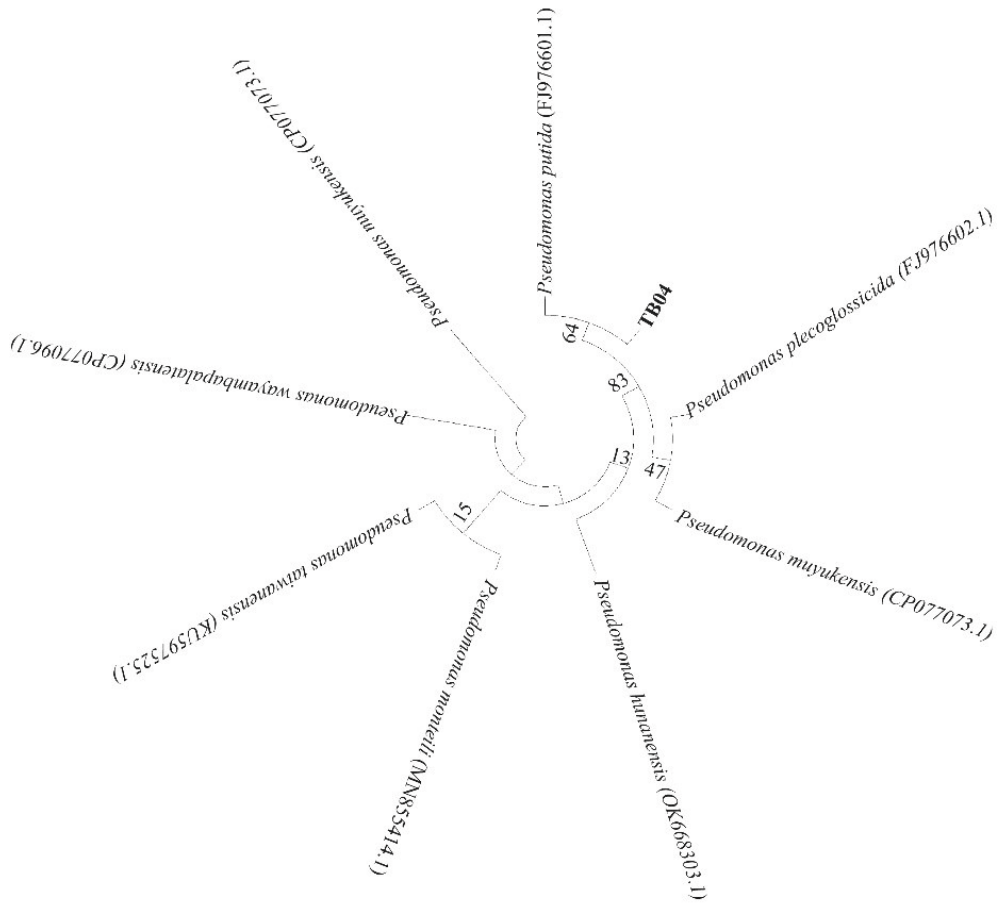


Figure 1: A neighbor-joining tree shows the phylogenetic relationships among 16S rDNA sequences of TB04 and their closely related sequences from NCBI

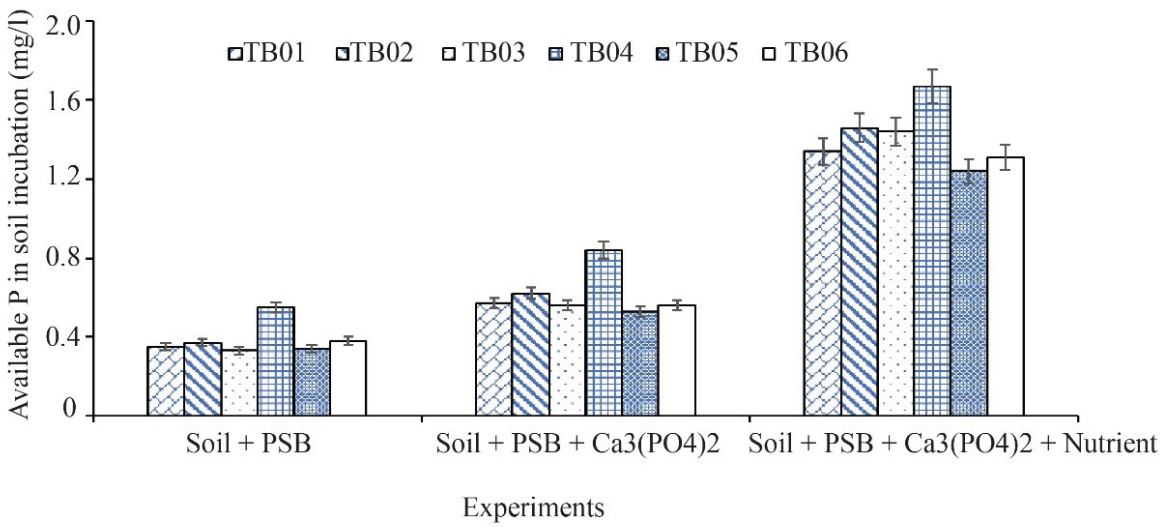


Figure 2: Evaluation of available P in soil incubation (mg l^{-1}). The presenting results are the mean value of three replicates. PSB: phosphorus solubilizing bacteria; TB01 to TB06 are phosphorus solubilizing bacteria

presented in soil (Teng et al., 2019). Presumably, the results indicated that TB04 showed a promising application in solubilizing insoluble phosphorus compounds in soil that increase soil health.

3.4 EVALUATION OF THE PHOSPHORUS SOLUBILIZATION ABILITY OF TB04 ISOLATES UNDER LEAD STRESS

In fact, the agricultural soil was contaminated with metals caused by the overuse of chemical fertilizer. Hence, we investigated the ability of strain TB04 to solubilize phosphorus compounds in the presence of lead with different concentrations. As shown in Figure 3, the amount of available P in treated soil was higher than that of initiated soil (about 0.19 mg g^{-1}). These results indicated that the TB04 strain could solubilize phosphorus compounds in soil and this ability was not affected by an increasing amount of Pb concentration.

3.5 INOCULATION OF TB04 STRAIN IMPROVES THE DEVELOPMENT AND PB UPTAKE OF WEED PLANT (*ECHINOCHLOA COLONA*)

The effect of TB04 inoculation on the growth properties of weed (*Echinochloa colona*) under greenhouse conditions was studied. The obtained results of greenhouse experiments were shown in Table 3. As can be seen, the TB04 strain significantly improved the plant growth parameters of *E. colona* compared to the control experiment, which used sterile water instead. Particularly, the length of TB04 inoculated plants was increased approximately 1.5 times compared to non-bacterized plants. Similarly, the increase in shoot and root dry mass observed for the plants bacterized with TB04 with 1.5 times higher than the control.

These data were not in agreement with some previous studies, which reported that plant development was inhibited when grown on heavy metal-contaminated soil (Tangahu et al., 2011). This might be due to the TB04 strain produced IAA (a plant up-regulator) and solubilized phosphorus compounds increasing the amount of available P in soil, and subsequently enhancing the plant development. Lin et al. (2018) demonstrated the growth of *Wedelia trilobata* (L.) H.Rob. & Cuatrec. cultivated in Cu^{2+}-contaminated soil was significantly upregulated when inoculated with *Paenibacillus polymyxa* (Prazmowski, 1880) Ash et al., 1994, a phosphate-solubilizing bacterium. Another example is the study of Yahaghi et al. (2018) who showed the inoculation of a bacterial mixture (*Brevibacterium frigoritolerans* Delaporte and Sasson, 1967 YSP40 and *Bacillus paralicheniformis* sp. nov. YSP151) improved the development of *Brassica juncea* (L.) Czern that grown in a soil contaminated with heavy metal by producing IAA, siderophores, and solubilizing inorganic phosphate.

The data in Table 3 also indicated that the Pb concentration in the shoot of bacterized *E. colona* plant was dramatically increased in the comparison with one of non-bacterized plants. The result also showed that the inoculation of TB04 was not clearly influenced by the amount of Pb in the root. In addition, the result also presented that the TB04-treated plants contained more amount of Pb uptake in the shoot than the control did.

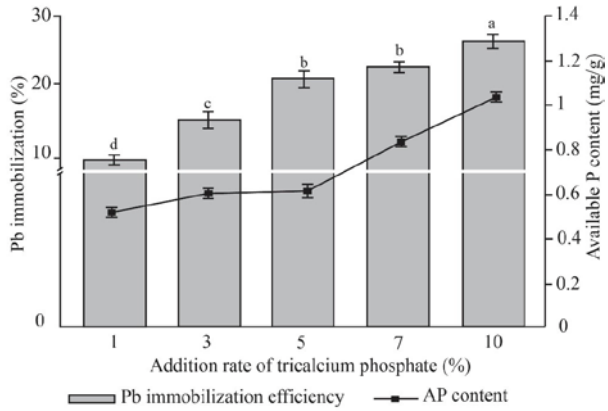


Table 3: Enhanced effect of TB04 strain on the development and Pb uptake of *Echinochloa colona*

Table 3: Enhanced effect of TB04 strain on the development and Pb uptake of Echinochloa colona

Phosphorus solubilizing bacteria	Plant length (cm)	Shoot dry mass (g/plant)	Root dry mass (g/plant)	Pb concentration in shoot (mg kg^{-1})	Pb concentration in root (mg kg^{-1})	Pb uptake by shoot (μg/pot)
SDW[a]	52.37 ± 1.79 a[b]	18.32 ± 2.73 a	14.05 ± 2.27 a	40.27 ± 3.02 a	94.17 ± 2.79 a	71.28 ± 11.53 a
TB04	73.51 ± 3.73 b	29.21 ± 3.72 b	22.07 ± 1.17 b	73.17 ± 5.27 b	84.92 ± 2.76 a	223.72 ± 18.74 b

[a] TB04: selected phosphorus solubilizing bacteria; SDW: Sterile distill water

[b] Presenting values the mean ± standard deviation. Values with a different letter in the same column indicated a significant difference according to HSD ($p < 0.05$)

The data showed that the bacterial inoculation increased the Pb concentration in the shoot of bacterized *E. colona* plant and was not clearly influenced by the amount of Pb in the root. The increase in Pb^{2+} absorption could be due to the inoculated PSB produced metabolites (such as organic acids) that enhanced the bioavailability of Pb^{2+} in the root rhizosphere, and subsequently improved the Pb^{2+} absorption of root (Aransiola et al., 2019; Xiao et al., 2021). In addition, the result also indicated a higher amount of Pb uptake in the shoot than the control did. This might be the result of the improvement in shoot biomass and the Pb^{2+} translocation caused by the TB04 inoculation. Yahaghi et al. (2018) reported that the Pb^{2+} uptake in the shoot of *B. juncea* inoculated with *Brevibacterium frigoritolerans* YSP40 and *Bacillus paralicheniformis* YSP151 strains was increased 3 and 4 times, respectively.

4 CONCLUSIONS

This study demonstrated the potential application of PSB isolated from paddy soil collected from Thai Binh province for enhancing the removal efficiency of Pb^{2+} pollutants from metal-contaminated soil by *E. colona*. The inoculation of PSB isolated into the Pb-contaminated soil not only promoted the plant growth of *E. colona* but also enhanced the Pb^{2+} uptake by the root of *E. colona*. These data suggest a potential application of isolated PSB combined with a phytoremediator for improving the phytoremediation of metal pollutants from metal-polluted soil.

5 REFERENCES

Aransiola, S.A., Ijah, U.J.J., Abioye, O.P. and Bala, J.D. (2019). Microbial-aided phytoremediation of heavy metals contaminated soil: a review. *European Journal of Biological Research*, *9*(2), 104-125.

Adhikari, A., Lee, K.E., Khan, M.A., Kang, S.M., Adhikari, B., Imran, M., Jan, R., Kim, K.M. and Lee, I.J. (2020). Effect of silicate and phosphate solubilizing rhizobacterium *Enterobacter ludwigii* GAK2 on *Oryza sativa* L. under cadmium stress. *Journal of Microbiology and Biotechnollogy, 30*(1), 118-126. https://doi.org/10.4014/jmb.1906.06010

Fankem, H., Nwaga, D., Deubel, A., Dieng, L., Merbach, W. and Etoa, F.X. (2006). Occurrence and functioning of phosphate solubilizing microorganisms from oil palm tree (*Elaeis guineensis*) rhizosphere in Cameroon. *African Journal of Biotechnology, 5*(24), 2450-2460.

Himani, S. and Reddy, M.S. (2011). Effect of inoculation with phosphate solubilizing fungus on growth and nutrient uptake of wheat and maize plants fertilized with rock phosphate in alkaline soils. *European Journal of Soil Biology, 47*(1), 30–34. https://doi.org/10.1016/j.ejsobi.2010.10.005

Jones, J.B. and Case, V.W. (1990). Sampling, handling, and analyzing plant tissue samples. In Westerman RL (ed.). *Soil Testing and Plant Analysis*. Soil Science Society of America, Inc., Madison, WI, pp. 389-447. https://doi.org/10.2136/sssabookser3.3ed.c15

Kumar, A. and Rai, L.C. (2015). Proteomic and biochemical basis for enhanced growth yield of *Enterobacter* sp. LCR1 on insoluble phosphate medium. *Microbiology Research*, *170*, 195-204. https://doi.org/10.1016/j.micres.2014.06.006

Katiyar, V. and Goel, R. (2003). Solubilization of inorganic phosphate and plant growth promotion by cold tolerant mutants of *Pseudomonas fluorescens*. *Microbiology Research*, *158*(2), 163-168. https://doi.org/10.1078/0944-5013-00188

Liu, Z., Li, Y.C., Zhang, S., Fu, Y., Fan, X., Patel, J. S. and Zhang, M, (2015). Characterization of phosphate-solubilizing bacteria isolated from calcareous soils. *Applied Soil Ecology*, *96*, 217–224. https://doi.org/10.1016/j.apsoil.2015.08.003

Lin, M., Jin, M., Xu, K., He, L. and Cheng, D. (2018). Phosphate-solubilizing bacteria improve the phytoremediation efficiency of *Wedelia trilobata* for Cu-contaminated soil. *International Journal of Phytoremediation*, *20*(8), 813-822. https://doi.org/10.1080/15226514.2018.1438351

Marra, L.M., de Oliveira-Longatti, S.L., Cláudio R.F.S.S., Fábio L.O. and de Souza Moreira F.M. (2019). The amount of phosphate solubilization depends on the strain, C-source, organic acids and type of phosphate. *Geomicrobiology Journal*, *36*(3), 232-242. https://doi.org/10.1080/01490451.2018.1542469

Nakhro, N. and Dkhar, M.S. (2010). Impact of organic and inorganic fertilizers on microbial population and biomass carbon in paddy field soil. *Journal of Agronomy*, *9*(3), 102-110. https://doi.org/10.3923/ja.2010.102.110

Noble, A., Banakuna, F., Tanee, G. and Osuji, J. (2018). The effect of ripe plantain peels waste on the phytoextraction of Pb and Cd by *Echinochloa colona* (L.) Link. *International Journal of Natural Resource Ecology and Management*, *3*(1), 19. https://doi.org/10.11648/j.ijnrem.20180301.13

Pikovskaya, R.I. (1948). Mobilization of phosphorus in soil in connection with the vital activity of some microbial species'. *Mikrobiologiya*, *17*, 362–370.

Pandey, A., Trivedi, P., Kumar, B. and Palni, L.M. (2006). Characterization of a phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (B0) isolated from a sub-Alpine location in the Indian Central Himalaya. *Current Microbiology*, *53*(2), 102-7. https://doi.org/10.1007/s00284-006-4590-5

Steadman, K., Ellery, A., Chapman, R., Moore, A. and Turner, N. (2004). Maturation temperature and rainfall influence seed dormancy characteristics of annual ryegrass (*Lolium rigidum*). *Australian Journal of Agricultural Research*, *55*(10), 1047-1057. https://doi.org/10.1071/AR04083

Stevenson, F.J. (2005). *Cycles of Soil: Carbon, Nitrogen, Phosphorus, Sulfur, Micronutrients*. John Wiley and Sons, Hoboken.

Subhashini, V. and Swamy, A.V.V.S. (2016). Efficiency of *Echinochloa colona* in the removal of heavy metals from contaminated soils. *International Journal of Scientific Research*, *5*(3), 689. https://doi.org/10.36106/ijsr

Sánchez-Cruz, N.D., Meza-Contreras, J.C., Froylán, M.E., Macías-Rodríguez, M.E., Salcedo-Perez, E. and González-García, Y. (2020). Phosphate solubilization and indole-like compounds production by bacteria isolated from forest soil with plant growth promoting activity on pine seedlings. *Geomicrobiology Journal*, *37*(10), 909-918. https://doi.org/10.1080/01490451.2020.1797945

Tangahu, B.V., Abdullah, S.R.S., Basri, H., Idris, M., Anuar, N. and Mukhlisin, M.A. (2011). Review on heavy metals (As, Pb, and Hg) uptake by plants through phytoremediation. *International Journal of Chemical Engineering*, *2011*, 939161. https://doi.org/10.1155/2011/939161

Teng, Z., Shao, W., Zhang, K., Huo, Y. and Li, M. (2019). Characterization of phosphate solubilizing bacteria isolated from heavy metal contaminated soils and their potential for lead immobilization. *Journal of Environmental Management*, *231*,189-197. https://doi.org/10.1016/j.jenvman.2018.10.012

Walpola, B.C. and Yoon, M.H. (2013). *In Vitro* solubilisation of inorganic phosphates by phosphate solubilizing microorganisms. *African Journal of Microbiology Research*, *7*, 3534-3541.

Wan, W., Qin, Y., Wu, H., Zuo, W., He, H., Tan, J., Wang, Y. and He, D. (2020). Isolation and characterization of phosphorus solubilizing bacteria with multiple phosphorus sources utilizing capability and their potential for lead immobilization in soil. *Frontier Microbiology*, *11*, 752. https://doi.org/10.3389/fmicb.2020.00752

Waterlot, C. (2018). Alternative approach to the standard, measurements and testing program used to establish phosphorus fractionation in soils. *Analytic Climica Acta*, *1003*, 26–33. https://doi.org/10.1016/j.aca.2017.11.059

Xiao, C., Guo, S., Wang, Q. and Chi, R. (2021). Enhanced reduction of lead bioavailability in phosphate mining wasteland soil by a phosphate-solubilizing strain of *Pseudomonas* sp., LA, coupled with ryegrass (*Lolium perenne* L.) and sonchus (*Sonchus oleraceus* L.). *Environmental Pollution*, *274*, 116572. https://doi.org/10.1016/j.envpol.2021.116572

Yahaghi, Z., Shirvani, M., Nourbakhsh, F., de la Peña, T.C., Pueyo, J.J. and Talebi, M. (2018). Isolation and characterization of Pb-solubilizing bacteria and their effects on Pb uptake by *Brassica juncea*: Implications for microbe-assisted phytoremediation. *Journal of Microbiology and Biotechnology*, *28*(7), 1156-1167. https://doi.org/10.4014/jmb.1712.12038

doi:10.14720/aas.2022.118.3.1873 Original research article / izvirni znanstveni članek

Influence of Ag nanoparticles on physiological and biochemical aspects of callus of *Thymus* species and *Zataria multiflora* Boiss.

Nima MOSAVAT [1, 2, 3], Maryam YOUSEFIFARD [4], Pooran GOLKAR [5, 6], Rabia JAVED [7]

Received September 12, 2020; accepted August 31, 2022.
Delo je prispelo 12. septembra 2020, sprejeto 31. avgusta 2022

Influence of Ag nanoparticles on physiological and biochemical aspects of callus of *Thymus* species and *Zataria multiflora* Boiss.

Abstract: *Thymus* species have found remarkable importance in food and medicine industries. The present study investigates the potential effect of Ag nanoparticle elicitors on proliferation of callus, and production of carvacrol and thymol in *Zataria multiflora* and three *Thymus* species. Firstly, callus was induced on Murashige and Skoog (MS) medium containing 2 mg l^{-1} of 2, 4-dichlorophenoxy acetic acid (2,4-D) and 1 mg l^{-1} of kinetin (Kin)). Secondly, the effects of two different concentrations of Ag nanoparticles (4 and 8 mg l^{-1}) were studied on callus growth and its secondary metabolites production. Results elucidated that after elicitation by 8 mg l^{-1} of Ag NPs, significantly the highest callus growth rate (CGR) (0.02 mm day^{-1}), callus fresh mass (CFM) (0.99 g), and carvacrol (0.68 mg l^{-1}) and thymol (11.09 mg l^{-1}) content was achieved. Comparing different *Thymus* species, notably the greatest carvacrol and thymol amount was obtained in *.kotschyanus* Boiss. & Hohen. and *T. Daenesis Čelak.* at 8 mg l^{-1} concentration of Ag NPs. Hence, it is evident that the stimulation by NPs is dose-dependent. This study has potential to be commercially applied for the enhancement of pharmaceutical compounds in different species of *Thymus*.

Key words: Ag nanoparticles; *Thymus* species; *Zataria multiflora*; callus; carvacrol thymol

Abbreviations: 2,4-D, 2, 4-dichlorophenoxyacetic acid; Kin, kinetin; PGRs, plant growth regulators; HPLC, high performance liquid chromatography; NPs, nanoparticles

Vpliv nanodelcev srebra (Ag) na fiziološke in biokemične lastnosti kalusa dveh vrst materine dušice (*Thymus* sp.) in vrste *Zataria multiflora* Boiss.

Izvleček: Vrste iz rodu materine dušice (*Thymus* sp.) imajo velik pomen v prozivodnji hrane in zdravil. V raziskavi je bil preučevan potencialni učinek nanodelcev srebra kot eliciatorja na rast kalusa, tvorbo karvakrola in timola pri vrsti *Zataria multiflora* in treh vrstah materine dušice. Najprej je bil na Murashige in Skoog (MS) gojišču, ki je vsebovalo 2 mg l^{-1} 2,4-D in 1 mg l^{-1} kinetina, vzgojen kalus. Potem je bil preučevan učinek dveh različnih koncentracij srebrovih nanodelcev (4 in 8 mg l^{-1}) na rast kalusa in in tvorbo sekundarnih metabolitov. Rezultati, pridobljeni z visokotlačno tekočinsko kromatografijo (HPLC) so pokazali, da je bila po uporabi 8 mg l^{-1} srebrovih nanodelcev kot iliciatorjev dosežena značilno največja rast kalusa (CGR) (0,02 mm dan^{-1}), največja sveža masa kalusa (CM) (0,99 g) in največja vsebnost karvakrola (0,68 mg l^{-1}) in timola (11,09 mg l^{-1}). V primerjavi različnih vrst materine dušice je bila dosežena največja vsebnost karvakrola in timola pri vrstah *T. kotschyanus* Boiss. & Hohen in *T. daenesis* Čelak pri koncentraciji srebrovih nanodelcev 8 mg l^{-1}. Očitno je, da je stimulacijski učinek nanodelcev odvisen od doze. Izsledke raziskave bi lahko komercialno uporabili za povečanje tvorbe zdravilnih spojin pri različnih vrstah materine dušice.

Ključne besede: Ag nanodelci; vrste iz rodu *Thymus*; *Zataria multiflora*; kalus; karvakrol; timol

Okrajšave: 2,4-D: 2, 4-diklorfenoksi ocetna kislina; Kin: kinetin; PGRs: rastlinski rastni regulatorji; HPLC: visokotlačna tekočinska kromatografija; NPs: nanodelci

1 Department of Agricultural Biotechnology, Payame Noor University (PNU), Tehran, Iran
2 Institute of Biotechnology, School of Agriculture, Shiraz University, Shiraz, Iran
3 Corresponding author, e-mail: nima.mosavat@gmail.com
4 Department of Engineering and Technology, Payame Noor University (PNU), Tehran, Iran
5 Department of Natural Resources, Isfahan University of Technology, Isfahan, Iran
6 Research Institute for Biotechnology and Bioengineering, Isfahan University of Technology, Isfahan, Iran
7 Department of Biotechnology, Quaid-i-Azam University, Islamabad, Pakistan

1 INTRODUCTION

Nanobiotechnology has enormous applications in diverse fields including agriculture, cosmetics, pharmaceutics, and food industry) Kim et al., 2017; Rastogi et al., 2017). Nanoparticles (NPs) are employed as elicitors of various cell signaling pathways in metabolism of plants (Kim et al., 2017; Marslin, et al., 2017). NPs have been found to play crucial role in enhancement of plant secondary metabolites (SMs) by imposing oxidative stress and increasing cell membrane permeability (Jasim et al., 2017; Marslin et al., 2017; Ahmad et al., 2020). The effects of NPs on growth rate of plants (Sharma et al., 2012), germination of seeds (Zaka et al., 2016), production of SMs (Marslin et al., 2017; Mosavat et al., 2019; Zaka et al., 2016; Golkar et al., 2021) and plant physiology (Jasim et al., 2017; Sharma et al., 2012) have been studied recently. Although there are few studies related to effect of NPs on callus culture development, physiology and secondary metabolism (Dykman & Shchyogolev, 2017; Kokina et al., 2013; Marslin et al., 2017; Sanzari et al., 2019; Zuverza-Mena et al., 2017), still this research domain needs to be explored further. Silver (Ag) NPs possess unique properties in terms of toxicity and alteration of yield, development, antioxidant activities, and SMs production of plants due to their high catalysis and reactivity (Rastogi et al., 2017; Sadak 2019). Furthermore, the influence of Ag NPs on the callus cultures of *Solanum nigrum* L. (Ewais et al., 2015), *Prunella vulgaris* L. (Fazal et al., 2019) and *Caralluma tuberculata* N.E. Brown (Ali et al., 2019) has recently been studied.

Thymus L., belonging to Lamiaceae family, has world-wide distribution (Sajed et al., 2013). However, it is dominantly present in Asia, Europe and North Africa (Zarshenas & Krenn, 2015). The essential oils and SMs enhance the commercial value of flowers and leaves of *Thymus* making it a valuable crop in cosmetics, pharmaceutics, and food and agriculture industry (Miraj & Kiani, 2016). A thyme-like plant, *Zataria multiflora* Boiss., belonging to Lamiaceae family, is wild plant found in only southern and central Pakistan, Afghanistan, and Iran (Sajed et al., 2013). Tissue culture propagation of thyme plant is well-known because it is a wide source of ingredients of pharmacology. Both *Thymus* sp. and *Z. multiflora* possess anti-cancerous, anti-inflammatory, anti-oxidant, anti-bacterial, anti-fungal, and anti-spasmodic properties (Mathela et al., 2010; Sajed et al., 2013). Naturally occurring terpenoid thymol (2-isopropyl-5-methyl phenol) and its phenol isomer, carvacrol/cymophenol are the phenolic compounds that play an important role in inducing these properties to *Thymus* sp. and *Z. multiflora* (Kianersi et al., 2021; Mathela et al., 2010). Some other properties like their use as additive in perfumes, deodorant, toothpastes, soaps, etc. and as important flavoring agent in foods are also attributed to these compounds (Sajed et al., 2013).

The defense system of plants is activated by chemical, biological or physical elicitors (Asadollahei et al., 2022; Zhao et al., 2005). The gene expression is then modulated that transcribes the formation of SMs (Ajungla et al., 2009). A more efficient method could be the use of callus cultures for extracting SMs naturally from *Thymus* sp. and *Z. multiflora* in a sustained manner (Ramakrishna & Ravishankar, 2011). Previously, there is no report concerning the carvacrol and thymol production by imposing Ag NPs in cell cultures of *Thymus* sp. and *Z. multiflora.* Hence, the current study investigates proliferation of callus and SMs production in three *Thymus* species, i.e., *T. vulgaris* L., *T. daenensis Čelak,* and *T. kotschyanus* Boiss. & Hohen as well as *Z. multiflora* Boiss. after Ag NPs exposure.

2 MATERIALS AND METHODS

The seeds of *Thymus* species, i.e., *T. vulgaris, T. daenensis, T. Kotschyanus* and *Zataria multiflora* (two accessions) (Table 1) were deposited at Botanic Herbarium of Research Institute for Biotechnology and Bioengineering, Isfahan University of Technology (IUT), Isfahan, Iran, after collection from different geographical regions and identification by using Flora Iranica (Rechinger, 1982). Their characteristics is shown in Table 1. The seeds of four different species were surface sterilized with 70 % (v/v) ethanol for 1 min, followed by the addition of 3 % (v/v) sodium hypochlorite for 20 min, and then rinsing in sterile distilled water thrice. After surface sterilization, the seeds were grown in Murashige and Skoog (MS) (1962) medium (Duchefa, Netherland). These were incubated for germination and growth of plantlets. The leaflet explants from about 1-month old plantlets were cultured in MS medium containing 2,4-D (2 mg l^{-1}) and Kin (1 mg l^{-1}) supplemented with 3 % (w/v) sucrose (Sigma-Aldrich, USA), 0.8 % (w/v) agar (Sigma-Aldrich, USA) and 0.1 mg l^{-1} myoinositol for callus induction. The pH was adjusted at 5.7. The samples were exposed to 16h/8h (light/dark) photoperiod for a period of 2 months at 23 ± 2 °C.

Silver (Ag) nano-powder was purchased from US Research Nanomaterials Inc., Houston, TX, USA having an average size of 30–50 nm and purity of 99.99 %. The nanoparticles were characterized by x-ray diffraction (XRD) and scanning electron microscopy (SEM) techniques by following the protocols of Javed et al. (2016). XRD was performed using Carlo ERBA Model EA 1108 analyzer and the instrument for getting SEM image was

Table 1: The geographical origins of *Thymus* sp. and *Z. multiflora* with their geographical traits collected from Iran

Species	Abbreviation	Origin	Genotype code	Latitude (m)	Longitude (m)	Altitude (m)
Zataria multiflora (1)	*Zm* (1)	Dehbala, Yazd, Iran	RIBB/ZM01/2016	31°59' N	54°11' E	2600
Zataria multiflora (2)	*Zm* (2)	Abadeh, Fars, Iran	RIBB/ZM02/2016	31°45' N	51°21' E	2030
Thymus vulgaris	Tv	Marvdasht, Fars, Iran	RIBB/TV01/2016	35°56' N	52°10' E	1620
Thymus daenensis	Td	Aligoodarz, Lorestan, Iran	RIBB/TD01/2016	33°24' E	49°41' E	2022
Thymus kotschyanus	Tk	Lahijan, Gilan, Iran	RIBB/TK01/2016	37°12' N	50°14' E	396

Hitachi S4800 (Japan). These NPs were added to MS medium after filter sterilization. The 2-months old friable callus (0.25 g) was transferred to MS containing 2,4-D (2 mg l^{-1}) and Kin (1 mg l^{-1}) under Ag NPs stress of 4 and 8 mg l^{-1}. This callus material was placed at 23 ± 2 °C under a photoperiod of 16h/8h. After incubation for 21 days of callus with Ag NPs, the callus growth rate (CGR) and callus fresh mass (CFM) was calculated. CGR was measured according to Afshar et al. (2016) every 7 days in 21 days period.

Later on, the quantity of carvacrol and thymol was obtained by high performance liquid chromatography (HPLC). The method of Castro et al. (2016) was utilized for preparing callus extracts. The process involved drying of 200 mg of callus from each treatment in an oven at 50 °C for 24 h, and then soaking it in 5 ml of diethyl ether for a period of 24 h. In order to prevent the evaporation of diethyl ether, the vials were kept closed and extraction was performed in a cold room. After adding the 80 % of methanol (1 ml) to remaining solid material, the extracts were filtered (0.22 µm pore size) into clean vials and prepared for injection to HPLC instrument. The HPLC (SY-8100 series, Beijing Beifan-Ruili Analytical Instrument, China) was performed by UV-VIS detector, a flow rate of 0.9 ml min-1, injection volume of 20 µl at 28 °C, C18 column (25 cm × 4.6 mm, partial size 5 µm), mobile phase methanol-water (80:20; v/v), and flow rate of 0.9 ml min-1. The detection was carried out at 280 nm of wavelength and a pressure of 12 atm. The UV spectra of phenolic compounds were recorded at 280 and 320 nm. The content of carvacrol and thymol were determined based on the calibration curve of standard compounds, including carvacrol (Sigma-Aldrich, USA) and thymol (Sigma-Aldrich, USA). For this purpose, 4 concentrations (10, 25, 50, and 100 mg l^{-1}) of carvacrol and 3 concentrations (25, 100, and 400 mg l^{-1}) of thymol were examined by HPLC. The retention time for carvacrol and thymol were appeared at 3-4 min and 14-15 min, respectively. After calibration of the standards with HPLC, the quantities of carvacrol and thymol in different samples were calculated.

2.1 STATISTICAL ANALYSIS

The experimentation was conducted with three replications in completely randomized design and the statistics was determined using two-way analysis of variance (ANOVA). LSD test ($p \leq 0.05$) in SAS software (SAS 9.1 Inc. USA) was applied to determine significant difference among the treatments.

3 RESULTS AND DISCUSSION

Callus growth and development as well as formation of secondary metabolic compounds is positively or negatively influenced by supplementing the growth medium with abiotic or biotic stress elicitors (Ajungla et al., 2009; Zaka et al., 2016). The contents of SMs show significant changes under elicitation of callus by different stresses (Fazal et al., 2016; Mosavat et al., 2019; Sanzari et al., 2019). NPs, specifically the metallic oxide NPs like ZnO, CuO, TiO_2 act as oxidative abiotic stress elicitors (Lala 2021). According to Al-jibouri et al. (2012), thymol amount was increased by proline in *Origanum vulgare* L. Similarly, the production of significantly the highest content of hyperforin in *Hypericum perforatum* L. (Sharafi et al., 2013), rebaudioside A and stevioside in *Stevia rebaudiana* Bertoni (Javed, et al., 2018), and proline in *Triticum aestivum* L. (Barbasz, et al., 2016) under ZnO NPs elicitation has been previously documented.

Ag NPs show significant effect on the production of SMs in callus culture, resulting in their ultimate increase (Fazal et al., 2016). For instance, Ali et al. (2019) reported that various concentrations of Ag NPs significantly affected the callus proliferation and substantially increased the callus biomass and SMs in *Caralluma tuberculata*. In another study, Fazal et al. (2019) reported the positive effects of Ag NPs and Au NPs on the production of biomass and SMs in the cell culture of *Prunella vulgaris* L.

XRD of Ag NPs is given in Figure 1 which shows 100 % phase purity by the sharpness of peaks. Similar XRD pattern was obtained by Kim et al. (2006). The

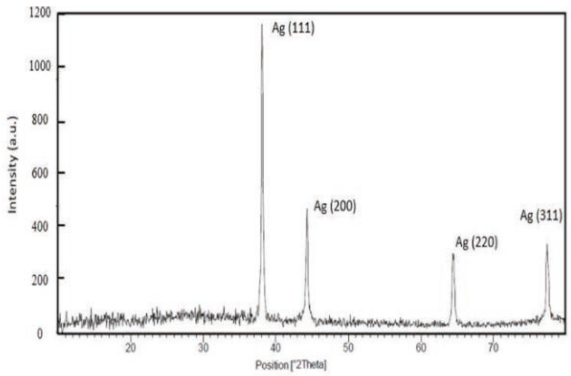


Figure 1: X-ray diffractogram (XRD) of Ag nanoparticles

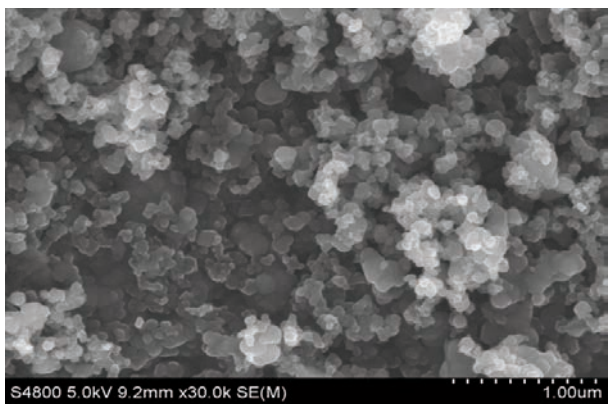
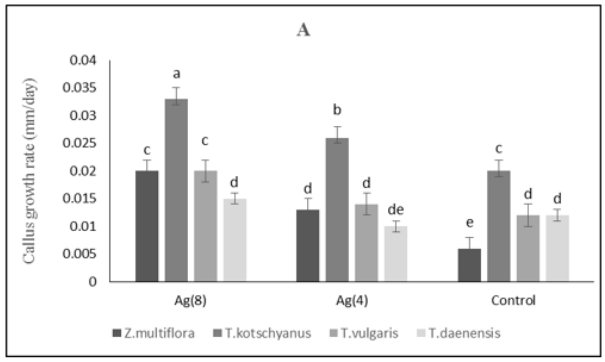


Figure 2: Scanning electron micrograph (SEM) of Ag nanoparticles



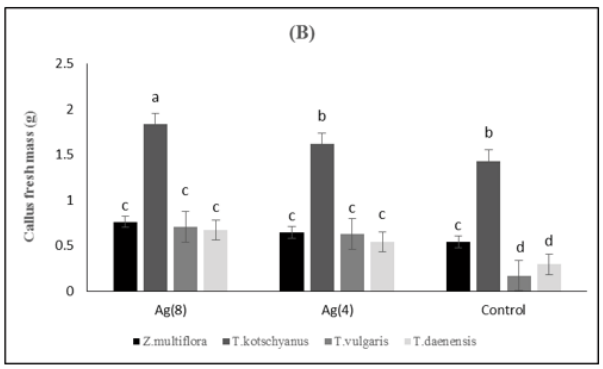


Fig 3: Effect of Ag nanoparticles on callus growth rate (A) and callus fresh mass (B) content of different *Thymus* species and *Z. multiflora* under callus culture

spherical shape of Ag NPs was illustrated by SEM image given in Figure 2 which is coinciding with the results of Elumalai et al. (2010).

The size, shape, surface, concentration and chemical composition of NPs cause stimulatory or inhibitory effects on the growth of callus cells (Al-Jibouri et al., 2012). The synthesis and accumulation of SMs in cells is enhanced by an increased surface area of NPs as a result of reduced size and transport of NPs in to the cells apoplastically which increases electrostatic interactions between living cell membranes (Javed et al., 2017). Table 2 and Figure 3 shows the effect of Ag NPs on callus growth of *Thymus* sp. and *Zataria multiflora*.

Compact calli with white and greenish colour were obtained after 10 days upon control culture (no AgNPs), whereas friable watery calli with white, greenish or yellowish colour were observed after 10-13 days upon culture supplemented with AgNPs (Figure 4.)

A significant effect is produced on callus traits by different concentrations of Ag NPs in this study that is coherent with the reports about effects of Ag NPs on

Table 2: Effect of different concentrations of Ag NPs on color, texture, growth rate, and fresh mass of callus cultures of *Thymus* sp. and *Zataria multiflora*

Nanoparticle	Concentration (mg l^{-1})	Color and Texture	CGR[I] (mm day^{-1})				CFM [II] (g)
			7	14	21	Mean	
Ag	8	Green, friable	0.045	0.06	0.015	0.022 [a]	0.99 [a]
Ag	4	Green, friable	0.07	0.06	0.032	0.015 [b]	0.85 [ab]
Control	-	White to yellow, soft	0.02	0.01	0.006	0.012 [c]	0.61 [b]

Mean values followed by the same letter in each column are not significantly different at $p<0.05$ (Least Significant Difference Test). I: CGR: Callus growth rate, II: CFM: Callus fresh mass

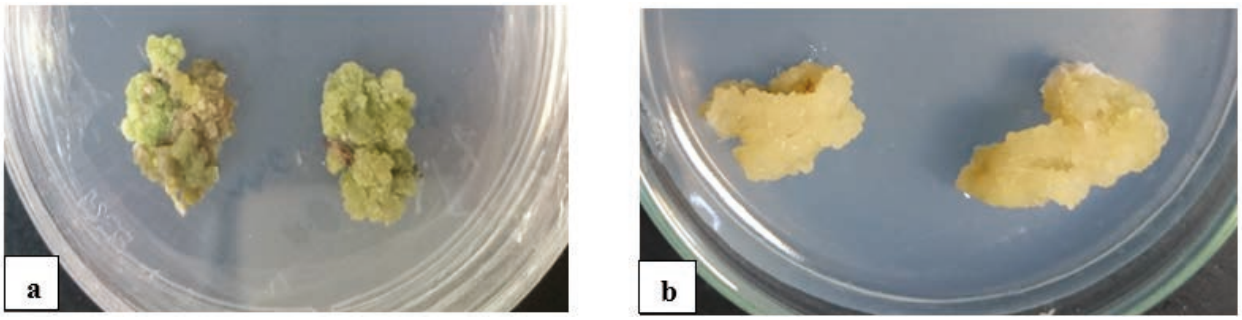


Fig 4: Friable calli with greenish or yellowish colour of *Thymus* species and *Z. multiflora* after culture treatment with $AgNP_s$ (a) and under control culture (b)

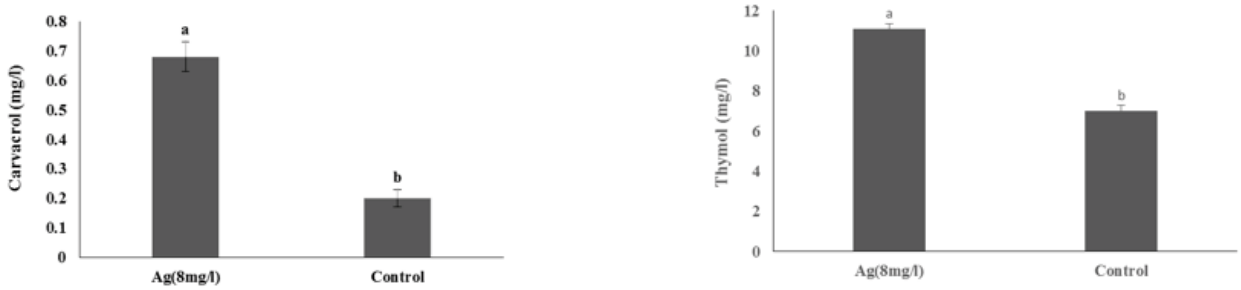


Fig 5: Effect of 8 mg l^{-1} Ag NPs on *in vitro* production of carvacrol and thymol in *Thymus* sp. and *Zataria multiflora*

callus culture of *Solanum nigrum* L. (Ewais et al., 2015) which gain friable watery calli with greenish or yellowish colour were observed after 10-13 days upon culture supplemented with AgNPs and effects of TiO_2 NPs on the callus of *Hordeum vulgare* L. (Mandeh et al., 2012). The higher TiO_2 NPs concentration influenced callogenesis of *Hordeum vulgare* explants in this study. Also according to Kokina et al. (2013) elicitation with Ag and Au NPs shows positive effects on callus width and length in *Linum usitatissimum* L. The positive effects of different NPs on callus growth of *Prunella vulgaris* L. is reported by Fazal et al. (2019). Callus growth traits affected by ZnO NPs in *Solanum lycopersicum* Mill. have also been reported (Alharby et al., 2016).

The effects of elicitation by Ag NPs on production of carvacrol and thymol under *in vitro* conditions are presented in Figure 5. The production of thymol and carvacrol was determined at 8 mg l^{-1} concentration of Ag NPs and control treatment. The carvacrol (0.68 mg l^{-1}) and thymol (11.09 mg l^{-1}) quantity was enhanced under 8 mg l^{-1} of Ag NPs. The chromatographic separation of the methanolic extracts in *Zataria multiflora* for carvacrol and thymol by HPLC is given (Figure 6). Asadollahei et al. (2022) employed different concentrations of CuNPs in *in vitro* culture medium and observed significant rise in thymol and carvacrol content compared to control in *Zataria multiflora*. This study elucidated that the selection of appropriate plant species and suitable elicitor is crucial for increasing the production of bioactive compounds as well as antioxidants of *Zataria multiflora*. This can be done by inducing expression changes in the biosynthetic pathways of thymol and carvacrol. In fact, the gene expression patterns of the pathways of formation of thymol and carvacrol were greatly influenced by the Ag NPs in our study which is the phenomenon well explained by the studies of Kianersi et al. (2021).

The interactive effects of NPs and genotypes/species of *Thymus* and *Zataria multiflora* for production of SMs

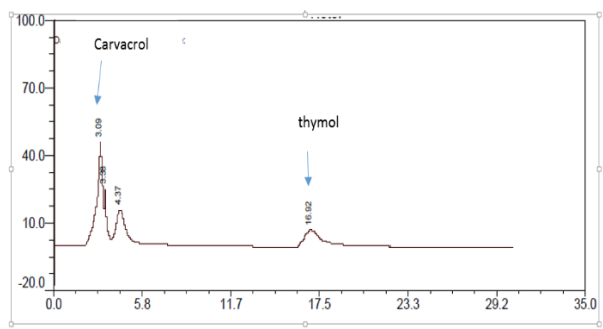


Fig 6: Representative HPLC chromatograms of thymol and carcacrol of *Z. multiflora*. Peak identifications were performed by matching retention time and UV spectra against commercially available reference compounds

have been presented in Table 3. The exposure of Ag NPs has significantly increased the content of two SMs of callus compared to control. Moreover, *in vitro* synthesis of thymol was notably greater than that of carvacrol which is also evident from the molecular studies of Kianersi et al. (2021). The highest content of carvacrol (1.06 mg l^{-1}) was observed at 8 mg l^{-1} concentration of Ag NPs in *T. kotschyanus*, whereas the least amount (0.10 mg l^{-1}) was observed in control treatment of *T. daenensis*. Furthermore, the highest concentration of thymol was obtained at 8 mg l^{-1} of Ag NPs in callus of *T. daenesis* (19.75 mg l^{-1}), while the least thymol content (3.95 mg l^{-1}) was achieved in *T. daenesis* under control condition.

This result can be well supported by the phenomenon that NPs trigger thymol synthetic pathways and/or transcription factors more than the carvacrol pathways (Mosavat et al., 2019). Taking into account of concurrent studies, the phenolics and flavonoids production is activated by ZnO NPs in seedlings of *Brassica nigra* L. (Zafar et al., 2016) Additionally, the significant rise in hyperforin content in cell suspension culture of *Hypericum perfolatum* L. under ZnO NPs stress is reported (Sharafi et al., 2013). A complex variety of elicitation effects on *in vitro* synthesis of SMs is obtained using different types of elicitors (Goswami et al., 2017; Marslin et al., 2017; Syu et al., 2014), plant tissues (Ajungla et al., 2009), and physiochemical environment of various species (Shakya et al., 2019).

4 CONCLUSION

The formation of callus from *Thymus* species and *Zataria multiflora* was performed in the presence of Ag NPs elicitors. Addition of abiotic elicitors, i.e., Ag NPs (8 mg l^{-1}) to the MS medium played a vital role in enhancing the thymol and carvacrol content in the callus cultures of different *Thymus* species and *Zataria multiflora*. In other words, Ag nano-elicitors applied to the *in vitro* callus cultures of *Thymus* species and *Zataria multiflora* in our study resulted in increase in SMs production at a concentration of 8 mg l^{-1}. Our finding opens the way for studies involving relationship between chemical elicitors and formation & accumulation of thymol/carvacrol. In future, transcriptomic and metabolomics studies should be performed to elucidate the regulation of SMs production under an elicitation of Ag NPs in these medicinal plants.

Table 3: The effect of species × NPs interaction on *in vitro* production of thymol and carvacrol in callus culture of *Thymus* species and *Z. multiflora*

Species	Nanoparticles (mg l^{-1})	Thymol (mg l^{-1})	Carvacrol (mg l^{-1})
Z. multiflora	Ag (8)	$12.76^{b} \pm 0.02$	$1.05^{a} \pm 0.03$
	Control	$7.06^{g} \pm 0.01$	$0.20^{bc} \pm 0.01$
T.kotschyanus	Ag (8)	$7.90^{e} \pm 0.01$	$1.06^{a} \pm 0.04$
	Control	$7.39^{f} \pm 0.01$	$0.26^{b} \pm 0.01$
T. vulgaris	Ag (8)	$11.06^{c} \pm 0.04$	$0.29^{b} \pm 0.01$
	Control	$9.64^{d} \pm 0.04$	$0.10^{c} \pm 0.002$
T. daenesis	Ag (8)	$19.75^{a} \pm 0.02$	$0.31^{b} \pm 0.01$
	Control	$3.95^{h} \pm 0.03$	$0.23^{bc} \pm 0.02$

Each value represents Mean ± SE. Mean values followed by the same letter are not significantly different at $p < 0.05$ (Least Significant Difference Test)

5 ACKNOWLEDGMENT

The authors wish to express their gratitude to the Research Institute for Biotechnology and Bioengineering, Isfahan University of Technology, Isfahan, Iran and Payame Noor University, Isfahan, Iran.

6 DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

7 REFERENCES

Afshar, B., & Golkar, P. (2016). Mucilage synthesis by in vitro cell culture in different species of Alyssum. *BioTechnologia. Journal of Biotechnology Computational Biology and Bionanotechnology*, *97*(2), 79–86. https://doi.org/10.5114/bta.2016.60778

Ahmad, M. A., Javed, R., Adeel, M., Rizwan, M., Ao, Q., & Yang, Y. (2020). Engineered ZnO and CuO nanoparticles ameliorate morphological and biochemical response in tissue culture regenerants of candyleaf (*Stevia rebaudiana*). *Molecules*, *25*(6), 1356. https://doi.org/10.3390/molecules25061356

Ajungla, L., Patil, P. P., Barmukh, R. B., & Nikam, T. D. (2009). Influence of biotic and abiotic elicitors on accumulation of hyoscyamine and scopolamine in root cultures of *Datura metel* L. *Indian Journal of Biotechnology*, *8*(3), 317–322.

Alharby, H. F., Metwali, E. M. R., Fuller, M. P., & Aldhebiani, A. Y. (2016). Impact of application of zinc oxide nanoparticles on callus induction, plant regeneration, element content and antioxidant enzyme activity in tomato (*Solanum lycopersicum* mill.) under salt stress. *Archives of Biological Sciences*, *68*(4), 723–735. https://doi.org/10.2298/ABS151105017A

Ali, A., Mohammad, S., Khan, M. A., Raja, N. I., Arif, M., Kamil,

A., & Mashwani, Z. ur R. (2019). Silver nanoparticles elicited *in vitro* callus cultures for accumulation of biomass and secondary metabolites in Caralluma tuberculata. *Artificial Cells, Nanomedicine and Biotechnology*, *47*(1), 715–724. https://doi.org/10.1080/21691401.2019.1577884

Al-Jibouri, A. M. J., Abd, A. S., Majeed, D. M., & Ismail, E. N. (2012). Influence of abiotic elicitors on accumulation of thymol in callus cultures of *Origanum vulgare* L. *Journal of Life Sciences*, *6*(10), 1094.

Asadollahei, M. V., Yousefifard, M., Tabatabaeian, J., Nekonam, M. S., & Mahdavi, S. M. E. (2022). Effect of elicitors on secondary metabolites biosynthesis in *Zataria multiflora* Boiss. *Industrial Crops and Products*, *181*, 114789. https://doi.org/10.1016/j.indcrop.2022.114789

Barbasz, A., Kreczmer, B., & Oćwieja, M. (2016). Effects of exposure of callus cells of two wheat varieties to silver nanoparticles and silver salt ($AgNO_3$). *Acta Physiologiae Plantarum*, *38*(3), 1–11. https://doi.org/10.1007/s11738-016-2092-z

Castro, A. H. F., Braga, K. de Q., de Sousa, F. M., Coimbra, M. C., & Chagas, R. C. R. (2016). Callus induction and bioactive phenolic compounds production from Byrsonima verbascifolia (L.) DC. (Malpighiaceae). *Revista Ciencia Agronomica*, *47*(1), 143–151. https://doi.org/10.5935/1806-6690.20160017

Dykman, L. A., & Shchyogolev, S. Y. (2017). Interactions of plants with noble metal nanoparticles. *Agricultural Biology*, *52*(1), 13. https://doi.org/10.15389/agrobiology.2017.1.13eng

Elumalai, E. K., Prasad, T. N. V. K. V., Hemachandran, J., Viviyan Therasa, S., Thirumalai, T., & David, E. (2010). Extracellular synthesis of silver nanoparticles using leaves of *Euphorbia hirta* and their antibacterial activities. *Journal of Pharmaceutical Sciences and Research*, *2*(9), 549–554.

Ewais, E. ., Desouky, S., & Elshazly, E. . (2015). Evaluation of callus responses of *Solanum nigrum* L. exposed to biologically synthesized silver nanoparticles. *Nanoscience and Nanotechnology*, *5*(3), 45–56.

Fazal, H., Abbasi, B. H., Ahmad, N., & Ali, M. (2016). Elicitation of medicinally important antioxidant secondary metabolites with silver and gold nanoparticles in callus cultures of *Prunella vulgaris* L. *Applied Biochemistry and Biotechnology*, *180*(6), 1076–1092. https://doi.org/10.1007/s12010-016-2153-1

Fazal, H., Abbasi, B. H., Ahmad, N., Ali, M., Shujait Ali, S., Khan, A., & Wei, D. Q. (2019). Sustainable production of biomass and industrially important secondary metabolites in cell cultures of selfheal (*Prunella vulgaris* L.) elicited by silver and gold nanoparticles. *Artificial Cells, Nanomedicine and Biotechnology*, *47*(1), 2553–2561. https://doi.org/10.1080/21691401.2019.1625913

Golkar, P., Bakhtiari, M.A. & Bazarganipour, M., (2021). The effects of nanographene oxide on the morpho-biochemical traits and antioxidant activity of *Lepidium sativum* L. under *in vitro* salinity stress. *Scientia Horticulturae*, 288, p.110301. https://doi.org/10.1016/j.scienta.2021.110301

Goswami, L., Kim, K. H., Deep, A., Das, P., Bhattacharya, S. S., Kumar, S., & Adelodun, A. A. (2017). Engineered nano particles: Nature, behavior, and effect on the environment. *Journal of Environmental Management*, *196*, 297–315. https://doi.org/10.1016/j.jenvman.2017.01.011

Jasim, B., Thomas, R., Mathew, J., & Radhakrishnan, E. K. (2017). Plant growth and diosgenin enhancement effect of silver nanoparticles in Fenugreek (*Trigonella foenum-graecum* L.). *Saudi Pharmaceutical Journal*, *25*(3), 443–447. https://doi.org/10.1016/j.jsps.2016.09.012

Javed, R., Ahmed, M., Haq, I. ul, Nisa, S., & Zia, M. (2017). PVP and PEG doped CuO nanoparticles are more biologically active: Antibacterial, antioxidant, antidiabetic and cytotoxic perspective. *Materials Science and Engineering C*, *79*, 108–115. https://doi.org/10.1016/j.msec.2017.05.006

Javed, R., Usman, M., Tabassum, S., & Zia, M. (2016). Effect of capping agents: Structural, optical and biological properties of ZnO nanoparticles. *Applied Surface Science*, *386*, 319–326. https://doi.org/10.1016/j.apsusc.2016.06.042

Javed, R., Yucesan, B., Zia, M., & Gurel, E. (2018). Elicitation of secondary metabolites in callus cltures of *Stevia rebaudiana* Bertoni grown under ZnO and CuO nanoparticles stress. *Sugar Tech*, *20*(2), 194–201. https://doi.org/10.1007/s12355-017-0539-1

Kianersi, F., Pour-Aboughadareh, A., Majdi, M., & Poczai, P. (2021). Effect of methyl jasmonate on thymol, carvacrol, phytochemical accumulation, and expression of key genes involved in thymol/carvacrol biosynthetic pathway in some Iranian Thyme Species. *International jJournal of Molecular Sciences*, *22*(20), 11124. https://doi.org/10.3390/ijms222011124

Kim, D. H., Gopal, J., & Sivanesan, I. (2017). Nanomaterials in plant tissue culture: the disclosed and undisclosed. *RSC advances*, *7*(58), 36492-36505. https://doi.org/10.1039/C7RA07025J

Kim, D., Jeong, S., & Moon, J. (2006). Synthesis of silver nanoparticles using the polyol process and the influence of precursor injection. *Nanotechnology*, *17*(16), 4019–4024. https://doi.org/10.1088/0957-4484/17/16/004

Kokina, I., Gerbreders, V., Sledevskis, E., & Bulanovs, A. (2013). Penetration of nanoparticles in flax (Linum usitatissimum L.) calli and regenerants. *Journal of Biotechnology*, *165*(2), 127–132. https://doi.org/10.1016/j.jbiotec.2013.03.011

Lala, S. (2021). Nanoparticles as elicitors and harvesters of economically important secondary metabolites in higher plants: A review. *IET Nanobiotechnology*, *15*(1), 28-57. https://doi.org/10.1049/nbt2.12005

Mandeh, M., Omidi, M., & Rahaie, M. (2012). In Vitro influences of TiO_2 nanoparticles on barley (Hordeum vulgare L.) tissue culture. *Biological Trace Element Research*, *150*(1–3), 376–380. https://doi.org/10.1007/s12011-012-9480-z

Marslin, G., Sheeba, C. J., & Franklin, G. (2017). Nanoparticles alter secondary metabolism in plants via ROS burst. *Frontiers in Plant Science*, *8*, 832. https://doi.org/10.3389/fpls.2017.00832

Mathela, C. S., Singh, K. K., & Gupta, V. K. (2010). Synthesis and in vitro antibacterial activity of thymol and carvacrol derivatives. *Acta Poloniae Pharmaceutica - Drug Research*, *67*(4), 375–380.

Miraj, S., & Kiani, S. (2016). Study of pharmacological effect of *Ocimum basilicum*: A review. *Der Pharmacia Lettre*, *8*(9), 315–320.

Mosavat, N., Golkar, P., Yousefifard, M., & Javed, R. (2019). Modulation of callus growth and secondary metabolites in different Thymus species and *Zataria multiflora* micropropagated under ZnO nanoparticles stress. *Biotechnology and Applied Biochemistry*, *66*(3), 316–322. https://doi.org/10.1002/bab.1727

Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Agsays with Tohaoco Tissue Cultures. *Physiologia Plantarum*, *15*(3), 474–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Ramakrishna, A., & Ravishankar, G. A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling and Behavior*, *6*(11), 1720–1731. https://doi.org/10.4161/psb.6.11.17613

Rastogi, A., Zivcak, M., Sytar, O., Kalaji, H. M., He, X., Mbarki, S., & Brestic, M. (2017). Impact of metal and metal oxide nanoparticles on plant: A critical review. *Frontiers in Chemistry*, *5*, 78. https://doi.org/10.3389/fchem.2017.00078

Rechinger, K. (1982). Flora Iranica, Vol. 150. *IC Hedge - Graz: Akademische Druck Und Verlagsanstalt*, *150*, 543–544.

Sadak, M. S. (2019). Impact of silver nanoparticles on plant growth, some biochemical aspects, and yield of fenugreek plant (*Trigonella foenum-graecum*). *Bulletin of the National Research Centre*, *43*(1), 1-6. https://doi.org/10.1186/s42269-019-0077-y

Sajed, H., Sahebkar, A., & Iranshahi, M. (2013). *Zataria multiflora* Boiss. (Shirazi thyme) - An ancient condiment with modern pharmaceutical uses. *Journal of Ethnopharmacology*, *145*(3), 686–698. https://doi.org/10.1016/j.jep.2012.12.018

Sanzari, I., Leone, A., & Ambrosone, A. (2019). Nanotechnology in plant science: to make a long story short. *Frontiers in Bioengineering and Biotechnology*, *7*, 120. https://doi.org/10.3389/fbioe.2019.00120

Shakya, P., Marslin, G., Siram, K., Beerhues, L., & Franklin, G. (2019). Elicitation as a tool to improve the profiles of high-value secondary metabolites and pharmacological properties of *Hypericum perforatum*. *Journal of Pharmacy and Pharmacology*, *71*(1), 70–82. https://doi.org/10.1111/jphp.12743

Sharafi, E., Fotokian, M. H., & Loo, H. (2013). Improvement of hypericin and hyperforin production using zinc and iron nano-oxides as elicitors in cell suspension culture of John'swort (*Hypericum perforatum* L). *Journal of Medicinal Plants and By-products*, *2*(2).

Sharma, P., Bhatt, D., Zaidi, M. G. H., Saradhi, P. P., Khanna, P. K., & Arora, S. (2012). Silver nanoparticle-mediated enhancement in growth and antioxidant status of *Brassica juncea*. *Applied Biochemistry and Biotechnology*, *167*(8), 2225–2233. https://doi.org/10.1007/s12010-012-9759-8

Syu, Y. yu, Hung, J. H., Chen, J. C., & Chuang, H. wen. (2014). Impacts of size and shape of silver nanoparticles on Arabidopsis plant growth and gene expression. *Plant Physiology and Biochemistry*, *83*, 57–64. https://doi.org/10.1016/j.plaphy.2014.07.010

Zafar, H., Ali, A., Ali, J. S., Haq, I. U., & Zia, M. (2016). Effect of ZnO nanoparticles on *Brassica nigra* seedlings and stem explants: Growth dynamics and antioxidative response. *Frontiers in Plant Science*, *7*, 535. https://doi.org/10.3389/fpls.2016.00535

Zaka, M., Abbasi, B. H., Rahman, L.-U., Shah, A., & Zia, M. (2016). Synthesis and characterisation of metal nanoparticles and their effects on seed germination and seedling growth in commercially important *Eruca sativa*. *IET Nanobiotechnology*, *10*(3), 1–7. https://doi.org/10.1049/iet-nbt.2015.0039

Zarshenas, M. M., & Krenn, L. (2015). A critical overview on Thymus daenensis Celak.: phytochemical and pharmacological investigations. *Journal of Integrative Medicine*, *13*(2), 91–98. https://doi.org/10.1016/S2095-4964(15)60166-2

Zhao, J., Davis, L., & Verpoorte, R. (2005). Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnology Advances*, *23*(4), 283–333. https://doi.org/10.1016/j.biotechadv.2005.01.003

Zuverza-Mena, N., Martínez-Fernández, D., Du, W., Hernandez-Viezcas, J. A., Bonilla-Bird, N., López-Moreno, M. L., Gardea-Torresdey, J. L. (2017). Exposure of engineered nanomaterials to plants: Insights into the physiological and biochemical responses-A review. *Plant Physiology and Biochemistry*, *110*, 236–264. https://doi.org/10.1016/j.plaphy.2016.05.037

doi:10.14720/aas.2022.118.3.2279 Original research article / izvirni znanstveni članek

Field resistance phenotyping of durum wheat to fusarium head blight in Algeria

Salah HADJOUT [1, 2, 3], Zouaoui BOUZNAD [2], Leila MEKLICHE [2], Mohamed ZOUIDI [1]

Received July 21, 2021; accepted September 06, 2022.
Delo je prispelo 21. julija 2021, sprejeto 6. septembra 2022

Field resistance phenotyping of durum wheat to fusarium head blight in Algeria

Abstract: In Algeria, several research studies point to the importance of the causative agents of fusarium head blight. Indeed, our research aims to study the phenotyping of the resistance of some durum wheat genotypes for their behavior to fusarium head blight, caused by four isolates of *Fusarium culmorum* (Wm.G.Sm.) Sacc.. For this purpose, the disease assessment is carried out in the field. The different evaluation criteria are: incubation period, measurement of the mass of a thousand grains and AUDPC (Area Under the Disease Progression Curve). The results obtained revealed that the varieties and lines resulting from crosses had a quite different level of susceptibility with regard to the four isolates studied and no genotype showed complete resistance (immunity) under our growing conditions. Among the tested material, the lines showed higher resistance than their parents. The reasons for this phenomenon is that crosses between genotypes implicated cultivars from Europe and Western Asia (Syria), where wheat domestication has occurred very early (between 12 000 and 10 000 years BP), which may be promising sources of resistance to fusarium head blight. The results also show a slight variability in behavior, also linked to the aggressiveness of the *Fusarium* species studied in this work.

Key words: durum wheat; phenotyping; fusarium head blight; resistance; susceptibility; aggressiveness

Ugotavljanje odpornosti trde pšenice na fuzariozo klasov na prostem v Alžiriji

Izvleček: Številne raziskave so poudarile pomen fuzarioz pšeničnih klasov v Alžiriji. Namen te raziskave je bil ugotoviti fenotipsko odpornost nekaterih genotipov trde pšenice na fuzarioze, ki jih povzročajo štiri izolati glive *Fusarium culmorum* (Wm.G.Sm.) Sacc.. Za oceno bolezni je bil izveden poljski poskus. Za oceno okužbe so bili uporabljeni naslednji kriteriji: inkubacijsko obdobje, meritev mase tisočih zrn in AUDPC (Območje pod naraščajočo krivuljo bolezni). Rezultati so pokazali, da so imele sorte in linije, ki so nastale s križanji zelo različno občutljivost na štiri v raziskavi uporabljene izolate glive, vendar ni imel noben genotip popolne odpornosti (imunosti) v razmerah potekanja poskusa. Med testiranimi vzorci pšenice so imele linije večjo odpornost kot njihovi starši. Razlog za ta fenomen je ta, da so bila križanja med genotipi sort iz Evrope in Zahodne Azije (Sirija), kjer je bila trda pšenica udomačena že zelo zgodaj (med 12 000 in 10 000 let pred sedanjostjo, t.j od začetka datiranja starosti na osnovi radioaktivnega ogljika), kar bi lahko bil obetajoč vir odpornosti na fuzarioze klasov. Izsledki so pokazali tudi manjšo variabilnost v odzivnosti med genotipi analizirane pšenice, kar je lahko povezano z različno ogresivnostjo v raziskavi uporabljenih sevov glive iz rodu *Fusarium*.

Ključne besede: trda pšenica; fenotipsko določanje; fuzarioza klasov; odpornost; občutljivost; agresivnost

1 Centre de Recherche en Aménagement du Territoire (CRAT), Campus Universitaire Zouaghi Slimane, Constantine, Algérie
2 Ecole Nationale Supérieure Agronomique (ENSA), El-Harrach, Algérie
3 Corresponding author, e-mail: hadjout.salah@gmail.com

1 INTRODUCTION

Durum wheat (*Triticum durum* Desf.) is one of the oldest and the most important cultivated cereal species in the world (Royo et al., 2009; Tidiane et al., 2019; Bouanaka et al., 2021). It is of great importance in the cereal-growing areas of the Mediterranean basin and North America, where most of the world production of this crop is concentrated (USDA, 2005; Xynias et al., 2020). However, durum wheat is no longer just a staple crop for food security, but has also become a major cash crop. Africa as a whole spends more than 4 billion euros per year for import of durum wheat to provide the raw material for its food industry (Tidiane et al., 2019). In Algeria, wheat consumption (both durum wheat and soft wheat) is far greater than its real production capacity. Consequently, the domestic market has been dependent on a significant level of imports in recent years. In addition, yields are quite low for locally grown wheat and should be improved (Touati-Hattab et al., 2016). Various reasons are at the origin of this situation such as precipitation and biotic (pests and diseases) and abiotic stresses (drought, sunshine, cold and salinity) (Xynias et al., 2020). Among the biotic constraints to wheat production, fusarium head blight (FHB) (Ghimire et al., 2020).

Fusarium head blight, reported by several species of the genus *Fusarium* (Bouanaka et al., 2020; Saharan, 2020), is one of the most destructive diseases of wheat (Dweba et al., 2017; Wachowska et al., 2020), particularly affecting durum wheat (Moreno-Amores et al., 2020) and thus leading to significant reductions in yield and quality throughout the world (Touati-Hattab et al., 2016; Dweba et al., 2017; Saharan, 2020). In addition, FHB poses additional food and animal safety concerns due to the contamination of grains with mycotoxins (Ghimire et al., 2020). Among the most important species associated with the disease worldwide is *Fusarium culmorum*.

The cereal pathogen *Fusarium culmorum* (Wm.G.Sm.) Sacc. is a ubiquitous soil fungus (ascomycete) (Bilska et al., 2018), considered a chronic fungus of economic interest worldwide, including in African countries from the North like Algeria. This pathogen produces a wide range of mycotoxins, including the trichothecene-B type deoxynivalenol (DON) (Yekkour et al., 2015), which constitutes a potential health hazard (Bilska et al., 2018). Previous studies carried out in Algeria have shown that *Fusarium culmorum* appears to be the major pathogen associated with fusarium head blight (Yekkour et al., 2015; Touati-Hattab et al., 2016; Laraba et al., 2017).

The use of various methods to limit the development of *Fusarium* cereal ear diseases and their contamination with mycotoxins, before and after harvest, is an important part of sustainable agriculture and the production of healthy foods (Mielniczuk & Skwaryło-Bednarz, 2020). Genetic resistance is the most effective and sustainable approach to manage diseases in wheat (Ghimire et al., 2020), in particular, reducing the problem of mycotoxins in farmers' fields affected by fusarium head blight (Saharan, 2020).

Today, FHB phenotyping performed by breeders is performed by visual examination (Serre et al., 2015). In this context, the main objective of our present study is to compare the phenotypic resistance to fusarium wilt of two new durum wheat lines of Algerian origin selected against those of three parental commercial varieties.

2 MATERIALS AND METHODS

2.1 VEGETAL MATERIAL

In our experience, five durum wheat genotypes were chosen. To this end, two pedigree lines (G1 and G4) selected in Algeria and three commercialized varieties (G9, G10 and G11) were tested in the field. These lines are composed of F15 seeds resulting from simple crosses between 4 parental varieties: Saadi, Siméto, Ardente and Waha (Mekliche et al., 2013). The main characteristics of these genotypes are shown in Table 1. The aim is to compare their levels of resistance.

2.2 FUNGAL MATERIAL

During our study, four isolates of *Fusarium culmorum* (F.C.T5, F.C.T7, F.C10.11 and F.C1.12) were used (Table 2). These isolates were obtained from the ears and crowns of the 'Vitron' variety of durum wheat, showing typical symptoms of the disease. The ears and collars were harvested in the area of Oued Semar (Algeria) in northern Algeria. The preliminary identification was made on the basis of the conidial morphology according to Leslie and Summerell (2006) then confirmed by

Table 1: F15 pedigree lines and parental varieties used during the experiment

Codes	Genotypes	Origin	Precocity
G1	Saadi × Waha	ENSA, Algeria	Early
G4	Ardente × Siméto	ENSA, Algeria	Early
G9	Siméto	Italy	Semi- Early
G10	Ardente	France	Early to very early
G11	Waha	ICARDA, Syria	Early

Table 2: *Fusarium culmorum* isolates used in the study

Code	Species	Origin	Isolation organ	Variety
$F.C.T_5$	*F. culmorum*	Oued Smar	Ear	Durum wheat ('Vitron')
$F.C.T_7$	*F. culmorum*	Oued Smar	Ear	Durum wheat ('Vitron')
$F.C_{10.11}$	*F. culmorum*	Oued Smar	Ear	Durum wheat ('Vitron')
$F.C_{1.12}$	*F. culmorum*	Oued Smar	Collar	Durum wheat ('Vitron')

molecular tools thus using classical PCR (Touati-Hattab et al., 2016; Hadjout et al., 2022).

2.3 INOCULUM PREPARATION

Fusarium isolates are cultured in Petri dishes containing PDA medium. They are then incubated in the dark and at a temperature of 25 °C until sporulation. After 20 days of incubation, a layer of sterile distilled water of 1 to 2 mm is placed on the colony contained in each Petri dish and then poured into a container. After counting in the Malassez cell, the inoculum is prepared from a suspension of conidia in water, adjusted to 5.10^4 spores per milliliter, prepared extemporaneously (Hadjout et al., 2017).

2.4 ARTIFICIAL FIELD INOCULATION METHOD

Inoculation in the field was done by spraying the ears of each genotype until the inoculum begins to run-off, approximately 200 ml m^{-2}. The inoculations were carried out at the flowering stage corresponding to a minimum of 10 % of the ears from which the stamens have emerged. The controls consist of plots where no artificial inoculation was carried out. In the field, the inoculations were carried out in the evening, after sprinkling irrigation for about 20 min before inoculation and then 10 min after, in order to maintain sufficient humidity on the plants during the night, but also to promote adhesion and the germination of conidia. Depending on climatic conditions, the plots are then irrigated regularly in the evening.

2.5 FIELD EXPERIMENTAL SET-UP

The experiment was carried out in the field, with the installation of five tests: a control test and four tests inoculated with the four isolates of *Fusarium culmorum* mentioned above. The experimental set-up was of the complete random block type, with three repetitions (Fig. 1). The spacing between the blocks was 1 m. The area of each microplot was 1 m^2, consisting of 5 lines of 1 meter (linear meter) 20 cm apart. The distance between each microplot was 50 cm. Lines of triticale were sown between trials to avoid cross-contamination.

2.6 FIELD DISEASE ASSESSMENT

2.6.1 Incubation period

The incubation period corresponds to the period between artificial inoculation and the appearance of a fusarium blighted spikelet in the plot.

2.6.2 Symptom scoring

In the case of our study, disease severity scoring was performed 21, 26 and 31 days after inoculation. The observation unit consisted of 25 ears selected at random from each microplot. On these spikes, the total number of spikelets per spike and the number of *Fusarium* colonized spikelets were counted. The proportion of spikelets showing symptoms is assessed using a logarithmic rating scale described by Michel (2001), ranging from 0 (no symptoms) to 9 (completely dead ear, generalized drying out).

2.6.3 Calculation of the area under the disease progression curve (AUDPC)

The AUDPC is calculated on the number of fused spikelets for all scoring dates according to the formula described by Shaner and Finney (1977):

$$\text{Standardized AUDPC} = \sum_i^n \left[\left(\frac{Xi + Xi-1}{2}\right)\right](t_i - t_{i-1})$$

Where: n: total number of observations; x_i: number of fusarium infected spikelets in 25 heads at each observation; $(t_i - t_{i-1})$: time separating two consecutive observations.

Fig. 1: Diagram of the open-field experimental set-up for each of the five trials

2.6.4 Evaluation of the Thousand Grains Mass (TGM) at harvest

TGM was measured to assess the impact of the disease on yield, all 25 heads were threshed using a threshing machine with poor ventilation.

2.7 STATISTICAL ANALYSIS OF DATA

The statistical analysis of the results in the field is carried out using statgraphics software version 15.1.0. Next, a multiple comparison of the means was performed using the ppds (least significant difference) test to determine the groups homogeneous at the 5 % significance level.

3 RESULTS AND DISCUSSION

3.1 MANIFESTATION OF THE DISEASE

Fusarium head blight of wheat was observed in the field. In fact, the inoculated plots showed symptoms of the disease, the attacks of which on wheat ears by this disease most often result in the scalding of certain groups of spikelets, part or all of the ear. Symptoms are manifested by the presence of one or more discolored spikelets on the green spikes (Fig. 2. a, b, c, d). The ripe kernels harvested were scalded, light, chalky white or sometimes pink (this is referred to as mummified or damaged kernels, fusarious kernels) (Fig. 2.e). It should be noted that the amount of symptoms depends on the stage of the plant at the time of inoculation; the peak of sensitivity

Fig. 2: Characteristic symptoms of fusarium head blight in durum wheat (personal photos)
a, b and c: fusarious ears, the orange tint denotes the presence of the pathogenic fungus
d: Hard wheat field almost completely fused; e: fusarium grains

corresponds to the flowering of the varieties. Burrows et al. (2008) report that the initial infection is characterized as a discolored lesion at the base of the glume and the rachis which then spreads in both directions of the ear. Previous data were obtained using a spray inoculation method, frequently used to screen for resistance to fusarium head blight in wheat (Prat et al. 2014). According to Miedaner et al. (2003), spray inoculation, compared to single flower inoculation, is more adequate to reproduce the natural conditions of infection.

According to Touati-Hattab et al. (2016) and Laraba et al. (2017), *F. culmorum* is the main fungal pathogen associated with fusarium head blight in Algeria. In addition, *F. culmorum*, the causative agent of various diseases of the ear and crown of cereals, is considered a chronic fungus of economic concern worldwide, including North African countries such as Algeria. (Yekkour et al., 2015).

3.2 SSESSMENT OF GENOTYPE BEHAVIOUR BY INCUBATION PERIOD

The results obtained show that the appearance of the first symptoms on the ears estimated by the incubation time varies according to the genotype / isolate interaction (Fig. 3). Analysis of variance for all four trials revealed a significant difference for genotypes ($p < 0.001$) and for treatments (isolates) ($p < 0.001$); on the other hand, the interaction (genotypes / treatments) has no significant effect ($p > 0.05$) (Fig. 3.a, b). In our trials, we were able to characterize the behavior of genotypes with respect to the incubation period. This criterion allowed us to observe that the G1 line ranked well compared to other genotypes, due to the long incubation period recorded, 18 days after contamination. This reflects a good level of type I resistance for this line, linked to a cellular mechanism that slows the expression of the first symptom and therefore the onset of the disease. In contrast, varieties G9 and G11 recorded a shorter incubation period, approximately 10 and 11 days respectively after contamination. They are considered to be the sensitive controls chosen during our experiments; G4 and G10 genotypes had a very comparable average incubation period, an average of 13 days after contamination. It should be noted that the resistant behavior of G1 line expressed by a longer incubation period is in agreement with the work of Trottet and Saur (1994) who also used this parameter. From these results, we can say that the period of onset of symptom onset is directly related to the level of resistance of the genotypes, but also to the aggressiveness of the isolates. The mechanisms of resistance in plants to fusarium wilt are very complex (Mesterhazy et al., 1999). It is generally accepted that resistance to fusarium wilt is controlled by a polygenic system, which is known to slow the development of individual infections, the spread of the disease in fields, and the rate of spread of the fungus in adjacent plant tissues (Qi et al., 1999; Lindhout, 2002).

3.3 EVALUATION OF GENOTYPE BEHAVIOUR BY AUDPC VALUE OF THE NUMBER OF *FUSARIUM* INFECTED SPIKELETS

AUDCP analysis of variance for the number of *Fusarium* infected spikelets showed a significant difference for genotypes ($p < 0.001$) (Fig. 4.a) and trials ($p < 0.001$) (Fig. 4. b). On the other hand, the interaction between genotypes and trials is not significant ($p > 0.05$). Our results show that the AUDPC of the number of *Fusarium* infected spikelets for the G1 line is very low (9.75), while the two susceptible varieties (G9 and G11)

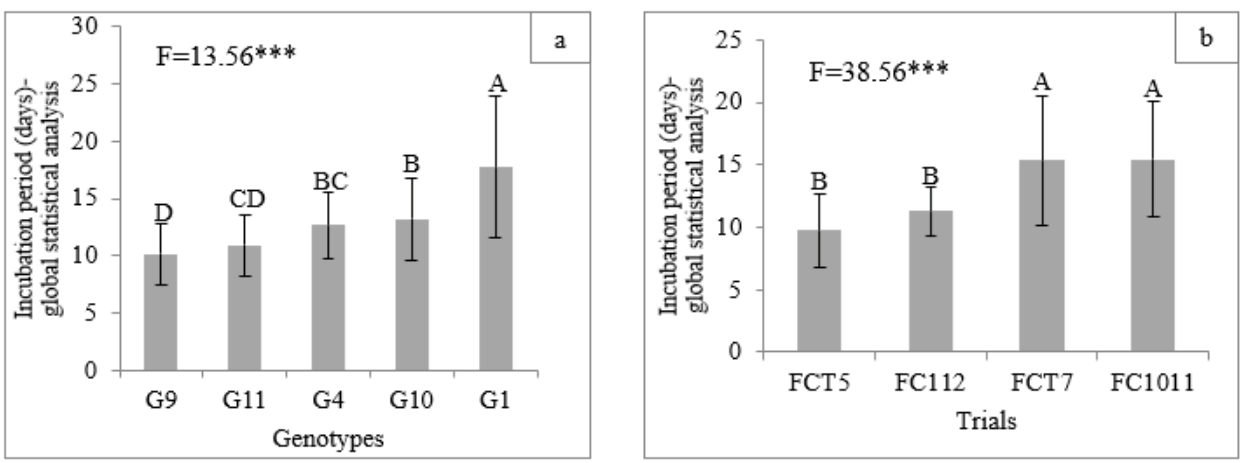


Fig. 3: Behavior of genotypes towards isolates according to incubation time

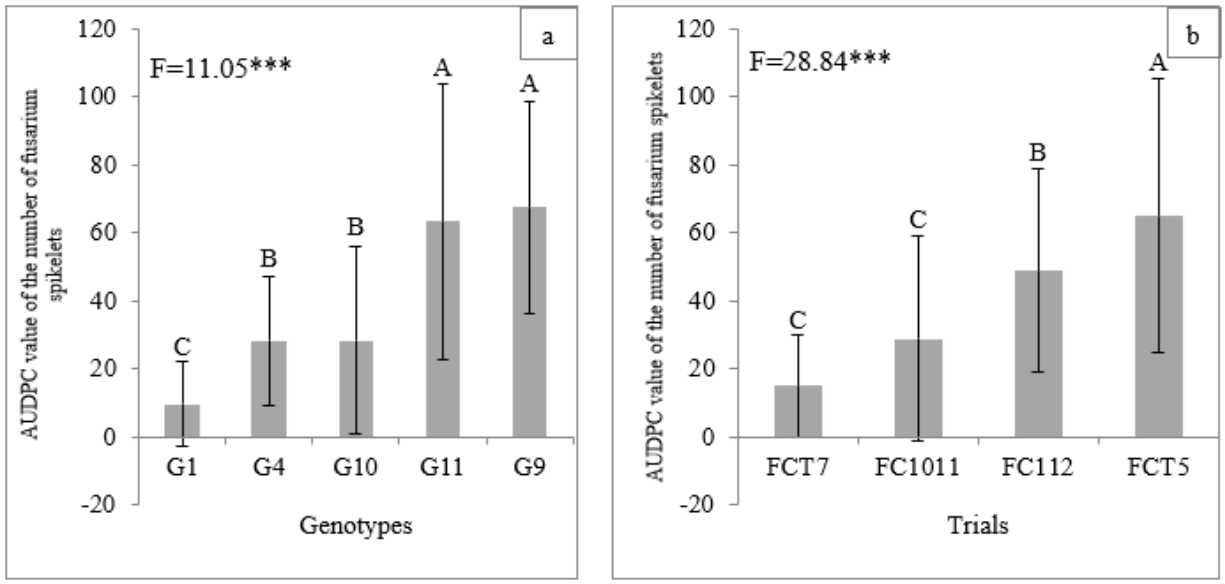


Fig. 4: Average AUDPC values of the number of *Fusarium* infected spikelets

recorded very high AUDPC values (67.57 and 63.49); the G4 and G10 genotypes marked AUDPCs intermediate between the resistant and the susceptible, namely 28.23 and 28.64 respectively. It is therefore clearly established that the G1 line behaves resistant to the progression of symptoms after inoculation. This variability in the behavior of durum wheat genotypes is most likely the result of the presence or absence of genes for resistance to this pathogen, but also the presence or absence of virulence or non-virulence genes in the pathogen. On the pathogenic side, the four isolates show different aggressiveness indicating that they differ in their pathogenicity. In many studies, the assessment of the severity of the disease in the field is essentially based on the calculation of the values of the AUDPC (Hadjout, 2013, Hadjout et al., 2017).

3.4 EFFECT OF DIFFERENT *FUSARIUM* ISOLATES AND SPECIES ON THOUSAND GRAIN MASS (TGM)

Analysis of variance integrating all trials showed the effects of genotypes and trials to be statistically significant ($p < 0.001$), while the genotypes / trials interaction showed a non-significant effect ($p > 0.05$) (Fig. 5 a, b). Analysis of the losses of the main component of yield showed that the different isolates affect all genotypes by decreasing TGW. According to our results, it is the susceptible varieties G9 and G11 which recorded the greatest losses in TGM (44.37 g and 45.30 g) followed just after by the moderately resistant variety G10 (47.41 g). The treatments affected the G1 (resistant) and G4 (moderately resistant) lines with relatively very low losses, namely 48.68 g and 54.82 g respectively, this reflects their good level of resistance, probably those of type II linked to the progression of the pathogen in the ear. The fact remains that the G10 variety showed more losses (47.41 g) than the two lines, something which was observed in previous work by Hadjout (2013). In addition, the symptoms observed explained part of the losses in TGM, this is in agreement with current knowledge on the epidemiology of fusarium head blight. The fact that the pathogen develops after the flowering stage, at the onset of the disease, the number of kernels per ear is already fixed, while the kernel filling has only just begun. The disease therefore affects this parameter and results in a large drop in TGM, especially in susceptible varieties (G9 and G11). The work of Gate et al. (1991) showed that a low TGM can be the result of end-of-life diseases (fusarium wilt), or late rains associated with high heat, and to a lesser extent with lodging. Fusarium head blight reduces grain yield and quality at the end of the crop's growth cycle, when non-diseased wheat kernels normally develop into fleshy, healthy kernels (McMullen et al., 2012).

4 CONCLUSIONS

The growing interest of the cereal sector for the sanitary quality of grains and particularly for mycotoxin contamination, strongly increases the demand for productive genotypes that accumulate few mycotoxins in their grains. In the absence of reliable information on the ability of genotypes to limit the accumulation of these

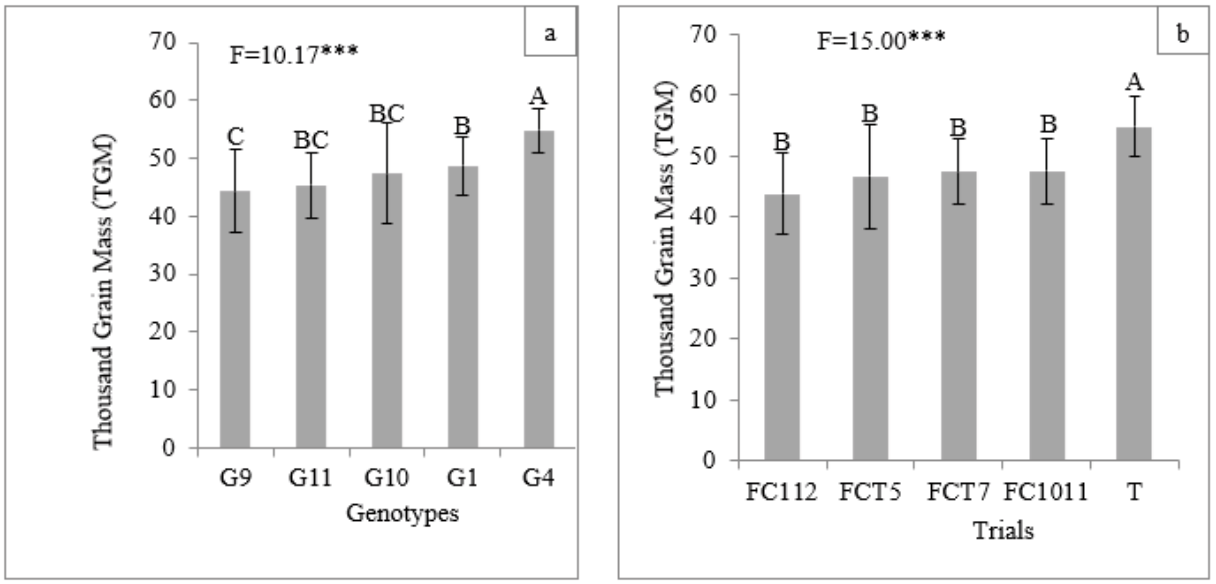


Fig. 5: Thousand grain mass for each genotype and in each trial

molecules, attention is focused on finding varieties with a good level of resistance to fusarium head blight. Indeed, the use of resistant genotypes linked to good agronomic practices remains the most satisfactory solution for farmers. Therefore, our study falls within the overall framework of the genetic control against fusarium head blight and this by the selection of genotypes resistant to the disease. To this end, the behavior of tested durum wheat genotypes with respect to fusarium head blight is evaluated under open field conditions. This behavior indicates that the G1 line exhibits longer incubation times, lower AUDPC values and thus exhibiting low disease yield losses compared to other genotypes and therefore it is of interest from a standpoint seen resistance to the appearance of the first symptoms and to the rate of spread of the fungus inside the ear.

Our results open up very important research perspectives on fusarium head blight in Algeria, in particular the search for mycotoxins as possible causes of poorly understood human diseases and the factors that contribute to their accumulation in grains.

5 REFERENCES

Bilska, K., Kulik, T., Ostrowska-Kołodziejczak, A., Buśko, M., Pasquali, M., Beyer, M., Baturo-Cieśniewska, A., Juda, M., Zaluski, D., Treder, K., Denekas, J., & Perkowski, J. (2018). Development of a highly sensitive FcMito qPCR assay for the quantification of the toxigenic fungal plant pathogen *Fusarium culmorum*. *Toxins*, *10*(5), 211. https://doi.org/10.3390/toxins10050211

Bouanaka, H., Bellil, I., Harrat, W., Boussaha, S., Benbelkacem, A., & Khelifi, D. (2021). On the biocontrol by *Trichoderma afroharzianum* against *Fusarium culmorum* responsible of Fusarium head blight and crown rot of wheat in Algeria. *Egyptian Journal of Biological Pest Control*, *31*(1), 1-13. https://doi.org/10.1186/s41938-021-00416-3

Burrows, M., Grey, W., & Dyer, A. (2008). *Fusarium head blight (scab) of wheat and barley.* Montana State University Extension, MT200806AG. Bozeman, Montana.

Dweba, C. C., Figlan, S., Shimelis, H. A., Motaung, T. E., Sydenham, S., Mwadzingeni, L., & Tsilo, T. J. (2017). Fusarium head blight of wheat: Pathogenesis and control strategies. *Crop Protection*, *91*, 114-122. https://doi.org/10.1016/j.cropro.2016.10.002

Gate, P., Dagneaud, J., & Vignier, L. (1991). Bilan climatique des céréales: Principaux faits marquants et comportement variétal. *Revue Perspecties Agricoles*, *163*, 77-86.

Ghimire, B., Sapkota, S., Bahri, B. A., Martinez-Espinoza, A. D., Buck, J. W., & Mergoum, M. (2020). Fusarium head blight and rust diseases in soft red winter wheat in the southeast United States: state of the art, challenges and future perspective for breeding. *Frontiers in Plant Science*, *11*, 1080. https://doi.org/10.3389/fpls.2020.01080

Hadjout, S. (2013). *Etude comparative (in vitro et in situ) de quelques lignées sélectionnées de blé dur et de variétés cultivées pour leur comportement à la fusariose de l'épi causée par Fusarium culmorum (WG Sm.) Sacc. et Fusarium graminearum Schwabe*. Mém. Magis. El-Harrach, Alger, 101 p.

Hadjout, S., Chéreau, S., Atanasova-Penichon, V., Marchegay, G., Mekliche, L., Boureghda, H., Barreau, C., Touati-Hattab, S., & Richard-Forget, F. (2017). Phenotypic and biochemical characterization of new advanced durum wheat breeding lines from Algeria that show resistance to fusarium head blight and to mycotoxin accumulation. *Journal of Plant Pathology*, *99*(3), 671-680.

Hadjout, S., Chéreau, S., Mekliche, L., Marchegay, G., Ducos, C., Boureghda, H., Zouidi, M., Barreau, C., Bouznad, Z., & Richard-Forget, F. (2022). Molecular identification of some *Fusarium* isolates and their chemotypes involved in fusarium head blight on durum wheat in Algeria. *Archives of Phytopathology and Plant Protection*, *55(*4), 499-513. https://doi.org/10.1080/03235408.2022.2034363

Laraba, I., Boureghda, H., Abdallah, N., Bouaicha, O., Obanor, F., Moretti, A., Geiser, M. D., Kim, H., Mccormick, S. P., Proctor, R. H., Kelly, A. C., Ward, T. J., & O'Donnell, K. (2017). Population genetic structure and mycotoxin potential of the wheat crown rot and head blight pathogen *Fusarium culmorum* in Algeria. *Fungal Genetics and Biology*, *103*, 34-41. https://doi.org/10.1016/j.fgb.2017.04.001

Leslie, J. F., Summerell, B. A. (2006). *The Fusarium laboratory manual*. Blackwell Publishers, Ames, Iowa, USA. 388 pp. (In: Abedi-Tizaki, M., Sabbagh, S. K. (2012). Morphological and molecular identification of *Fusarium* head blight isolates from wheat in north of Iran. *Australian Journal of Crop Science*, *6*(9), 1356-1361). https://doi.org/10.1002/9780470278376

Lindhout, P. (2002). The perspectives of polygenic resistance in breeding for durable disease resistance. *Euphytica*, *124*(2), 217-226. https://doi.org/10.1023/A:1015686601404

McMullen, M., Bergstrom, G., De Wolf, E., Dill-Macky, R., Hershman, D., Shaner, G., & Van Sanford, D. (2012). A unified effort to fight an enemy of wheat and barley: Fusarium head blight. *Plant Disease*, *96*(12), 1712-1728. https://doi.org/10.1094/PDIS-03-12-0291-FE

Mekliche, A., Dahlia, F., & Hanifi-Mekliche, L. (2013). Agro-morphological diversity and stability of durum wheat lines (*Triticum durum* Desf.) in Algeria. *Acta Agronomica Hungarica*, *61*(2), 149-159. https://doi.org/10.1556/AAgr.61.2013.2.6

Mesterházy, Á., Bartók, T., Mirocha, C. G., & Komoroczy, R. (1999). Nature of wheat resistance to fusarium head blight and the role of deoxynivalenol for breeding. *Plant Breeding*, *118*(2), 97-110. https://doi.org/10.1046/j.1439-0523.1999.118002097.x

Miedaner, T., Moldovan, M., & Ittu, M. (2003). Comparison of spray and point inoculation to assess resistance to fusarium head blight in a multienvironment wheat trial. *Phytopathology*, *93*(9), 1068-1072. https://doi.org/10.1094/PHYTO.2003.93.9.1068

Michel, V. (2001). La sélection de variétés de blé et de triticale résistantes aux maladies. *Revue Suisse d'Agriculture*, *33*(4), 133-140.

Mielniczuk, E., & Skwaryło-Bednarz, B. (2020). Fusarium head blight, mycotoxins and strategies for their reduction. *Agronomy*, *10*(4), 509. https://doi.org/10.3390/agronomy10040509

Moreno-Amores, J., Michel, S., Miedaner, T., Longin, C. F. H., & Buerstmayr, H. (2020). Genomic predictions for fusarium head blight resistance in a diverse durum wheat panel: An effective incorporation of plant height and heading date as covariates. *Euphytica*, *216*(2), 1-19. https://doi.org/10.1007/s10681-019-2551-x

Prat, N., Buerstmayr, M., Steiner, B., Robert, O., & Buerstmayr, H. (2014). Current knowledge on resistance to fusarium head blight in tetraploid wheat. *Molecular Breeding*, *34*(4), 1689-1699. https://doi.org/10.1007/s11032-014-0184-2

Qi, X., Jiang, G., Chen, W., Niks, R. E., Stam, P., & Lindhout, P. (1999). Isolate-specific QTLs for partial resistance to *Puccinia hordei* in barley. *Theoretical and Applied Genetics*, *99*(5), 877-884. https://doi.org/10.1007/s001220051308

Royo, C., Elias, E. M., & Manthey, F. A. (2009). Durum wheat breeding. In *Cereals* (pp. 199-226). Springer, New York, NY. https://doi.org/10.1007/978-0-387-72297-9_6

Saharan, M. S. (2020). Current status of resistant source to fusarium head blight disease of wheat: a review. *Indian Phytopathology*, *73*(1), 3-9. https://doi.org/10.1007/s42360-019-00186-x

Serre, F., Faure, M., Abadi, M., Desray, P., Roche, S., Joannin, M., Vinot, M. - Petit, S., & Tourvieille de Labrouhe, D. (2015). Cereal field phenotyping for Fusarium head blight by automatic image analysis [Conference poster]. In *11e Conférence Internationale sur les Maladies des Plantes, Tours, France, 7 au 9 décembre 2015 (pp. 285-294)*. Association Française de Protection des Plantes (AFPP).

Shaner, G., & Finney, R. E. (1977). The effect of nitrogen fertilization on the expression of slow-mildewing resistance in knox wheat. *Phytopathology*, *67*(8), 1051-1056. https://doi.org/10.1094/Phyto-67-1051

Tidiane Sall, A., Chiari, T., Legesse, W., Seid-Ahmed, K., Ortiz, R., Van Ginkel, M., & Bassi, F. M. (2019). Durum wheat (*Triticum durum* Desf.): Origin, cultivation and potential expansion in Sub-Saharan Africa. *Agronomy*, *9*(5), 263. https://doi.org/10.3390/agronomy9050263

Touati-Hattab, S., Barreau, C., Verdal-Bonnin, M. N., Chereau, S., Richard-Forget, F., Hadjout, S., Mekliche, L., & Bouznad, Z. (2016). Pathogenicity and trichothecenes production of *Fusarium culmorum* strains causing head blight on wheat and evaluation of resistance of the varieties cultivated in Algeria. *European Journal of Plant Pathology*, *145*(4), 797-814. https://doi.org/10.3390/agronomy9050263

Trottet, M., & Saur, L. (1994). *Effet des septorioses et de la fusariose de l'épi sur l'élaboration du rendement et sur la qualité de quelques variétés de blé tendre*. Compte rendu de la réunion scientifique du groupe céréales de l'INRA, Dijon.

USDA (United States Department of Agriculture). 2005. (http://www.fas.usda.gov/pecad/highlights/2005/07/durum2005/).

Wachowska, U., Kucharska, K., Pluskota, W., Czaplicki, S., & Stuper-Szablewska, K. (2020). Bacteria associated with winter wheat degrade *Fusarium* mycotoxins and triazole fungicide residues. *Agronomy*, *10*(11), 1673. https://doi.org/10.3390/agronomy10111673

Xynias, I. N., Mylonas, I., Korpetis, E. G., Ninou, E., Tsaballa, A., Avdikos, I. D., & Mavromatis, A. G. (2020). Durum wheat breeding in the Mediterranean region: Current status and future prospects. *Agronomy*, *10*(3), 432. https://doi.org/10.3390/agronomy10111673

Yekkour, A., Toumatia, O., Meklat, A., Verheecke, C., Sabaou, N., Zitouni, A., & Mathieu, F. (2015). Deoxynivalenol-producing ability of *Fusarium culmorum* strains and their impact on infecting barley in Algeria. *World Journal of Microbiology and Biotechnology*, *31*(6), 875-881. https://doi.org/10.1007/s11274-015-1841-2

doi:10.14720/aas.2022.118.3.2599 Original research article / izvirni znanstveni članek

Flight activity of *Bactrocera oleae* (Rossi, 1790) (Diptera: Tephritidae) infesting two Algerian olive varieties in north-west Algeria

Zineb BOURAKNA [1, 2], Kada RIGHI [1], Fatiha ASSIA [1], Abdelkader ELOUISSI [1]

Received March 08, 2022; accepted September 16, 2022.
Delo je prispelo 8. marca 2022, sprejeto 16. september 2022

Flight activity of *Bactrocera oleae* (Rossi, 1790) (Diptera: Tephritidae) infesting two Algerian olive varieties in north-west Algeria

Abstract: *Bactrocera oleae* (Rossi, 1790) (Diptera: Tephritidae) is the most dangerous insect pest of the olive tree in the Mediterranean region. This study was conducted in the Mascara region (North-West Algeria) during 2019-2020 season, in order to monitoring the flight activity of *B. oleae* by using Mc Phail type traps and evaluating the infestation rate on two olive varieties (Sigoise and Chemlal) by fruits sampling. The data obtained indicated that the flight activity of *B. oleae* developed five peaks of the abundance. The General Linear Model (GLM) showed that infestation rate and fruit caliber varied considerably among varieties and across the sampling date, which gradually increased with time. 'Sigoise' having the highest caliber and was more infested than 'Chemlal'. The northern cardinal orientation of the tree was the least attacked by this pest. The GLM function showed that there was relationship between the infestation rate and fruit size.

Key words: *Bactrocera oleae*; flight activity; infestation; caliber; 'Sigoise'; 'Chemlal'

Let oljčne muhe (*Bactrocera oleae* (Rossi, 1790), Diptera: Tephritidae) na dveh alžirskih sortah oljke v severozahodni Alžiriji

Izvleček: Oljčna muha (*Bactrocera oleae* (Rossi, 1790), Diptera: Tephritidae) je najškodljivejša žuželčja vrsta na oljkah v Sredozemlju. Raziskava je bila izvedena na območju Mascare (severozahodna Alžirija) v rastni dobi 2019-2020, z namenom načrtnega spremljanja leta oljčne muhe z uporabo Mc Phailovih pasti in ovrednotenja stopnje napada dveh sort oljke ('Sigoise' in 'Chemlal') z vzorčenjem plodov. Pridobljeni podatki nakazujejo, da je imela oljčna muha pet vrhov pojavljanja. Splošni linearni model je pokazal, da sta se stopnja napada in debelina plodov znatno spreminjala glede na sorto in datum vzorčenja in sta s časom naraščali. Sorta Sigoise je imela najdebelejše plodove in je bila bolj napadena kot sorta Chemlal. Na sever orientirani deli krošenj so bili najmanj napadeni. Splošni linearni model je pokazal, da obstaja povezava med stopnjo napada oljčne muhe in debelino plodov oljk.

Ključne besede: *Bactrocera oleae*; aktivnost izletov; napadi; debelina plodov; 'Sigoise'; 'Chemlal'

1 Biology Systems and Geomatics Laboratory, Department of Agronomy, Faculty of Natural Sciences and Life, Mascara University, Algeria
2 Corresponding author, e–mail: Zineb.bourakna@univ-mascara.dz

1 INTRODUCTION

Algeria is one of the main olive (*Olea europaea* L., Oleaceae) producing countries. In 2019 it took the ninth class in world olive production with a production of 868,754 tons on an area of 431,634 ha (FAO Stat, 2021). Algerian olive oil production was 90,000 tons in the 2020/2021 campaign. This crop is attacked by various pests and diseases. The olive fly *Bactrocera oleae* (Rossi, 1790) (Diptera: Tephritidae), is the most serious and economically harmful insect pest of commercial olive production worldwide (Ras et al., 2017; Torrini et al., 2020). This fly is multivoltine and homodynamic, i.e. their population dynamics, number of generations and the length of their life cycles depend mainly on the climate (temperature and humidity), but also vary according to other factors: geographic regions , availability and quality of olive fruits (Daane & Johnson, 2010; Malheiro et al., 2015; Pertíñez & Vélez, 2020). This pest causes the severe qualitative and quantitative damage, where economic losses can reach 100% due to uncontrolled infestation and oil losses of up to 80% (Rice, 2000; Genç & Nation, 2008; Zalom et al., 2009). Also, the formation of tunnels inside mesocarp and exit holes allowing the introduction of bacteria and fungi that rot the fruit and increase the acidity of the oil (Athar, 2005; Zalom et al., 2009). The infestation of olives caused by *B. oleae* varies greatly between years, regions and olive varieties (Goncalves et al., 2012). Gaouar and Debouzi (1991) found that the level of infestation was quite high near 100% in orchards close to the coast, in the province of Tlemcen (North West Algeria) on two local varieties (Sigoise and Chemlal). Other authors have also shown a fruit infestation level of up to almost 100% in Portugal (Bento et al., 2009) and in California (Burrak et al., 2011).

The preference and sensitivity of olive cultivars by the *B. oleae* vary by three factors: physical, chemical and molecular. The physical factor remains the most influencing, which includes size, mass, volume, fruit color and hardness of the exocarp (Malheiro et al., 2015). The female of *B. oleae* prefers to oviposite on cultivars with large, unripe olives (Neuenschwander et al., 1985). Several studies (Burrack & Zalom, 2008; Goncalves et al., 2012; Garantonakis et al., 2017; Medjkouh et al., 2018) have confirmed that the oviposition preference by the female *B. oleae* was positively correlated with the maturity index, mass and volume on the other hand oviposition was negatively correlated with the hardness of the exocarp.

The olive varieties studied 'Sigoise' and 'Chemlal' are two Algerian varieties renowned for their excellence in quality and productivity. In order to preserve the quantity and the marketable quality of these two varieties against the attacks of such a pest, it was imperative to determine its population dynamics and its infestation rates in relation to the size of the fruits than with the four cardinal orientations of the tree in the region of Mascara (North-West Algeria).

2 MATERIAL AND METHODS

2.1 STUDY AREA

This study was carried out in Oued Taghia region at an altitude of 471 m (35 ° 6 '35 "N, 0 ° 5' 19" E) in the province of Mascara (North-West of Algeria), during the

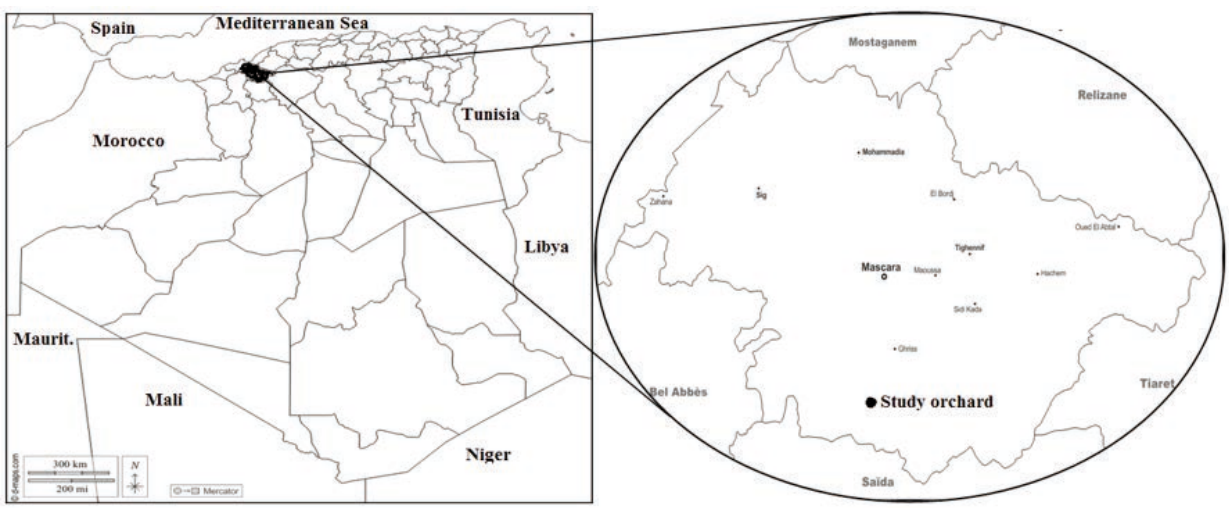


Figure 1: The geographical localization of study area (Mascara: Algeria) and the situation of study orchard

Figure 2: The study orchard (Oued Taghia, Mascara)

period that extends from June 2019 to April 2020. The study region is characterized by a semi-arid, dry and cold climate, far from the sea by a distance of about 120 km (Figure 1). The olive orchard has been planted with two varieties, Sigoise (intended for the production of table olives) and Chemlal (intended for the production of oil). The trees were medium in size, about 16 years old, and were spaced about 10 m × 8 m, being irrigated artificially by gravity and pruned bi-annually. The olive orchard has not received any treatment against diseases and pests for the past three years, but chemical fertilizers are applied every winter (Figure 2).

2.2 SAMPLING METHODOLOGY

The flight activity of adults of *B. oleae* was monitored using 4 plastic Mc Phail traps with a transparent upper half and a yellow lower half, baited with a 3 % aqueous solution of di ammonium phosphate which is attractive to both sexes. The traps are installed at the beginning of June 2019. The solution has been renewed every 10 to 20 days. The traps were tied under the shade of the branches inside the foliar crown in the southwest direction of the tree, at a human height. The traps were distributed randomly in the olive orchard with 50 m distance between them. Which were checked every 10 days and the olive flies were counted, sexed and removed. The total number of individuals captured in the McPhail traps was used to estimate the population index (Pi) which was expressed as the total number of captures per trap per day in each date (Goncalves et al., 2012). Sex ratio was estimated by the ratio (male / total and female / total).

Every 10 days, from the appearance of the first stings (beginning of September which corresponds to the slight drop in temperature and after the setting of the olives) until the harvest (end of December), fruit samples were taken from 5 trees of each variety of olive tree, to assess the infestation rate of the olive tree and the size of the fruit. 40 olives per tree were harvested at head height from 4 cardinal orientations of each tree (north, south, east, and west), due to 10 olives for each orientation. The olives collected were brought to the laboratory and were observed under a binocular stereo-microscope (EUROMEX, The Netherlands) to check for the presence of oviposition stings and exit holes insect. The *B. oleae* infestation rate was expressed as a percentage of the infested olives relative to the total number of olives collected. According to Burrack et al. (2011), olives with oviposition stings were considered infested.

To estimate the caliber of the fruit, 50 olives were chosen by chance for each variety (10 olives per tree). Using a digital caliper (OEM, China), the widest dimension was measured in mm.

2.3 DATA ANALYSIS

The statistical software SPSS (version 21) was used to analysis the data on infestation rate and fruit caliber with General Linear Model (GLM): Repeated Measures with "variety" and "sampling date" as effects. ANCOVA was used to study the effect of cardinal orientation on infestation rate in both varieties. Tukey post-hoc test was applied to compare the infestation rate of different cardinal orientations. The GLM function in R environment (R Core Team, 2021) was used to build the relation between the infestation rate and the fruits caliber. The significance level for all analyses was 0.05.

3 RESULTS

3.1 POPULATION DYNAMICS OF THE OLIVE FLY AND ENVIRONMENTAL CONDITIONS

Olive fly flight activity was distributed throughout the year (Figure 3). The dynamic of adult flights was showed five major peaks, which correspond to the number of generations. The first flies in our study area were captured on 23/06/2019 with a Pi population index of 0.12 flies / trap /day. So that, the first peak appeared on 13/07/2019. From this date, the number of individuals decreased and coincided with the increasing in temperature and the falling in humidity (summer period). In September, the population returns to increase relatively with the decreasing in temperature and the increasing in humidity, forming a succession of 3 autumn-winter generations; September 03 (0.70 flies / traps / day), November 03 (2.4

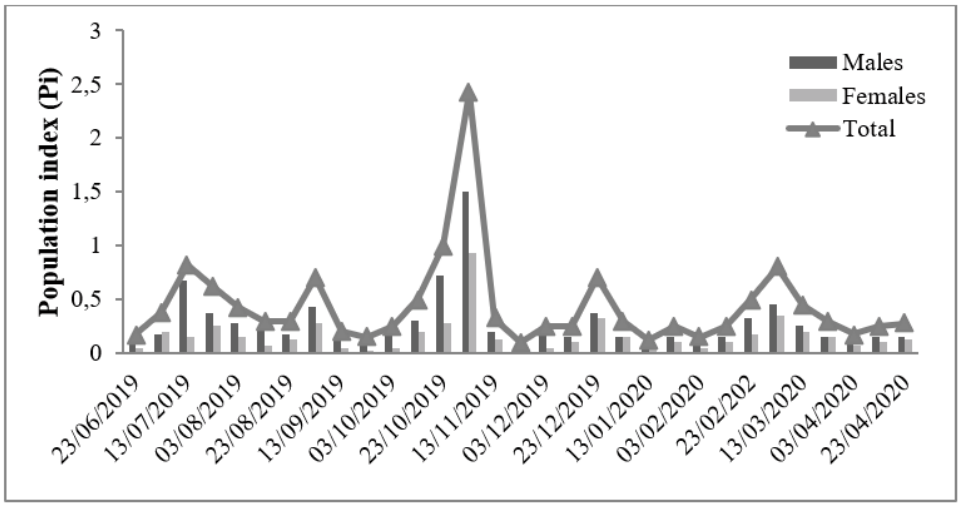


Figure 3: Population indexes Pi (total, male and female), during the study period

flies / traps / day), December 23 (0.70 flies / traps / day) respectively. The value of the population index decreased from the end of December to February, where the temperature in this period is ≤ 10 °C which corresponds as a limiting factor (Fig 3 and 4). The 5th generation is spring generation that appeared at 03/03/2020 (0.80 flies / traps / day). The sex ratio of captured flies was constantly in favor of males (0.63 males and 0.37 females).

3.2 INFESTATION RATE

Infestation rate differed significantly between varieties and across the sampling date as well as the interaction of them (Table 1). Infestation rate of 'Sigoise' was higher than that of 'Chemlal' (Figure 5). At the beginning of September, the infestation rate was low (11.50 ± 1.66 %, 7.00 ± 1.27 %) respectively for the two varieties Sigoise and Chemlal. As of October 03, the infestation increased for 'Sigoise' variety, while for 'Chemlal', the increase of the infestation was moderate. In December, the infestation in 'Chemlal' was intensified and reached high value at the time of harvest (78 %), which is near to 'Sigoise' infestation rate (84 %) (Figure 5).

Fruit calibers differed significantly among varieties, the sampling date and the interaction between the two factors (Table 1). The fruit caliber was higher in 'Sigoise' than 'Chemlal' throughout the study period. We noted a rapid increase of caliber in 'Sigoise' variety and a slight increase in 'Chemlal' variety (Figure 6).

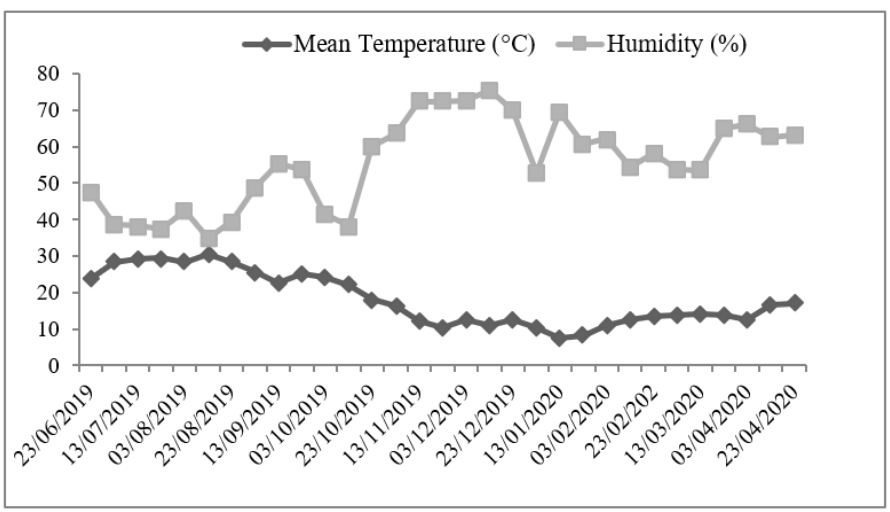


Figure 4: Average daily data of the temperature and relative humidity from June 23, 2019 to April 23, 2020 in the Mascara region

Table 1: Effects of variety and sampling date on infestation rate and fruits caliber for the year 2019 (GLM: Repeated measures)

Variable	Factor	*df*	*F*	*p*
Infestation rate	Sampling date	6.46	238.02	<0.0001
	Variety	1	26.28	<0.0001
	Interaction	6.46	7.79	<0.0001
Fruit caliber	Sampling date	10.16	295.62	<0.0001
	Variety	1	1743.06	<0.0001
	Interaction	10.16	49.87	<0.0001

In order to study the influence of the fruit size factor on both varieties susceptibility to *B. oleae*, a relationship was estimated between the infestation rate and the caliber of the fruits by the GLM function in R (R Core Team, 2021). The results showed that size coefficient is highly significant (p <0.001), while the variety coefficient is not significant (p = 0.14). This mean that the infestation rate is not linked to the variety but to the size. The relation is written in the following form:

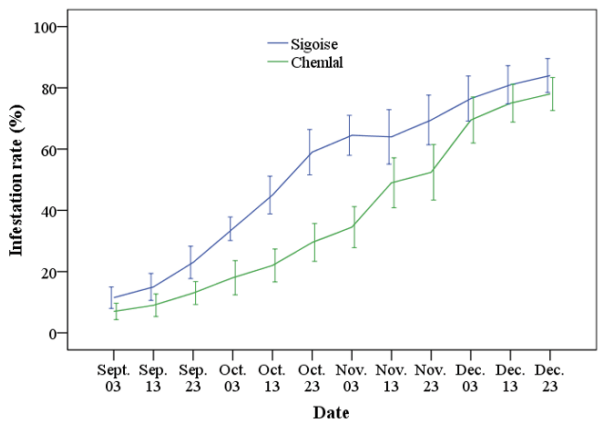


Figure 5: Infestation rate (mean ± S.E) of the two varieties by the olive flies (September to December 2019)

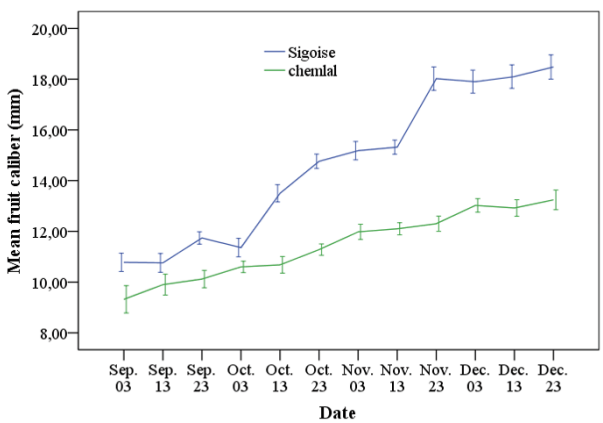


Figure 6: Mean fruit caliber (mean ± S.E) of the two varieties by the olive flies (September to December 2019)

*Infestation rate = -15.414 + -2.32 * Variety + 5.17 * Size* (1)

On the other hand, ANCOVA analysis revealed that there is a significant difference of the infestation between the cardinal orientations of the tree throughout the study period (F = 44.03, df = 3, p = 0.006) and between varieties (F = 111.28, df = 1, p = 0.002), while their interaction did not found significant for infestation (F = 0.29, df = 3, p = 0.83). Posthoc tests of Tukey's confirmed that the North direction is the least infested by the olive fly in both varieties, but there is no significant difference between other orientations (East, South and West) (Figure 7).

4 DISCUSSION

4.1 STUDY OF THE POPULATION DYNAMICS OF THE OLIVE FLY

The presence and fluctuation of the fly throughout the year are well demonstrated by our results with an important number of generations (five peaks per year), this latter depends on several factors, mainly the climate which is closely linked to the longevity of this pest, fruits

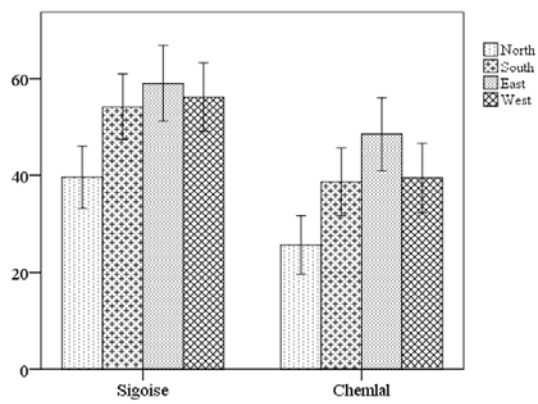


Figure 7: Variations in the infestation rate (mean ± E.S) of olives in relation to cardinal orientation of the tree throughout the study period

damaged which remain after harvest which ensures the maintaining and continuity of the species in the orchards (Jimenez et al., 1994). Daane & Johnson (2010) claim that even if the olive tree is unsuitable for oviposition, adults have the ability to reproduce and survive when their nutrition is available, which constitutes a true danger to olive orchard.

In the study area, the first generation was reported in July. Also, Gaouar (1996) in the Tlemcen region in Algeria, Yokoyama et al. (2006) in southern California, Goncalves et al. (2012) in Portugal, Ait Mansour et al. (2015) in Morocco and Pertíñez & Vélez (2020) in Madrid (Spain) found a generation was marked at the end of June or the beginning of July in the olive orchards close to the sea (fresh and humid), however in inland areas which far of coastline, the summer generation was absent. This can be explained by the hot, arid conditions and the unavailability of fruit. We reported the second generation on September 03, 2019 where the temperature and humidity conditions become ambient also the receptive olives are available and premature. It was followed by the third at the beginning of November and the fourth at the end of December. This succession of three generations in the autumn was similar to the results of Goncalves et al. (2012) and Ait Mansour et al. (2015). However, Gaouar (1996) and Yokoyama et al. (2006) found two generations in this period and Pertíñez & Vélez (2020) found only one generation in the fall. This overlap of generations was explained by the contribution of each generation to the coexistence of the future generation. The fourth generation in our result was absent in most of the studies, this can be explained by the late harvest of the fruits until the end of December. Generally according to several researchers, in most regions, autumn is the season best suited to the development of the olive fly, when its larval food is available (Daane & Johnson, 2010). Besides, Yokoyama et al. (2006) explained that the unusually large number of adults captured from March to April is due to the presence of fruits in the orchard of the previous year, which provides oviposition sites and food for the development of this pest. This ascertainment explains well and justifies the appearance of the fifth generation (March 03, 2020) in our study region. Also, the population density is closely related to climatic conditions (temperature and humidity). According to Marchi et al. (2016), interannual variations of the population are explained by temperature and according to Broufas et al. (2009) Relative humidity can lead to increased longevity of the fly and the fertility of their females. Concerning the study of the sex ratio, it was noticed from the results that the number of catches of males was greater than that of females (0.63 males and 0.37 females), this can be justified by the color yellow traps and nature of bait. This ascertainment is similar to that of Katsoyannos & Kouloussis (2001) who explain that catches are strongly influenced by the color of the traps, where he reported that males of olive flies are attracted to the yellow, orange and white color traps, while females by the colors red and black. Rice et al. (2003) revealed that traps baited only with ammonium bicarbonate, more male than female flies were collected.

4.2 INFESTATION RATE

The fruit infestation started on September 03, 2019 where the olives reached the fruit enlargement and stone hardening stage, which is considered the receptive stage for oviposition olive fly (Civantos, 1999), thus coinciding with the period of ovarian maturation of females (Tzanakakis, 2003). Our results are similar to those of (Ibnsouda et al., 2004; Goncalves et al., 2012). The significant increase of the infestation over time was justified by the increase in the number of captures. Pertíñez & Vélez (2020) mentioned that the proportional increase of damage was caused by the increase of the population size, while all reductions in population size maintained the total amount of damaged olives. The study of the influence of the cardinal orientation of the tree on the level of infestation revealed that the northern cardinal orientation of the tree is less attacked by the fly, according to Goncalves et al. (2012) the olive fly prefers to oviposite on the coldest areas of the tree. While Gaouar & Debouzi (1991) indicated that the cardinal orientation in olive trees did not influence the infestation.

Not only the number of captures by olive flies is responsible for of the damage importance to the olive tree, but also the different aspects of cultivars play an important role in their susceptibility to oviposition. The olive flies preference for oviposition appears to lie in the interaction and correlation of three aspects: physical, chemical and molecular (Malheiro et al., 2015). Certain physical characteristics of fruits including color, elongation, hardness and volume affect their susceptibility to this pest (Rizzo et al., 2012). Several studies have evaluated a good correlation between fruit size and olive tree infestation (Neuenschwander & Michelakis, 1979; Burrak & Zaloum, 2008; Rizzo et al., 2012; Garantonakis et al., 2017; Medjkouh et al., 2018). We have reported in our case that there is an important relationship between fruit size and infestation rate. The difference between the infestations of the two varieties was justified by the sensitivity of the table variety (Sigoise) which has a larger size than that of the 'Chemlal' variety with small fruits and high oil content, where this latter is less infested. Our results are in agreement with those of Jerraya et al. (1982), Arambourg (1984) and of Gaouar & Debouzi (1991). A

similar infestation rate in the two varieties studied was observed at the end of the season (84.00 ± 2.65 % 'Sigoise', 78.00 ± 2.57 % 'Chemlal'), despite that the fruits calibers are different (18.48 ± 0.24 mm for the 'Sigoise' and 13.24 ± 0.19 mm for 'Chemlal'). These rates which seem the same important mark the third generation with a number of adults which reaches its maximum (2.4 flies / traps / day), we can say that the adults of this generation could oviposite their eggs in almost all the fruits not infested (regardless of caliber) (Gaouar & Debouzi, 1991).

5 CONCLUSION

The trapping of the olive fly adults allowed us to determine their population dynamic and to evaluate the number of generations in Mascara region, this pest is present throughout the year with five generations. The overlapping of the autumn generations causes important damage to the olives. The evaluation of the infestation rate showed that at the beginning of the season, the olives of the Sigoise variety are more attacked by *B. oleae* than the olives of 'Chemlal'. This difference remain linked to the size of the fruits, where the Sigoise variety had large caliber olives (table variety) compared to the small-fruited Chemlal variety (olive intended for oil). However, at the end of the season, despite the two varieties were different in the size of their olives, but the infestation rate is high for both.

The knowledge the dynamics of *B. oleae* populations and the determination the level of infestation that can inflict on different olive varieties remains the key to obtain better integrated control strategy against such parasites in an area as important as Mascara (Algeria) recognized by its olive vocation and its national and international fame.

6 ACKNOWLEDGEMENTS

The authors are grateful to the olive orchard owner who permitted access for this study, and to Professor A. Hammimed (Mascara University) who provided us with climatic data for the study region. My thanks also to my colleague Elhouacine Houcine for his invaluable help during the experiment.

7 REFERENCES

Ait-Mansour, A., Kahime, K., Chemseddine, M. & Boumezzough, A. (2015). Study of the population dynamics of the olive fly *Bactrocera oleae* Rossi. (Diptera, Tephritidae) in the region of Essaouira. *Open Journal of Ecology, 5*, 174-186. https://doi.org/10.4236/oje.2015.55015

Arambourg, Y. (1984). La faune entomologique de I 'olivier. *Olivae, 4*, 14-37.

Athar, M. (2005). Infestation of olive fruit fly, *Bactrocera oleae*, in California andtaxonomy of its host trees. *Agriculturae Conspectus Scientificus, 70* (4), 135-138.

Bento, A., Pereira, J. A., Cabanas, J., Pinto, A. & Torres, L. (2009). Sensibility of different olive cultivars to infestations by the olive fly, *Bactrocera oleae*, and the olive moth, *Prays oleae*. *Actas Portuguesas de Horticultura, 13*, 134-140.

Broufas, G. D., Pappas, M. L. & Koveo,s D. S. (2009). Effect of relative humidity on reproduction, longevity and ovarian maturation of the tephritid fly *Bactrocera oleae*. *Annals of the Entomological Society of America, 102*, 70-75. https://doi.org/10.1603/008.102.0107

Burrack, H. J., & Zalom, F. G. (2008). Olive fruit fly (Diptera: Tephritidae) ovipositional preference and larval performance in several commercially important olive varieties in California. *Journal of Economic Entomology, 101*(3), 750–758. https://doi.org/10.1603/0022-0493(2008)101[750:OFFDTO]2.0.CO;2

Burrack, H. J., Bingham, R., Price, R., Connell, J. H., Phillips, P. P., Wunderlich, L., Vossen, P. M., O'Connell, N. V., Ferguson, L. & Zalom, F. G. (2011). Understanding the seasonal and reproductive biology of olive fruit fly is critical to its management. *California Agriculture, 65*, 14-20. http://dx.doi.org/10.3733/ca.v065n01p14

Civantos, M. L. (1999). *Olive pest and disease management*, Collection: Practical Handbooks. International Olive Oil Council, Madrid, 207 p.

Daane, K. M., & Johnson, M. W. (2010). Olive fruit fly: Managing an ancient pest in modern times. *Annual Review of Entomology*, *55*(1), 151–169. https://doi.org/10.1146/annurev.ento.54.110807.090553

FAO. (2021). consulted [10/10/2021]. http://http://www.fao.org/faostat/en/#rankings/commodities_by_country

Gaouar, N. (1996). *Apport de la biologie des populations de la mouche de l'olive Bactrocera (=Dacus) olaea (Gmelin) à l'optimisation de son contrôle dans la région de Tlemcen*. Ph D thesis, Biology institute, Tlemcen University, Algeria.

Gaouar, N., & Debouzie, D. (1991). Olive fruit fly *Dacus oleae* Gmel (Diptera: Tephritidae) damage in Tlemcen region Algeria. *Journal of Applied Entomology, 112*, 288-297. http://dx.doi.org/10.1111/j.1439-0418.1991.tb01058.x

Garantonakis, N., Varikou, K. & Birouraki, A. (2017). Influence of olive variety on biological parameters of *Bactrocera oleae* (Diptera: Tephritidae). *Applied Entomology and Zoology*, *52*, 189–196. https://doi.org/10.1007/s13355-016-0467-7

Genç, H., & Nation, J. L. (2008). Maintaining *Bactrocera oleae* (Gmelin) (Diptera: Tephritidae) colony on its natural host in the laboratory. *Journal of Pest Science, 81*, 167–174. https://doi.org/10.1007/s10340-008-0203-3

Goncalves, M. F., Malheiro, R., Casal, S., Torres, L. & Pereira, J. A. (2012). Influence of fruit traits on oviposition preference of the olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), on three Portuguese olive varieties (Cobrancosa, Madural and Verdeal Transmontana). *Scientia Horticulturae, 145*, 127–135. https://doi.org/10.1016/j.scienta.2012.08.002

Ibnsouda-Koraichi, S., El-Haidani, A., Khila, A., Houari, A., Haggoud, A. & Vincent, A. (2004). Study of the olive fruits infestation by *Bactrocera oleae*in the area of Fez in Morocco and their fertility in the laboratory. *Moroccan Journal of Biology, 1*, 21-31.

Jarraya, A., Jardak, T., Khalif, M. & Germazi, T. (1982). La mouche de l'olivier *Dacus oleae* (Gmel) Dipt., Tephritidae) et son impact sur la production oléicole dans la région de Sfax. Les *Annanles de l'INRAT, 55***,** 5-55.

Jiméne, A., Castillo, E. & Esteban-Durán, J. R. (1994). Niveles de población larvaria de *Bactrocera (Dacus) oleae* Gmelin, descendientes de hembras en presencia de bajas cantidades de frutos receptivos. *Boletín de Sanidad Vegetal. Plagas, 20*(1), 119-130.

Katsoyannos, B. I., & Kouloussis, N. A. (2001). Captures of the olive fruit fly *Bactrocera oleae* on spheres of different colours. *Entomologia Experimentalis et Applicata, 100*, 165–172. https://doi.org/10.1046/j.1570-7458.2001.00860.x

Malheiro, R., Casal, S., Baptista, P. & Pereira, J. A. (2015). A review of *Bactrocera oleae* (Rossi) impact in olive products : From the tree to the table. *Trends in Food Science and Technology, 44*, 226-242. https://doi.org/10.1016/j.tifs.2015.04.009

Marchi, S., Guidotti, D., Ricciolini, M. v Petacchi, R. (2016). Towards understanding temporal and spatial dynamics of *Bactrocera oleae* (Rossi) infestations using decadelong agrometeorological time series. *International Journal of Biometeorology, 60*(11), 1681-1694. https://doi.org/10.1007/s00484-016-1159-2.

Medjkouh, L., Costa, A., Tamendjari, A., Bekdouche, F., Bouarroudj, K. & Oliveira, M. B. P. P. (2018). Susceptibility of eight Algerian olive cultivars to *Bactrocera oleae* infestation – a pomological and nutritional quality perspective. *Phytoparasitica, 46*, 595–605. https://doi.org/10.1007/s12600-018-0697-z.

Neuenschwander, P., & Michelakis, S. (1979). McPhail trap captures of *Dacus oleae* (Gmel.) (Diptera: Tephritidae) in comparison to the fly density and population composition as assessed by sondage technique in Crete, Greece. *Bulletin de la Societe Entomologique Suisse, 52*, 343–357.

Neuenschwander, P., Michelakis, S., Holloway, P. & Berchtold, W. (1985). Factors affecting the susceptibility of fruits of different olive varieties to attack by Dacus oleae (Gmel.) (Diptera: Tephritidae). *Zeitschrift für Angewandte Entomologie*, *100*, 174–188. https://doi.org/10.1111/j.1439-0418.1985.tb02770.x

Pertíñez, Á. M., & Vélez, P. M. (2020). A *Bactrocera oleae* (Rossi) damage estimation model to anticipate pest control strategies in olive production. *Crop Protection, 137*(2), 105-281. doi: https://doi.org/10.1016/j.cropro.2020.105281

Ras, E., Beukeboom, L. W., Cáceres, C. & Bourtzis, K. (2017). Review of the role of gut microbiota in mass rearing of the olive fruit fly, *Bactrocera oleae*, and its parasitoids. *Entomologia, 164*, 237–256. https://doi.org/10.1111/eea.12609

R Core Team (2021). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rice, R. E. (2000). Bionomics of olive fruit by *Bactrocera (Dacus) oleae. University of California Plant Protection Quarterly, 10*, 1-5.

Rice, R. E., Phillips, P. A., Stewart-Leslie, J. & Sibbett, G. S. (2003). Olive fruit fly populations measured in Central and Southern California. *California Agriculture, 57*(4), 122–7. https://doi.org/10.3733/ca.v057n04p122

Rizzo, R., Caleca, V. & Lombardo, A. (2012). Relation of fruit color, elongation, hardness, and volume to the infestation of olive cultivars by the olive fruit fly, *Bactrocera oleae. Entomologia Experimentalis et Applicata*, *145*(1), 15–22. https://doi.org/10.1111/j.1570-7458.2012.01311.x

Rizzo, R., Verde, G. L., Sinacori, M., Maggi, F., Cappellacci, L., Petrelli, R., Vittori, S., Morshedloo, M. R., Fofie, N'G. B. Y. & Benelli, G. (2020). Developing green insecticides to manage olive fruit flies? Ingestion toxicity of four essential oils in protein baits on *Bactrocera oleae. Industrial Crops & Products, 143*, 1-7. https://doi.org/10.1016/j.indcrop.2019.111884

Sivropoulou, A., Haritidou, L., Vasara, E., Aptosoglou, S. & Koliais, S. (2000). Correlation of the insecticidal activity of the *Bacillus thuringiensis* A4 strain against *Bactrocera oleae* (Diptera) with the 140-kDa crystal polypeptide. *Current Microbiology, 41*, 262–266. https://doi.org/10.1007/s0028432708

Torrini, G. Mazza, G., Benvenuti, C., Simoncini, S., Landi, S., Frosinini, R., Rocchini, A. & Roversi, P. F. (2020). Entomopathogenic nematodes as potential biocontrol agents against *Bactrocera oleae* (Diptera: Tephritidae). *Biocontrol Science and Technology, 30*(1), 1-11. https://doi.org/10.1080/09583157.2020.1775177

Tzanakakis, M. E. (2003). Seasonal development and dormancy of insects and mites feeding on olive: a review. *Netherlands Journal of Zoology, 52*(2–4), 87–224. https://doi.org/10.1163/156854203764817670

Yokoyama, V. Y., Miller, G. T., Stewart-Leslie, J., Rice, R. E. & Phillips, P. A. (2006). Olive fruit fly (Diptera: Tephritidae) populations in relation to region, trap type, season, and availability of fruit. *Journal of Economic Entomology*, *99*, 2072–2079. https://doi.org/10.1093/jee/99.6.2072

Zalom, F. G., Van-Steenwyk, R. A., Burrack, H. J. & Johnson, M. W. (2009). Olive fruit fly: Integrated Pest Management for Home Gardeners and Landscape Professionals. In: *ANR, Pest notes. University of California Agriculture and Natural Resources*, p. 1-4.

doi:10.14720/aas.2022.118.3.2437 Original research article / izvirni znanstveni članek

Phytochemical analysis, antioxidant and photoprotective activities of aqueous extract of *Euphorbia retusa* Forssk. different parts from Algeria

Selwa LAHMADI [1, 2, 3], Mohamed Seif Allah KECHEBAR [1], Samira KAROUNE [1], Chawki BENSOUICI [4], Lynda GALI [4], Latifa KHATTABI [4], Hasna BOURAL [4], Amina CHOUH [4], Somia SAAD [1]

Received November 25, 2021; accepted September 17, 2022.
Delo je prispelo 25. novembra 2021, sprejeto 17. september 2022

Phytochemical analysis, antioxidant and photoprotective activities of aqueous extract of *Euphorbia retusa* Forssk. different parts from Algeria

Abstract: *Euphorbia retusa* is an endemic medicinal plant of Sahara. This study aimed to determine the total phenolic and flavonoid contents of *Euphorbia retusa* seed, capsule and leaves aqueous extracts as well as to evaluate the antioxidant and photoprotective activities. The correlations between these activities and the different contents were also performed. The antioxidant activity was estimated by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-3-ethyl benzthiazoline-6-sulfonic (ABTS) scavenging, β-carotene bleaching, cupric-reducing activity (CUPRAC) and reducing power essays. In addition, the sun protection factor (SPF) was reported for the first time and measured according to the Mansur equation. Results showed that, seeds exhibit a higher total phenolics and flavonoids contents. This organ showed the highest capacity in DPPH (IC_{50} = 50.79 ± 1.87 μg ml^{-1}), ABTS (IC_{50} < 6.25 μg ml^{-1}), β-carotene bleaching (IC_{50} < 6.25 μg ml^{-1}), reducing power ($A_{0.50}$ = 6.97 ± 0.75 μg ml^{-1}) and CUPRAC ($A_{0.50}$ = 7.64 ± 0.30 μg ml^{-1}) essays. Accordingly, seed extracts characterized by a high sun protection factor (SPF = 38.26 ± 0.07). Nevertheless, the Pearson correlation coefficients calculated show the highest positive correlation between total phenolic and flavonoids contents and photoprotective activity, while no correlations were found between SPF and other antioxidant activity. This plant could be used as alternative adjuncts in sunscreen product preparation.

Key words: *Euphorbia retusa* Forssk.; polyphenols; antioxidant activity; sun protection factor; Pearson correlation

Kemična analiza, antioksidacijska in fotoprotektivna aktivnost vodnih izvlečkov iz različnih delov vrste mlečka *Euphorbia retusa* Forssk. iz Alžirije

Izvleček: Vrsta *Euphorbia retusa* Forssk. je endemična zdravilna rastlina iz Sahare. Namen raziskave je bil določiti vsebnost celokupnih fenolov in flavonoidov v vodnih izvlečkih semen, glavic in listov te rastline kot tudi ovrednotiti njihovo antioksidacijsko in fotoprotektivno aktivnost. Pokazale so se povezave med različnimi aktivnostmi in vsebnostmi analiziranih sestavin. Antioksidacijska aktivnost je bila ocenjena na osnovi redukcijske moči snovi kot so DPPH, ABTS, bledenja β-karotena in redukcijske aktivnosti bakra (CUPRAC). Dodatno je bil prvič izmerjen zaščitni faktor pred soncem po Mansurjevi enačbi. Rezultati so pokazali, da imajo semena veliko vsebnost celokupnih fenolov in flavonoidov. Izvlečki iz semen so pokazali tudi največjo sposobnost pri uporabi DPPH (IC_{50} = 50,79 ± 1,87 μg ml^{-1}), ABTS (IC_{50} < 6,25 μg ml^{-1}), bledenju β-karotena (IC_{50} < 6,25 μg ml^{-1}), redukcijski moči ($A_{0.50}$ = 6,97 ± 0,75 μg ml^{-1}) in pri preiskusu CUPRAC ($A_{0.50}$ = 7,64 ± 0,30 μg ml^{-1}). Sorazmerno temu je bil za izvlečke semen značilen velik zaščitni faktor pred soncem. (SPF = 38,26 ± 0,07). Kljub temu, da je izračunani Pearsonov koeficient korelacije pokazal največjo pozitivno korelacijo med vsebnostjo celokupnih fenolov in flavonoidov ter aktivnostjo zaščite pred soncem ni bilo nobene korelacije med zaščito pred soncem in drugimi antioksidacijskimi aktivnostmi. Iz izledkov sledi, da bi se ta rastlina lahko uporabljala kot alternativni dodatek pri izdelavi zaščitnih pripravkov pred soncem.

Ključne besede: *Euphorbia retusa* Forssk.; polifenoli; antioksidacijska aktivnost; zaščitni faktor pred soncem; Pearsonova korelacija

1 Scientific and Technical Research Center on Arid Regions (CRSTRA), Biskra, Algeria
2 Laboratory of Ecosystems Diversity and Dynamics of Agricultural Production Systems in Arid Zones, Mohamed Khider University, Biskra, Algeria
3 Corresponding author, e-mail: lahmadisalwa@yahoo.fr
4 Biotechnology Research Center, Constantine, Algeria

1 INTRODUCTION

Recently, a high demand of natural antioxidants has increased to replace synthetic antioxidants that are known by their undesirable side effects on human health (Megdiche et al., 2013). In addition, the demand for herbal cosmetics used in sunscreens has grown rapidly also to give better protection against UV radiations which can provoke more damages and develop a number of skin diseases (Napagoda et al., 2016).

However, plants are an important source for the development of new chemotherapeutic including antioxidant agents, which can be protect cell constituents against oxidative damage and withstand the risk of various diseases associated with oxidative stress (Herlina et al., 2018). Furthermore, phenolic compounds are known for their potential antioxidant to eliminate toxic reactive oxygen species (ROS) as well as flavonoids, which were characterized by a strong potential protection against UV radiations (Hopkins, 2003; Macheix et al., 2005). The usage of plant species in the traditional medicine for the treatment of a variety of diseases (El-haj et al., 2014; Nematy et al., 2015) may be an important way to facilitate research on the sources of natural additives. Besides, medicinal plants of Sahara have higher secondary metabolites contents including phenolic compounds (Trabelsi et al., 2010; Gasmi et al., 2019).

Euphorbia retusa Forssk. is an endemic species of northern and central Sahara. It is an annual plant which grows naturally up to 30 cm high in hard climatic conditions of Sahara (Quezel and Santa, 1962; Ozenda, 2004). This plant is known for its use in folk medicine particularly as a treatment of dermatosis in the central Algerian Sahara (Ghareeb et al., 2018; Abdallah, 2014; Sdayria et al., 2019; Hammiche and Maiza, 2005).

Thus, the aim of this study was to investigate the antioxidant and photoprotective activities of the selected plant and to provide also the relationship between these activities and total phenolics and flavonoids contents of the aqueous extracts of *E. retusa* seed, capsule and leaves.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL COLLECTION

Euphorbia retusa Forssk. plant parts were collected from the South-East arid region of Algeria (34°54'21.751"N, 005°38'27"E) in June 2016. The plant samples were identified based on the flora of Ozenda (2004). The plant samples were separated into different parts: seeds, capsules and leaves. Then, samples were cleaned, dried in shade and grounded to powder.

2.2 EXTRACTION METHODS

In order to extract the phenolic compounds present in our plant, 10 g of each part of plant (seeds, capsules or leaves) were extracted separately with 100 ml of distilled water using Soxhlet apparatus at 40 °C for 8 hours. After extraction, the solvent of each part extracts was evaporated using a rotary vacuum evaporator until dryness.

2.3 TOTAL PHENOLICS AND FLAVONOIDS CONTENTS DETERMINATION

The total phenolics content of aqueous extracts was determined using Folin-Ciocalteu method following the protocol of Singleton et al. (1999) with slight modification. Briefly, 20 µl of each sample was mixed with 100 µl Folin–Ciocalteu reagent (10 fold diluted) and 75 µl of 7.5 % sodium carbonate solution. This mixture was incubated for 2 h at room temperature and the absorbance was measured at 765 nm using a 96-well microplate multimode plate reader (En Spire, PerkinElmer, MA, USA). The phenolic compounds concentrations were expressed as gallic acid equivalents/mg solid dry extract (µg GAE/mg DE) and calibration equation was found as: $y = 0.002\times + 0.010$, ($R^2 = 0.989$).

The total flavonoids content of aqueous extracts was quantified according to Moreno et al. (2000) method. 20 µl of each diluted extract solution was mixed with 10 µl of 10 % aluminium nitrate, 10 µl of potassium acetate (1 M) and 130 µl of methanol. After 40 min incubation at room temperature, the absorbance was measured at 415 nm. The total flavonoid content concentrations were expressed as Quercetin equivalents/ mg solid dry extract (µg QE/mg DE) and calibration equation was determined as: $y\ 0.006\times - 0.006$, ($r^2 = 0.998$).

2.4 ANTIOXIDANT ACTIVITY EVALUATION

2.4.1 Antiradical activity

The free radical scavenging activity of the aqueous extracts of each part was evaluated using DPPH assay described by Bloi (1958). 40 µl of the each extract concentrations (6.25, 12.5, 25, 50,100, 200 and 400 $\mu g ml^{-1}$) was mixed with 160 µl of a methanolic DPPH solution. The mixture was incubated at room temperature for 30 min. Then, the absorbance was measured at 517 nm using 96 well microplate reader. Results were expressed as % inhibition and as IC_{50} values in $\mu g\ ml^{-1}$. Butylhydroxytoluène (BHT) was used as a positive control. The inhibi-

tion percentage was calculated according to the following formula;

% *Inhibition* = [(*A0* – *A1* / *A0*)] × 100

Where; *A0* is the absorbance of the negative control, and *A1* is the absorbance of the sample at 30 min.

2.4.2 ABTS$^{\bullet+}$ scavenging activity

ABTS radical-scavenging activity of aqueous extracts was assessed according to the method developed by Re et al. (1999). 40 µl of extract at different concentrations (6.25, 12.5, 25, 50,100, 200 and 400 µg ml^{-1}) were mixed with to 160 µl of ABTS$^{\bullet+}$ solution in micro plate 96 wells. After 10 min of incubation, the absorbance was recorded at 734 nm. Butylhydroxytoluène (BHT) was used as a positive control and the inhibition percentage was calculated.

% *Inhibition* = [(*A0* – *A1* / *A0*)] × 100

Where; *A0* is the absorbance of the negative control, and *A1* is the absorbance of the sample at 10 min.

2.4.3 β-carotene–linoleic acid bleaching assay

β-carotene–linoleic acid bleaching assay of seeds, capsules and leaves aqueous extracts of *E. retusa* Forssk was measured following the method of Marco (1968). 40 µl of each sample at seven different concentrations was added to 160 µl of the β-carotene–linoleic acid emulsion. The first absorbance was measured in the zero-time at 470 nm and the second absorbance was recorded after 120 min of incubation on the same wavelength. Butylhydroxytoluène (BHT) was used as a positive control and the inhibition percentage as measured as following:

$$\%\ Inhibition = [1-(A0_{Extract} - At_{Extract})/(A0_{Control} - At_{Control})] \times 100$$

Where; $A_{0\ Control}$ is the absorbance of the negative control at 0 min. $At_{Control}$ is the absorbance of the negative control at 120 min. $A0_{Extract}$ is the absorbance of the sample at0 min. $A_{t\ Extract}$ is the absorbance of the sample at 0 min.

2.4.4 Reducing power assay

The reducing power of studied extracts was determined following the method of Bouratoua et al. (2017). 10 µl of extract were added to 40 µl of phosphate buffer (0.2 M, pH 6.6) and 50 µl of potassium ferricyanide (1%). The plate was incubated at 50 °C for 20 min. Then, 50 µl of tricarboxylic acid (10 %), 40 µl of distilled water and 10 µl of ferric chloride (0.1%) were added to mixture. Butylhydroxytoluène (BHT) was used as a positive control and the absorbance was measured at 700 nm. Results were expressed as absorbance against reagent blank and as $A_{0.50}$ values (µg ml^{-1}) corresponding the concentration indicating 0.50 absorbance intensity.

2.4.5 Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant capacity of aqueous extracts was determined according to the method of Apak et al. (2004). 40 µl of the extracts were added to 50 µl of copper (II) chloride (10 mM), 50 µl of neocuproine at 7.5 mM, and 60 µl of ammonium acetate (NH4Ac) buffer (1 M, pH = 7.0) solutions. After 1 hour of incubation, the absorbance was measured at 450 nm and butylhydroxytoluène (BHT) was used as a positive control.

2.5 *IN VITRO* SUN PROTECTION FACTOR (SPF) DETERMINATION

In order to evaluate ultraviolet (UV) absorption ability of the aqueous extracts, the *in vitro* SPF is determined according to the spectrophotometric method of Mansur *et al.* (1986).

The aliquots prepared were scanned between 290 and 320 nm, and the obtained absorbance values were multiplied with the respective EE (λ) values. Then, their summation was taken and multiplied with the correction factor. Methanol was taking as blank.

$$SPF = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs\ (\lambda)$$

Where; *EE*: erythemal effect spectrum, *I*: solar intensity spectrum, *Abs*: absorbance of sunscreen product, *CF*: correction factor (= 10). The value of (EE × *I*) is constant and determined by Sayre et al. (1979) (Table 1).

2.6 STATISTICAL ANALYSIS

All values were expressed as the mean ± SD (standard deviation). Analysis of variance (ANOVA) test followed by Newman–Keuls test were performed to check significant differences between the studied samples using the statistical software Statistica version 6.0. $p < 0.05$ compared to control was considered to be statistically significant.

Table 1: Correlation between the erythemogenic effect (EE) and the radiation intensity at each wavelength (I)

Wavelength (λ nm)	EExI (λ) (Normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0837
320	0.0180

3 RESULTS AND DISCUSSION

3.1 TOTAL PHENOLICS AND FLAVONOIDS CONTENTS

Total phenolics and flavonoids contents of *E. retusa* different parts were described in Table 2. The results revealed that the seeds aqueous extract exhibited the highest amount of total phenolic contents (356.83 ± 3.69 µg GAE/mg DE) followed by the capsules (114.25 ± 0.35 µg GAE/mg DE) and the leaves (75.83 ± 8.96 µg GAE/mg DE) extracts. According to Öztürk et al. (2006) and Karoune et al. (2015), secondary metabolites inter-organs as well as phenolic compounds are more variable in plant organs. Moreover, this variability may be dependent on the endogenous and exogenous factors (Oueslati et al., 2012). Furthermore, the aqueous extracts of *E. retusa* capsule registered the highest content on total phenolics than the methanolic extracts (105.33 ± 7.75 µg GAE/mg DE) reported by Lahmadi et al. (2020*)*. Thus, the phenolic compounds of *E. retusa* capsules are very soluble in water in distilled water than methanol, which means that this organ is rich in polar polyphenol (Baldosano et al., 2015).

Flavonoids contents were higher in seeds aqueous extract followed by capsules and leaves extracts (194.38 ± 8.31, 30.7 ± 0.4 and 44.25 ± 5.9 µg QE/mg DE respectively). However, flavonoids contents in leaves reported in the present work were higher than that reported by Sdayria et al. (2019) (20.50 ± 0.107 µg QE/mg DE) extracted by the maceration method which suggest that extraction with water was more effective than with 96 % ethanol.

3.2 DPPH SCAVENGING ACTIVITY

DPPH free radical scavenging activity of aqueous extracts of *E. retusa* seed, capsule and leaves is shown in Table 3. The results were expressed as inhibition percentage at different concentrations (6.25, 12.5, 25, 50, 100, 200 and 400 µg ml^{-1}) and as IC_{50} values in µg ml^{-1}. Data revealed that DPPH scavenging capacity increases with the raise in concentration of each extract. Furthermore, our findings showed that seeds aqueous extract exhibited a high activity competing with the both other extracts at all concentrations. Likewise, research reports found that seeds phenolic compounds are capable more for donating hydrogen to a free radical to scavenge the potential damage (Ksouri et al., 2009; Saeed et al., 2012). Ashraf et al. (2015) reported also the DPPH free radical scavenging activity of roots aqueous extract of *E. royleana*. The comparing of our results with this study showed that our samples (seeds, capsule and leaves) at 100 µg ml^{-1}present a higher effective scavenger of hydroxyl radical (79.67 % ± 1.44, 57.87 % ± 0.76 and 36.09 % ± 1.11 respectively) than their samples (20.18 % ± 0.96).

3.3 ABTS SCAVENGING ACTIVITY

For the ABTS radical-scavenging activity, seed extracts have a stronger capacity to quench $ABTS^{\bullet+}$ at concentrations ≥ 50 µg ml^{-1}as well as BHT (Table 4). The inhibition percentage of seed, capsule and leaves aqueous extracts was significantly important (92.01 % ± 1.64, 92.44 % ± 0.25 and 52.63 % ± 0.09 respectively) compared to those reported by Alaklabi et al. (2018) for root aqueous extracts of *Saururus chinensis* (Lour.) Baill. with 19.07 % ± 0.12 at same concentration (100 µg ml^{-1}).

Table 2: Total phenolics and flavonoids contents of the aqueous extracts of *E. retusa* different parts

Extract	Seeds	Capsules	Leaves
Total phenolics (µg GAE/mg DE)	356.83 ± 3.69^{a}	114.25 ± 0.35^{b}	75.83 ± 8.96^{c}
Total flavonoids (µg QE/mg DE)	194.38 ± 8.31^{a}	30.7 ± 0.4^{b}	44.25 ± 5.9^{c}

Values expressed as mean ± SD (n = 3). Values in the same line followed by a different letter (a-c) are significantly different ($p < 0.05$). µg GAE/mg DE: microgram gallic acid equivalent per milligram of dry plant extract. *µg QE/mg DE*: microgram quercetin equivalent per milligram of dry plant extract

Table 3: Antioxidant activity of the aqueous extracts of *E. retusa* different parts by DPPH assay

Concentrations µg ml^{-1}	% Inhibition in DPPH scavenging assay			
	Seed	Capsule	Leaves	BHT
6.25	9.21 ± 1.25	1.44 ± 1.29	1.94 ± 1.80	18.55 ± 2.46
12.5	16.14 ± 2.34	4.60 ± 1.37	5.16 ± 0.35	32.60 ± 3.72
25	27.12 ± 1.10	11.47 ± 1.03	8.88 ± 1.15	53.80 ± 2.58
50	49.99 ± 1.62	28.19 ± 1.11	19.77 ± 0.35	74.97 ± 2.14
100	79.67 ± 1.44	57.87 ± 0.76	36.09 ± 1.11	83.41 ± 0.86
200	84.12 ± 0.34	82.20 ± 0.68	73.69 ± 0.95	84.59 ± 0.46
400	92.82 ± 0.41	92.83 ± 0.17	81.79 ± 0.42	85.76 ± 0.91
IC_{50} µg ml^{-1}	50.79 ± 1.87^{b}	87.38 ± 1.53^{c}	158.49 ± 3.24^{d}	23.54 ± 1.83^{a}

Values expressed as mean ± SD (n = 3). Values in the last line followed by a different letter (a-d) are significantly different ($p < 0.05$). BHT: butyl hydroxytoluene. IC50: half maximal inhibitory concentration expressed as the necessary concentration to decrease the initial absorbance of DPPH by 50 %

Table 4: Antioxidant activity of the aqueous extract of *E. retusa* different parts by ABTS assay

Concentrations µgml^{-1}	% Inhibition in ABTS assay			
	Seeds	Capsules	Leaves	BHT
6.25	69.10 ± 2.97	18.38 ± 1.51	-	61.38 ± 0.57
12.5	88.06 ± 2.60	33.88 ± 1.99	9.68 ± 2.78	62.02 ± 3.82
25	88.93 ± 1.51	56.27 ± 4.35	12.92 ± 3.96	76.50 ± 1.40
50	91.14 ± 0.37	86.05 ± 2.87	25.83 ± 1.78	82.55 ± 1.04
100	92.01 ± 1.64	92.44 ± 0.25	52.63 ± 0.09	88.60 ± 2.66
200	92.82 ± 0.41	92.83 ± 0.17	73.69 ± 0.95	90.38 ± 0.67
400	-	92.89 ± 0.19	90.33 ± 0.25	-
IC_{50} µg ml^{-1}	< 6.25^{a}	21.12 ± 0.76^{b}	95.92 ± 1.20^{c}	< 6.25^{a}

Values expressed as mean ± SD (n = 3). Values in the last line followed by a different letter (a-c) are significantly different ($p < 0.05$). BHT: butylhydroxytoluene. IC50: half maximal inhibitory concentration expressed as the necessary concentration to decrease the initial absorbance of DPPH by 50 %

Table 5: Antioxidant activity of the aqueous extract of *E. retusa* different parts by β-carotene–linoleic acid bleaching assay

Concentrations µg ml^{-1}	% Inhibition in β-carotene–linoleic acid bleaching assay			
	Seeds	Capsules	Leaves	BHT
6.25	97.83 ± 0.38	96.14 ± 0.43	92.94 ± 0.50	57.25 ± 3.1
12.5	97.42 ± 0.22	96.02 ± 0.37	89.37 ± 0.95	82.39 ± 2.79
25	96.29 ± 0.20	94.21 ± 0.41	82.29 ± 0.30	83.12 ± 2.82
50	94.67 ± 0.39	93.77 ± 1.52	72.53 ± 1.72	92.99 ± 3.26
100	93.07 ± 0.18	92.01 ± 0.14	57.08 ± 2.74	92.65 ± 3.19
200	89.6 ± 0.04	89.28 ± 0.23	30.34 ± 1.36	93.52 ± 0.00
400	79.83 ± 0.32	84.44 ± 0.18	14.24 ± 0.91	94.22 ± 0.30
IC_{50} µg ml^{-1}	< 6.25 ± 0.00^{a}	< 6.25 ± 0.00^{a}	23.82 ± 0.95^{b}	< 6.250 ± 00 ^{a}

Values expressed as mean ± SD (n = 3). Values in the last line followed by a different letter (a-b) are significantly different ($p < 0.05$). BHT: butylhydroxytoluene. IC50: half maximal inhibitory concentration expressed as the necessary concentration to decrease the initial absorbance of DPPH by 50 %

Table 6: Antioxidant activity of the aqueous extracts of *E. retusa* different parts by reducing power assay

Concentrations µg ml^{-1}	Absorbance in reducing power assay			
	Seeds	Capsules	Leaves	BHT
6.25	0.22 ± 0.01	0.08 ± 0.01	-	0.05 ± 0.02
12.5	0.31 ± 0.02	0.1 ± 0.00	-	0.07 ± 0.02
25	0.46 ± 0.03	0.14 ± 0.00	-	0.11 ± 0.03
50	0.7 ± 0.04	0.19 ± 0.02	-	0.19 ± 0.02
100	0.85 ± 0.11	0.29 ± 0.01	0.05 ± 0.00	0.30 ± 0.03
200	1.29 ± 0.09	0.35 ± 0.01	0.05 ± 0.00	0.74 ± 0.18
400	2.14 ± 0.00	0.57 ± 0.01	0.06 ± 0.02	1.07 ± 0.17
$A_{0.50}$ µg ml^{-1}	6.97 ± 0.75^{a}	84.49 ± 2.38^{c}	> 100	37.41 ± 3.89^{b}

Values expressed as mean ± SD (n = 3). Values in the last line followed by a different letter (a-c) are significantly different ($p < 0.05$). BHT**:** butyl-4-methylphenol ou butylhydroxytoluene. $A_{0.50}$: corresponding the concentration indicating 0.50 absorbance intensity

3.4 B-CAROTENE–LINOLEIC ACID BLEACHING ASSAY

The bleaching of β-carotene assay was used to evaluate the ability of the antioxidants to inhibit lipid peroxidation (Moualek et al., 2016). Furthermore, up to our knowledge, there are no reports on the bleaching of β-carotene assay of *E. retusa* organs. So this is the first report which deals with this effect. The results of this activity were expressed as inhibition percentage and as IC_{50} µg ml^{-1} (Table 5). Results showed that seed, capsule aqueous extracts and BHT as a standard have a stronger capacity to inhibit the coupled oxidation of β-carotene and linoleic acid (IC_{50} < 6.25 µg ml^{-1}).

3.5 REDUCING POWER ASSAY

For reducing power activity (Table 6), the absorbance of samples and BHT as a standard were increased by the rising of concentrations. However, the results showed that seeds aqueous extract had the strongest capacity to reduce ion at all concentrations compared with BHT or stems and leaves aqueous extracts. Moreover, seeds aqueous extract ($A_{0.50\ =}$ 6.97 ± 0.75 µg ml^{-1}) indicates a high $A_{0.50}$ value as compared with seeds methanolic extract ($A_{0.50\ =}$ 11.84 ± 1.72 µg ml^{-1}) reported by Lahmadi et al. (2019).

3.6 CUPRIC REDUCING ANTIOXIDANT CAPACITY (CUPRAC)

The CUPRIC reducing antioxidant capacity method described by Apak et al.(2004) measures the absorbance of Cu(II)- neocuproine (Nc) chelate formed by the redox reaction of chain-breaking antioxidants with the CUPRAC reagent. Cupric reducing antioxidant capacity

Table 7: Antioxidant activity of the aqueous extract of *E. retusa* different parts by CUPRAC assay

Concentrations µg ml^{-1}	Absorbance in CUPRAC assay			
	Seeds	Capsules	Leaves	BHT
6.25	0.44 ± 0.01	0.14 ± 0.01	-	0.44 ± 0.03
12.5	0.72 ± 0.03	0.20 ± 0.02	0.07 ± 0.00	1.32 ± 0.07
25	1.15 ± 0.05	0.30 ± 0.02	0.08 ± 0.00	1.80 ± 0.09
50	2.00 ± 0.19	0.53 ± 0.03	0.08 ± 0.00	1.82 ± 0.22
100	3.28 ± 0.03	0.81 ± 0.02	0.09 ± 0.00	2.39 ± 0.39
200	3.69 ± 0.12	0.98 ± 0.08	0.10 ± 0.00	2.71 ± 0.46
400	4.06 ± 0.02	1.45 ± 0.07	0.18 ± 0.05	2.76 ± 0.46
$A_{0.50}$ µg ml^{-1}	7.64 ± 0.30^{b}	49.50 ± 1.51^{c}	> 400	6.64 ± 0.18^{a}

Values expressed as mean ± SD (n = 3). Values in the last line followed by a different letter (a-c) are significantly different ($p < 0.05$). BHT: butyl-4-methylphenol ou butylhydroxytoluene. $A_{0.50}$: A0.5 (µg ml^{-1}) corresponding the concentration indicating 0.50 absorbance intensity

Table 8: SPF values of the aqueous extract of *E. retusa* different parts

Absorbance			CF× EE(λ)×I(λ)× Abs (λ)		
Seeds	Capsules	Leaves	Seeds	Capsules	Leaves
3.85	2.21	2.84	0.58	0.33	0 .43
3.86	1.85	2.48	3.15	1.51	2.02
3.85	1.61	2.26	11.07	4.62	6.48
3.82	1.50	2.17	12.53	4.90	7.10
3.80	1.46	2.15	7.08	2.71	4.00
3.78	1.46	2.14	3.17	1.22	1.79
3.75	1.50	2.12	0.68	0.27	0.38
Sun Protection Factor (SPF)			38.26 ± 0.07^{a}	15.57 ± 0.24^{b}	22.21 ± 0.56^{c}

Values expressed as mean ± SD (n = 3). Values in the last line followed by a different letter (a-c) are significantly different ($p < 0.05$). EE: erythemal effect spectrum, I: solar intensity spectrum, Abs: absorbance of sunscreen product, CF: correction factor

(CUPRAC) of the organ extracts and the BHT are shown in Table 7. The ranking order for CUPRAC test was BHT > seeds > capsules > leaves at all concentrations. Accordingly, the results of CUPRAC test showed that BHT have a higher activity ($A_{0.50}$ = 6.64 ± 0.18 µg ml^{-1}) followed by seeds ($A_{0.50}$ = 7.64 ± 0.30 µg ml^{-1}), capsule ($A_{0.50}$ = 49.50 ± 1.51 µg ml^{-1}) and leaves ($A_{0.50}$ = > 400 µg ml^{-1}) extracts.

3.7 IN VITRO SUN PROTECTION FACTOR (SPF) DETERMINATION

The sun protection factor (SPF) values of different part samples were shown in the table 8. The seeds extract has the higher SPF values (38.26 ± 0.07) followed by leaves and capsules extracts (SPF = 22.21 ± 0.56, 15.57 ± 0.24 respectively). In literature, the data about photoprotective activity of this plant is not available. According to Afssaps (2011), SPF is generally divided into four protection classes; low (SPF values: 6-15), medium or moderate (SPF values: 15-30), high (SPF values: 30 - 50) and very high (SPF values > 50). Thus, seed aqueous extract belongs to the range of good sunscreen activity while leaves and capsules aqueous extracts were characterized by moderate sunscreen activity. In comparison to other works on SPF values, seed extracts of *E. retusa* presented a higher SPF than *Mentha spicata* L. aerial parts methanolic extract reported by El Aanachi et al. (2021) with SPF = 35.76 ± 0.21 and the aerial parts methanolic extract *of Capnophyllum peregrinum* (L.) Lange reported by Lefahal et al. (2018) with SPF = 35.21 ± 0.18. In fact, SPF result shows that the aqueous extracts of seed have a good sun protection activity against ultraviolet radiation.

In order to analyze the relationship between total phenolics and flavonoids contents, antioxidant and photoprotective activities of *E. retusa* seed, capsule and leaves aqueous extracts, Pearson's correlations were applied (Table 9). A statistically significant positive correlation between total phenolic and flavonoid contents and photoprotective activity with Pearson's correlation coefficients $r > 0.90$, suggesting that the SPF was dependent not only on the total phenolic but also on the total flavonoid contents, which may be attributed to their synergistic action. These results support the hypothesis that flavonoid contents contribute to photoprotective activity (Macheix et al., 2005). However, total phenolics contents showed a significant negative correlation with antioxidant activities (DPPH and ABTS radical-scavenging capacity, β -carotene bleaching and cupric-reducing antioxidant capacity (CUPRAC) and reducing power) with $r > - 0.59$. Similarity, a statistically significant negative correlation between total phenolic contents, DPPH free radical scavenging capacity and β carotene bleaching was supported by Terpinc et al. (2012). Also, Kainama et al. (2020) found the negative correlation between total phenolic content and ABTS scavenging activity in Garcinia stem and bark ethyl acetate extracts ($r = – 0.91$), indicating that the antioxidant activity may be linked to the structure and the nature of the phenolic compounds (Oueslati, 2013). However, negative correlation between flavonoid contents and antioxidant activities (DPPH and ABTS cation radical scavenging, cupric-reducing antioxidant capacity (CUPRAC) and reducing power) was shown. On the other hand, reducing power showed a significant negative correlation with β carotene bleaching inhibition and SPF values with Pearson's correlation coefficients of -0.56 and -0.67 respectively. These results indicated the antagonist effects of reducing power with β carotene bleaching inhibition and with SPF. Furthermore, no correlations were found between SPF and other antioxidant activity. Similar results were found by Ebrahimzadeh et

Table 9: Correlations among *E. retusa* total phenolics and flavonoids contents, antioxidant and photoprotective activities

	TPC	TFC	$DPPH^{+}$	$ABTS^{+}$	β-CLAB	RP	CUPRAC	SPF
TPC	1							
TFC	0.97	1						
DPPH	- 0.84	- 0.70	1					
ABTS	- 0.76	- 0.61	0.99	1				
β-CLAB	- 0.60	- 0.43	0.94	0.98	1			
RP	- 0.31	- 0.5	- 0.25	- 0.38	- 0.56	1		
CUPRAC	- 0.71	- 056	0.98	0.1	0.99	- 0.44	1	
SPF	0.91	0.975	- 0.54	- 0.43	- 0.23	- 0.67	- 0.37	1

Correlation is significant at $p < 0.05$. TPC: total phenolics content, TFC: total flavonoids content, DPPH: 2,2-diphenyl-1-picrylhydrazyl test, ABTS: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt cation radical scavenging, *β-CLAB: β*-Carotene–linoleic acid bleaching, RP: reducing power, CUPRAC: cupric-reducing activity, SPF: sun protection factor

al. (2014). Hence, our results indicated no correlations between SPF and DPPH radical-scavenging activity.

4 CONCLUSIONS

Our results revealed that seed extract demonstrates the best total phenolics and flavonoids contents and SPF value. Also, this extract showed a great potential for antioxidant activity. Furthermore, the correlation analysis revealed that SPF is positively correlated with total phenolics and flavonoids contents. But, generally no correlations were found between SPF and antioxidant activity. According to the obtained results, *E. retusa* may be considered as a remarkable antioxidant and pharmaceutical source.

5 ACKNOWLEDGEMENT

This work was supported by MESRS-DGRSDT (Algeria).

6 REFERENCES

Abdallah, E.M. (2014). Antimicrobial properties and phytochemical constituents of the methanol extracts of *Euphorbia retusa* Forssk. and *Euphorbia terracina* L. from Saudi Arabia. *South Asian Journal of Experimental Biology*, *4*(2), 48-53. https://doi.org/10.38150/sajeb.4(2).p48-53

Afssaps, Recommandations de bon usage des produits de protection solaire à l'attention des utilisateurs. (2011) URL: http://ansm.sante.fr/var/ansm_site/storage/original/application/7dff1bdc58ff373048961896c9c72db5.pdf.

Alaklabi, A., Arif, I.A., Ahamed, A., Kumar, R.S., Idhayadhulla, A. (2018). Evaluation of antioxidant and anticancer activities of chemical constituents of the *Saururus chinensis* root extracts. *Saudi Journal of Biological Sciences*, *25*(7), 1387-1392. doi: 10.1016/j.sjbs.2016.12.021. https://doi.org/10.1016/j.sjbs.2016.12.021

Apak, R., Güçlü, K., Özyürek, M., Karademir, S.E. (2004). Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *Journal of Agricultural and Food Chemistry*, *52*, 7970-7981. https://doi.org/10.1021/jf048741x

Ashraf, A., Sarfraz, R.A., Rashid, M.A., Shahid, M. (2015). Antioxidant, antimicrobial, antitumor, and cytotoxic activities of an important medicinal plant (*Euphorbia royleana*) from Pakistan. *Journal of Food and Drug Analysis*, *23*, 109-115. https://doi.org/10.1016/j.jfda.2014.05.007

Baldosano, H.Y., Castillo, M.B.M.G., Elloran, C.D.H., Bacani, F.T. (2015). Effect of particle size solvent and extraction time on tannin extract from *Spondias purpurea* bark through soxhlet extraction. *Proceedings of the DLSU Research Congress*, *3*, 4-9.

Blois, M.S. (1958). Antioxidant determinations by the use of a stable Free Radical. *Nature*, *461*, 1119-1200. https://doi.org/10.1038/1811199a0

Bouratoua, A., Khalfallah, A., Bensouici, C., Kabouche, Z., Alabdul Magid, A., Harakat, D., Voutquenne-Nazabadioko, L., Kabouche, A. (2017). Chemical composition and antioxidant activity of aerial parts of *Ferula longipes* Coss. ex Bonnier and Maury. *Natural Product Research*, *32*, 1873-1880. https://doi.org/10.1080/14786419.2017.1353513

Ebrahimzadeh, M.A., Enayatifard, R., Khalili, K., Ghaffarloo, M., Saeedi, M., Charati, J.Z. (2014). Correlation between sun protection factor and antioxidant activity, phenol and flavonoid contents of some medicinal plants. *Iranian Journal of Pharmaceutical Research*, *13*, 1041-1047.

El Aanachi, S., Gali, L., Rammali, S., Bensouici, C., Aassila, H., Dari, k. (2021). *In vitro* study of the antioxidant, photoprotective, antityrosinase, and antiurease efects of methanolic extracts from leaves of six Moroccan Lamiaceae. *Journal of Food Measurement and Characterization*, *15*, 1785-1795. https://doi.org/10.1007/s11694-020-00759-9

El-haj, N., Bao, M.B.B., Goldstein, N. (2014). Pharmacology and therapeutics sun protection in a pill: the photoprotective properties of *Polypodium leucotomos* extract. *International Journal of Dermatology*, *54*(3), 1-5. https://doi.org/10.1111/ijd.12611

Gasmi, A., Benabderrahim, M.A., Guasmi, F., Elfalleh, W., Triki, T., Zammouri, T., Ferchichia, A. (2019). Phenolic profiling, sugar composition and antioxidant capacity of arta (*Calligonum comosum* L.), a wild Tunisian desert plant. *Industrial Crops and Products*, *130*, 436-442. https://doi.org/10.1016/j.indcrop.2019.01.003

Ghareeb, T.A., El-Toumy, S.A., El– Gendy, H., Haggag, E.G. (2018). Secondary metabolites and hepatoprotective activity of *Euphorbia retusa*. *Journal of Advanced Pharmacy Research*, *4*, 283-291. http://aprh.journals.ekb.eg/

Hammiche, V., Maiza, K. (2005). Traditional medicine in Central Sahara: Pharmacopoeia of Tassili N'ajjer. *Journal Ethnopharmacology*, *105*, 358-367. https://doi.org/10.1016/j.jep.2005.11.028

Herlina, N., Riyanto, S., Martono, S., Rohman, A. (2018). Antioxidant activities, phenolic and flavonoid contents of methanolic extract of *Stelechocarpus burahol* fruit and its fractions. *Dhaka University Journal of Pharmaceutical Sciences*, *17*, 153-159. https://doi.org/10.3329/dujps.v17i2.39170

Hopkins, W. (2003). *Physiologie Végétale*. 2nd ed. De Boeck.

Kainama, H., Fatmawati, S., Santoso, M., Mercy, Papilaya, P., Ersam, T. (2020). The relationship of free radical scavenging and total phenolic and flavonoid contents of *Garcinia lasoar* Pam. *Pharmaceutical Chemistry Journal*, *53*(12), 1151-1157. https://doi.org/10.1007/s11094-020-02139-5

Karoune, S., Falleh, H., Kechebar, M.S.A., Halis, Y., Mkadmini, K., Belhamra, M., Rahmoune, C., Ksouri, R. (2015). Evaluation of antioxidant activities of the edible and medicinal *Acacia albida* organs related to phenolic compounds. *Natural Product Research*, *29*(5), 452-454. https://doi.org/10.1080/14786419.2014.947497

Ksouri, R., Falleh, H., Megdiche, W., Trabelsi, M., hamdi, N., Chaieb, K., Bakroufd A., Magnéc, C. (2009). Antioxidant and antimicrobial activities of the edible medicinal halophyte *Tamarix gallica* L. and related polyphenolic constituents. *Food and Chemical Toxicology, 47*, 2083-2091. https://doi.org/10.1016/j.fct.2009.05.040

Lahmadi, S., Belhamra, M., Karoune, S., Kechebar, M.S.A., Bensouici, C., Kashi, I., Abdellya, C. (2019). In vitro antioxidant capacity of *Euphorbia retusa* Forssk. from Algerian desert. *Journal of Pharmacology and Pharmaceutical Research*, *7*, 356-366. http://jppres.com/jppres

Lahmadi, S., Belhamra, M., Karoune, S., Imad, k., Bensouici, C., Kechebar, M.S.A., Halis, Y., Ksouri, R. (2020). Phenolic constituents and antioxidant activity of *Euphorbia retusa* Forssk. *Natural Product Research*, *24*, 3545-3547. https://doi.org/10.1080/14786419.2019.1582040

Lefahal, M., Zaabat, N., Ayad, R., Makhloufi, E., Djarri, L., Benahmed, M., Laouer, H., Nieto, G., Akkal, S. (2018). In vitro assessment of total phenolic and flavonoid contents , antioxidant and photoprotective activities of crude methanolic extract of aerial parts of *Capnophyllum peregrinum* (L.) Lange (Apiaceae) growing in Algeria. *Medicines*, *2*(5), 2-10. https://doi.org/10.3390/medicines5020026

Macheix, J.J., Fleuriet, C., Jay-Allemand, C.(2005*). Les composés phénoliques des végétaux: Un exemple de métabolites secondaires d'importance éconoique*. Italie. Press polytechnique et universitaires romandes.

Mansur, J.S., Breder, M.N.R., Mansur, M.C.A., Azulay, R.D. (1986). Determinação do fator de proteção solar porespectrofotometria. *Anais Brasileiros Dermatologia*, *61*, 121-124.

Marco, G.J. (1968). A rapid method for evaluation of antioxidants. *Journal of the American Oil Chemists' Society*, *45*, 594-598. https://doi.org/10.1007/BF02668958

Megdiche, W., Medini, F., Mkadmini, K., Legault, J., Magné, C., Abdelly, C. (2013). MS identification of bioactive secondary metabolites involved in the antioxidant, anti inflammatory and anticancer activities of the edible halophyte *Zygophyllum album* Desf. *Food Chemistry*,*139*, 1073-1080. https://doi.org/10.1016/j.foodchem.2013.01.047

Moreno, M.I.V., Isla, M.I., Sampietro, A.R., Vattuone, M.A. (2000). Comparison of the free radical-scavenging activity of propolis from several regions of Argentina. *Journal of Ethnopharmacoloy*, *71*, 109-114. https://doi.org/10.1016/S0378-8741(99)00189-0

Moualek, I., Aiche, G.I., Guechaoui, N.M., Lahcene, S., Houali, K. (2016). Antioxidant and antiinflammatory, activities of *Arbutus unedo* aqueous extract. *Asian Pacific Journal of Tropical Biomedicine*, *6*, 937-944. https://doi.org/10.1016/j.apjtb.2016.09.002

Napagoda, M.T., Malkanthi, B.M.A.S., Abayawardana, S.A.K., Qader, M.M., Jayasinghe, L. (2016). Photoprotective potential in some medicinal plants used to treat skin diseases in Sri Lanka. *BMC Complementary Medicine and Therapie, 16*, 1-6. https://doi.org/10.1186/s12906-016-1455-8

Nematy, M., Mehdizadeh, M.D., Razmpour, F. (2015). A review on nutrition and skin aging. *Iranian Journal of Dermatology*, *18*, 20-24.

Oueslati, S. (2013). *Exploration de la variabilité de la composition phénolique et des activités biologiques chez quatre halophytes du genre Suaeda*. These de doctorat en sciences biologiques. Universite de Tunis El Manar, 134p.

Oueslati, S., Trabels, N., Boulaaba, M., Legault, J., Abdelly, C., Ksouri, R. (2012). Evaluation of antioxidant activities of the edible and medicinal *Suaeda* species and related phenolic compounds. *Industrial Crops and Products, 36*, 513-518. https://doi.org/10.1016/j.indcrop.2011.10.006

Ozenda, P., (2004). *Flore du Sahara*. 2nd ed. Paris. CNRS.

Öztürk, M., Aydog˘mus-Öztu¨rk, F., Emin Duru, M., Topçu, G. (2006). Antioxidant activity of stem and root extracts of Rhubarb (*Rheum ribes*): An edible medicinal plant. An edible medicinal plant. *Food Chemistry*, *103*, 623-630. https://doi.org/10.1016/j.foodchem.2006.09.005

Quezel ,P., Santa, S. (1962). *Nouvelle flore de l'Algérie et des régions désertiques méridionales*. Paris. CNRS. T1.

Re , R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*, 1231-1237. https://doi.org/10.1016/S0891-5849(98)00315-3

Saeed, N., Khan, M.R., Shabbir, M. (2012). Antioxidant activity,

total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L. *BMC Complementary Medicine and Therapie, 221*, 1-12. http://www.biomedcentral.com/1472-6882/12/221

Sayre, R.M., Agin, P.P., Levee, G.J., Marlow, E.A. (1979). Comparison of in vivo and in vitro testing of sunscreening formulas. *Photochemistry and Phytobiology, 29*(3), 559-566. https://doi.org/10.1111/j.1751-1097.1979.tb07090.x

Sdayria, J., Rjeibi, I., Feriani, A., Ncib, S., Bouguerra, W., Hfaiedh, N., Elfeki, A., Allagui M.S. (2019). Chemical composition and antioxidant, analgesic, and anti-inflammatory effects of methanolic extract of *Euphorbia retusa* in mice. *Pain Research and Management, 2018*, 1-11. https://doi.org/10.1155/2018/4838413

Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology*, *299*, 152-178. https://doi.org/10.1016/S0076-6879(99)99017-1

Terpinc, P., Barbara, C., Poklar Ulrih, N., Abramovičc, H. (2012). Studies of the correlation between antioxidant properties and the total phenolic content of different oil cake extracts. *Industrial Crops and Products, 39*, 210-217. https://doi.org/10.1016/j.indcrop.2012.02.023

Trabelsi, N., Megdiche, W., Ksouri, R., Falleh, H., Oueslati, S., Bourgou, S., Hajlaouic, H., Abdellya, C. (2010). Solvent effects on phenolic contents and biological activities of the halophyte *Limoniastrum monopetalum* leaves. *LWT - Food Science and Technology*, *43*(4), 632-639. https://doi.org/10.1016/j.lwt.2009.11.003

Türkoğlua, A., Durub, M.E., Mercan, N. (2007). Antioxidant and antimicrobial activity of *Russula delica* Fr: An Edidle Wild Mushroom. *Eurasian Journal of Analytical Chemistry*, *2*(1), 54-67. https://doi.org/10.12973/ejac/78055

doi:10.14720/aas.2022.118.3.2690 Original research article / izvirni znanstveni članek

Pollen quality and sensory attributes of Algerian jujube (*Ziziphus lotus* (L.) Lam.) honeys

Scherazad MEKIOUS [1, 2, 3], Carine MASSEAUX [4], Wahida LOUCIF-AYAD [5, 6], Monica VERCELLI [7]

Received May 14, 2022; accepted September 23, 2022.
Delo je prispelo 14. maja 2022, sprejeto 23. septembra 2022

Pollen quality and sensory attributes of Algerian jujube (*Ziziphus lotus* (L.) Lam.) honeys

Abstract: Honey bees and beekeeping activity are of huge importance for the crop production and biodiversity conservation as well as for the economic impact due to ecosystem services. In the south of Algeria, the jujube blooming is an essential forage source for honey bees. The aim of this study was to determine the melissopalynological and sensory characteristics of Algerian jujube honey. Nineteen samples of jujube honey collected in south Algeria over the period from 2016 to 2018 were analyzed. The unifloral designation attributed to the honey was confirmed by a pollen analysis following the established standard methods. Sensory analysis is carried out testing the color, the odor and aromas. The results showed that *Ziziphus lotus* pollen was predominant in all samples, and in terms of sensory analysis, color ranged from amber yellow to light brown; the determined scent classes were warm, floral and woody with medium intensity; the aroma was represented by medium intensity with the warm caramelized, floral fruity and woody classes. Sweet flavor is perceived at medium intensity, acidic flavor is weak, astringent sensation is average and the piquant note is perceived with a low intensity. This work proves to be important for improving the knowledge in typical honeys.

Key words: jujube; *Ziziphus lotus*; honey; melissopalynology; sensory analysis; pollen

Kakovost peloda in senzorične lastnosti medu iz alžirske vrste čičimaka (*Ziziphus lotus* (L.) Lam.)

Izvleček: Medonosne čebele in njihova reja imajo velik pomen za pridelavo gojenih rastlin in ohranjanje biodiverzitete kot tudi velik ekonomski pomen pri ekosistemskih storitvah. Na jugu Alžirije je cvetenje alžirskega čičimaka (žižole) najpomembnejša paša za medonosne čebele. Namen raziskave je bil določiti sestavo in senzorične lastnosti medu, nabranega na tej rastlini. Analiziranih je bilo 19 vzorcev čičimakovega medu, nabranih na jugu Alžirije v obdobju 2016-2018. Pripadnost medu tej medonosni vrsti je bila potrjena s pelodno analizo in in drugimi uveljavljenimi standardnimi metodami. Pri senzorični analizi so bili preiskušeni barva, vonj in aroma. Rezultati so pokazali, da je v vseh vzorcih medu prevladoval pelod te vrste čičimaka. Barva medu je bila od oranžnorumene do svetlorjave, vonj je bil srednje močan, določen kot topel, cvetlični do lesni. Aroma medu je bila srednje močna, po toplih karamelah, cvetno-sadna z odtenki po lesu.Sladkost je bila srednja, kislost šibka, trpkost srednja, pikantnost je bila zaznana kot šibke jakosti. Raziskava je pomembna, ker prispeva k poznavanju tipičnih medov.

Ključne besede: čičimak; *Ziziphus lotus*; med; melisopalinologija; senzorična analiza; pelod

1 University of Djelfa, Faculty of Nature and Life Sciences, Djelfa, Algeria
2 University of Blida, Faculty of Nature and Life Sciences, Medicinal and Aromatic Plants Laboratory, Blida, Algeria
3 Corresponding author, e-mail: mekious.sch.uni.djelfa@gmail.com
4 Catholic University of Louvain, Beekeeping Research and Information Center (CARI), Louvain-la-Neuve, Belgium
5 Faculty of Medicine, Badji Mokhtar University, Annaba, Algeria
6 Laboratory of Applied Animal Biology, Faculty of Science, Badji Mokhtar University, Annaba, Algeria
7 Department of Agricultural, Forest and Food Sciences (DISAFA), University of Torino, Torino, Italy

1 INTRODUCTION

Bees are the primary animal pollinators in most ecosystems (Neff & Simpson, 1993) and honey bees (*Apis mellifera* L., 1758) in particular play a key role as providers of pollination services essential for agricultural productivity and biodiversity conservation (Potts et al., 2003; Klein et al., 2007; Salami et al., 2016; Ferrazzi et al., 2017). An economic study demonstrated that the total economic value of pollination services globally amounts to approximately €153 billion annually (Gallai et al., 2009), corresponding to about 9 % of the total economic value of agricultural crops grown for human consumption. Moreover, beekeeping supplies market beehive products (honey, pollen, royal jelly, propolis and wax) and livestock (artificial swarms, packed bees, queens). Currently, a total of 92.3 million hives have been recorded around the world and the honey production amounts to around 1.8 million tons (Vercelli et al., 2021).

Mediterranean basin represents an important area for beekeeping activity and honey production characterized by typical unifloral and multifloral honeys (Battesti, 1990). However, in Mediterranean areas, climate change is a severe threat to honey bees (Le Conte &Navajas, 2008; IPBES, 2016; Novelli et al., 2021; Vercelli et al., 2021), influencing significantly other strictly related factors such as diseases, parasites, predators, parasitoids, viruses and pesticide use (Goulson et al., 2015; Zawislak et al. 2019).In arid and semi-arid areas of southern Algeria, the jujube (*Ziziphus lotus* L.) blooming is essential forage sources (nectar and pollen) for honey bees usually between May and June as well as other flowering present during the year (Mekious et al.,2015). Due to this important source, it is possible to produce unifloral honey that is highly sought and appreciated by beekeepers and consumers. As Jujube honey is exposed to a lot of fraud, particularly with regard to its unifloral appellation, the identification and characterization of this honey is essential to preserve the quality as well as geographical and botanical origins. Some studies have been targeted the characterization of this unifloral honey but there is an insufficient knowledge about its sensory properties (Song et al., 2012; Zhou et al., 2013; Mekious et al., 2015; Chakir, et al., 2016; Zerrouk et al., 2017; Mekious et al., 2020; Zerrouk et al., 2021).

Melissopalynological and sensory properties are one of the main ways used to identify unifloral and multifloral honeys (Ferrazzi&Medrzycki, 2002; Piana et al., 2004; Ferrazzi& Vercelli, 2014; Prđun et al., 2020). The aim of this study was to determine pollen characteristics and sensory properties in several of honey samples of the same floral origin. The study of the pollen profile is important to verify the unifloral attributed designation and to know the botanical origin of pollen collected by honey bees. This analysis provides valuable information on the foraging activity of honey bee (Battesti, 1992; Floris et al., 2020). When pollen of one species is highly present in a honey, it is very likely that that species gives an important contribution in terms of nectar in the process of elaboration of this honey except for some honeys derived from plants that do not provide much pollen (pollen under-represented, e.g. lavender honeys) or from plants that produce much more pollen than nectar (pollen over-represented, e.g. eucalyptus honey) (Louveaux et al., 1978; Von Der Ohe et al., 2004).

Furthermore, sensory properties describe the general physical characteristics of honey perceptible by our senses. Taste and aroma vary and depend on the plant origin. Usually, sensory evaluation is commonly used to complete physico-chemical and pollen analyses. It is used to confirm quality, to verify the absence of defects, to establish sensory profiles of unifloral honeys, and also to understand consumer preferences (Piana et al., 2004; Marcazzan, 2018). Organoleptic qualities are also considered as markers of floral origin (Amiot et al., 1989). In the absence of sensory standards specific to jujube honey, this study was based on the evaluation of the intensity of organoleptic attributes in 19 honey samples of the same floral origin in order to define the majority aromatic notes.

2 MATERIALS AND METHODS

2.1 STUDY AREA AND HONEY COLLECTION

During the years 2016-2018, in the regions of Djelfa (34° 40′ 0″ N, 3° 15′ 0″ E) and Laghouat (33° 48′ 23″ N, 2° 52′ 56″ E) located in the south of Algeria, characterized by a hot and dry climate with pastoral vegetation well adapted to pedoclimatic conditions of these regions. According to the information collected by the professional climatological stations of the national metrological office of Djelfa and Laghouat, in the years of our study, the maximum average temperature reached 33.82 °C in Djelfa and 41.09 °C in Laghouat Regarding the precipitation, 275 mmyear^{-1} and163 mmyear^{-1}were registered in Djelfa and in Laghouat, respectively.19 unifloral declared as *Z. lotus* honey samples were collected from hives belonging to professional beekeepers and placed in jujube blossom area.

2.2 QUALITATIVE MELISSOPALYNOLOGICAL ANALYSIS

In order to complete a qualitative melissopalynological analysis, the extraction and analysis of the pollen spectra were carried out using methods established by the International Commission of Apicultural Botany, described by Louveaux et al. (1978), Ferrazzi (1992) and Von der Ohe et al. (2004). Ten grams of honey was dissolved in 20 ml of distilled hot water (20-40 °C). The solution was centrifuged once for 10 min (30,000 rpm), and then another centrifugation was done for 5 min under the same conditions.

The sediment was put on a slide with an area of 24× 24 mm. 500 pollen grains were counted and their relative frequency classes were determined according to the international melissopalynological nomenclature: predominant pollen for pollen occurring for more than 45 % of the total pollen count, accompanying pollen or secondary pollen (16-45 %), important minor pollen (3-15 %) and minor pollen, occurring < 3 %.

The identification of pollen types was carried out by comparing the morphology and dimensions of the pollen grains present in our samples observed under a light microscope with those of microphotographs of reference pollens established by Ricciardelli d'Albore (1998), by our database (Laboratory of aromatic and medicinal plants, University of Blida1) and other pollen atlas. The pollen type includes species and/or genera present in the area, which have the same or similar pollen morphology microscopically.

2.3 SENSORY ANALYSIS

The sensory analysis, visual, olfactory, and gustatory characteristics was performed using the technique described by Piana et al. (2004). The samples were tested by a panel of three assessors from the CARI (Center for Beekeeping Research and Information) trained to identify a sensory stimulus on the basis of odor and aroma wheel developed by CARI laboratory (Bruneau et al., 2000).

The wheel synthesizes a common lexicon of reference and defines a list of descriptors with a precise meaning and the same meaning for all, and on the other hand to approve standardized aromatic references corresponding to each descriptor. Assessors recorded the color using the Pfund method. The intensity of odor and aroma is carried by a system of quantified evaluation on a scale of 1 to 3. 1: low intensity, 2: medium intensity, 3: high intensity.

3 RESULTS AND DISCUSSION

3.1 QUALITATIVE MELISSOPALYNOLOGICAL ANALYSIS

The unifloral declaration attributed to the honey samples was confirmed by a qualitative pollen analysis. The types of pollen present in honey samples divided into four classes of pollen frequencies are illustrated in Table 1.

The pollen grains of nectarless species from the families such as Poaceae, Chenopodiaceae, Pinaceae, Cistaceae, Plantaginaceae and Oleaceae were calculated separately (Louveaux et al., 1978 and Von Der Ohe et al., 2004). The taxa *Ziziphuslotus, Thapsia garganica, Centaurea, Carduus, Euphorbia bupleuroides, Retama retam and Peganum harmala* have a large distribution (>50 %) in the honey samples. Usually, these pollens are markers of the floristic environment, having a high pollen frequency

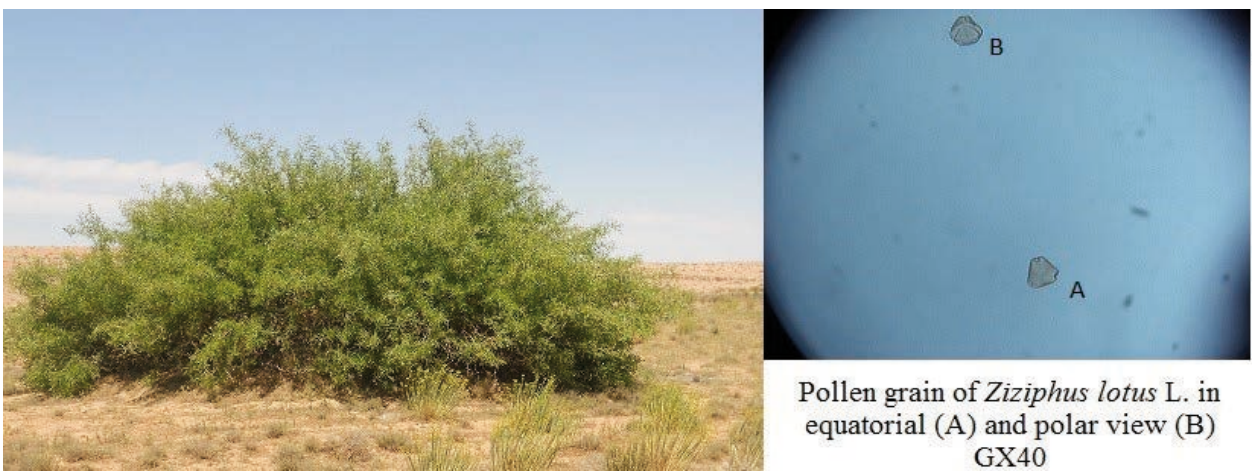


Figure1: The jujube tree in the steppe region of Djelfa and the pollen grain of *Ziziphus lotus* (photo credit: Scherazad Mekious)

Table1: Frequency of distribution of taxa and their frequency classes in honey samples

Family	Taxon (Pollen type)	F (%)	M (<3 %)	I (3-15 %)	S (16-45 %)	P (>45 %)	Max
Anacardiaceae	*Pistacia lentiscus* L.*	10.53	10.53	-	-	-	1.1
Apiaceae	*Thapsia garganica* L.	89.47	42.11	36.84	10.53	-	17.3
Apiaceae A	*Daucus carota* L.	36.84	36.84	-	-	-	7.6
Arecaceae	Arecaceae	21.05	21.05	-	-	-	1.6
Asteraceae	*Echinops*	47.37	47.37	-	-	-	2.5
	Centaurea	68.42	52.63	15.79	-	-	6.1
	Carthamus	10.53	10.53	-	-	-	1.1
Asteraceae S	*Carduus*	57.89	31.58	26.32	-	-	15.2
	Calendula	42.11	42.11	-	-	-	2.7
	Scolymus	15.79	15.79	-	-	-	1.2
Asteraceae T	*Taraxacum*	26.32	26.32	-	-	-	1.2
Boraginaceae	*Echium*	15.79	15.79	-	-	-	2.7
Brassicaceae	*Sinapis* form	21.05	21.05	-	-	-	1.7
Brassicaceae	*Brassica* form	21.05	21.05	-	-	-	1.2
Caprifoliaceae	Caprifoliaceae	31.58	31.58	-	-	-	1.1
Chenopodiaceae	Chenopodiaceae*	15.79	15.79	-	-	-	2.8
Cistaceae	*Cistus**	21.05	21.05	-	-	-	1.4
Ericaceae	*Erica*	10.53	10.53	-	-	-	1.2
Euphorbiaceae	*Euphorbia bupleuroides* (Desf.) Soják,	57.89	42.11	-	15.79	-	23.7
Fabaceae	*Acacia*	10.53	10.53	-	-	-	1.7
	Vicia	21.05	21.05	-	-	-	1.1
	Medicago	10.53	10.53	-	-	-	2.5
	Ononis	31.58	31.58	-	-	-	1.7
	Retama retam (Forssk.) Webb & Berthel.	42.11	31.58	5.26	5.26	-	19.2
Fagaceae	*Quercus**	10.53	10.53	-	-	-	1.1
Lamiaceae	*Thymus*	26.32	26.32	-	-	-	1.7
Liliaceae	*Asphodelus microcarpus* Parl.	10.53	10.53	-	-	-	1.2
Malvaceae	*Malva*	15.79	15.79	-	-	-	2.7
Myrtaceae	*Eucalyptus*	10.53	10.53	-	-	-	1.7
Oleaceae	*Olea**	21.05	21.05	-	-	-	1.4
Papaveraceae	*Papaver rhoeas* L.*	10.53	10.53	-	-	-	2.2
Pinaceae	Pinaceae*	5.26	5.26	-	-	-	1.1
Plantaginaceae	Plantaginaceae*	15.79	15.79	-	-	-	5.2
Poaceae	Poaceae*	31.58	31.58	-	-	-	6.3

Continued on the next page

Ranunculaceae	Ranunculaceae	26.32	26.32	-	-	-	1.7
Resedaceae	*Reseda*	10.53	10.53	-	-	-	1.2
Rhamnaceae	*Ziziphus lotus* (L.) Lam.	100.00	-	-	-	100	97.1
Rosaceae	Rosaceae	15.79	15.79	-	-	-	1.2
Tamaricaceae	*Tamarix*	36.84	36.84	-	-	-	7.2
Nitrariaceae	*Peganum harmala* L.	68.42	31.58	26.32	10.53	-	27.6

*: nectarless species. F: Frequency of distribution of taxa in honey samples, M: Minor pollen (< 3 %), I: important minor pollen (3-15 %), A: Secondary pollen (16-45 %), P: Predominant (> 45 %), Max: maximum recorded of pollen frequency

in relation to the widespread distribution in the region (Battesti, 1990). Qualitative pollen analysis highlighted the dominance of *Z. lotus* (Figure 1) in honey samples as predominant with a maximum frequency of 97.1 %. The determination of botanical origin was based on the relative frequencies of the pollen types of nectariferous species. In general, a honey is considered unifloral when the relative pollen frequency of onetaxon exceeds 45 % (Von Der Ohe et al., 2004). *Z. lotus* constitutes an abundant source of nectar frequently collected by honey bees justifying the unifloral designation "Jujube honey" attributed by beekeepers. Jujube is a species also visited for the pollen harvest. Moreover, we noted the secondary presence of pollens from *Thapsia garganica, Euphorbia bupleuroides, Retama retam* and *Peganum harmala*, important sources of nectar and pollen, with respective maximum levels of 17.3 %, 23.7 %, 19.2 % and 27.6 % (Table 1).

3.2 SENSORY ANALYSIS

Regarding visual analysis, slight variations in color, ranging from 61 to 99 mm Pfund, were observed, which corresponds to a color range from amber yellow to light brown. Studies reported that the color interval of Algerian honeys ranged from 18 to 119 mm Pfund corresponding to a color range varying from very light to dark brown. The very light honey samples are dominated by *Citrus* and *Hedysarum* while dark brown honeys are characterized by predominance of*Eucalyptus*, Apiaceae as *Daucus* and *Rubus* (Ouchemoukhet al., 2007; Benaziza et al., 2010).

The profile of the aromas and flavors perceived in the 19 honey samples analyzed were shown in Table 2. The odor classes determined by the tasters were warm, floral and woody, with medium intensity. In terms of the perception of aromas, the general intensity is medium, and the aromatic classes perceived were warm caramelized, floral fruity and woody. The perception of chemical aroma was found only in two samples with low intensity. The sweet flavor was perceived with medium to high intensity.

Z. lotus honey samples are characterized by higher fructose contents than glucose, and pH values between 5.17-5.8 for jujube honey from North Africa (Mekious et al., 2015; Chakir et al., 2016; Zerrouket al., 2017), and an average value of pH equal to 6.71 for jujube honey from China (Zhou et al., 2013). In all samples, the acidic flavor was weak, and the astringent sensation was medium.

The piquant note was perceived in all the samples, but with a lower rate in 84.21 % of honey samples and

Table 2: Sensory characteristics of *Ziziphus lotus* honey samples

Sensory characteristics		
Olfactory assessment	Intensity: medium	Description: warm, floral, woody
	Intensity: low	Description: chemical
Tasting assessment	Intensity	Medium
	Sweetness	Medium to high
	Acidity	Low
Aroma	Intensity: medium	Description: warm caramelised, floral-fruity, woody
	Persistence	Medium
Other sensations	Intensity: low to medium	Description: astringent, piquant
	Crystallization	Absent

with medium rate in the rest of the samples. Pollen analysis of these samples showed the presence of *Euphorbia bupleuroides* pollen, either as an accompaniment with a maximum pollen frequency of 23.7 % or in rare, isolated cases at <3 %(Table 1). The nectar of this species gives a piquant note to honey; this is confirmed by the reference system for unifloral honeys established by the laboratory specifying that the honey samples were obtained from nectars of the Euphorbiaceae family species. Some notes are linked to the flora foraged by bees without exogenous contamination (Guyot-Declerck, 2001).

The unifloral honey samples derived mainly from a single plant species (at least 45 % of pollen grain), may considerably differ in their sensory properties with highly prominent flavor and aroma (Lippolis, 2020).In all honey samples crystallization was absent.

4 CONCLUSION

This study describes the melissopalynological and sensory characteristics of honey from the same floral origin "*Ziziphus lotus*" produced in arid and semi-arid areas in Algeria. Overall, all the honey samples were characterized by the predominance of *Z. lotus* pollen. This type of honey has slight variations in color, ranging from amber yellow to light brown. The perceived odor and aromatic classes were warm caramelized, floral fruity and spicy woody, with medium intensity. Sweet flavor, astringent sensation, acid flavor and spicy notes were also perceived in the honey samples. This work proves to be important for improving the knowledge in typical Algerian honeys and in particular in Jujube honey. In this context, the protection and the promotion of regional honeys will be ensured effectively when the various physicochemical, pollen and aromatic components can be described quite precisely. The definition of floral and regional appellations should be supported, and they are necessary for any quality approach to honey. It is therefore necessary to define a set of pollen and sensory physicochemical standards characteristic of unifloral honey. The acquisition of new data regarding melissopalynological and sensory analysis of honey allows the protection and the promotion of specific regional honeys. In this context, more studies based on the characterization of *Z. lotus* L. are needed to provide a normative framework and to determine future specific standards composition for this type of honey.

5 ACKNOWLEDGMENTS

We would like to thank the collaboration of the CARI laboratory, in particular the tasters Beatrice Perat, Laurence Leclercq and Christine Delcourt. We also thank the professional beekeepers of the Professional Beekeepers Association (ANAP) for their collaboration in the sampling part.

6 REFERENCES

Amiot, MJ., Aubert, S., Gonnet, M. & Tacchini M. (1989). Les composés phénoliques des miels: étude préliminaire sur l'identification et la quantification par famille. *Apidologie*, *20*(2), 115-125. https://doi.org/10.1051/apido:19890202

Battesti, M.J. (1990). *Contribution à la mélissopalynologie méditerranéenne: les miels corses.* Thèse doctorat, Université d'Aix, Marseille (France).

Battesti, M-J., Goeury, C. (1992). Efficacité de l'analyse mélissopalynologique quantitative pour la certification des origines géographiques et botaniques des miels: le modèle des miels corses. *Review of Palaeobotany and Palynology*, *75*, 77-102. http://dx.doi.org/10.1016/0034-6667(92)90151-6

Benaziza-Bouchema,D.,Schweitzer,P.(2010).Caractérisation des principaux miels des régions du Nord de l'Algérie. *Cahiers Agricultures*, *19*,432-438. https://doi.org/10.1684/agr.2010.0432

Bruneau,E.,Barbier,E.,Gallez,L.M., &Guyot-Declerck,C.(2000). La roue des arômes des miels [The honeyaromaswheel]. *Abeilles & Cie,77*,16-23.

Chakir, A., Romane, A., Marcazzan, L.G., Ferrazzi, P. (2016). Physicochemical properties of some honeys produced from different plants in Morocco. *Arabian Journal of Chemistry*, *9*, S946-S954. https://doi.org/10.1016/j.arabjc.2011.10.013

Ferrazzi, P. (1992). Caratterizzazione dei mieli: analisi melissopalinologiche. *Apicolura Moduli*, *83*, 129-138.

Ferrazzi, P. &Medrzycki, P. (2002). Primo approccio di applicazione della cluster analysis alle analisi melissopalinologiche per la determinazione dell'origine geografica dei mieli. In: *Atti Convegno A.M.A., Bologna, 14-16 marzo 2002*, pp. 223-228.

Ferrazzi, P. & Vercelli,M. (2014). Melissopalynological characterization of the Lanzo Valleys honeys (Piedmont, northwestern Italy) as a reference for alpine productions and biodiversity conservation. *2nd ApiEcoFlora & Biodiversity, Apimondia Symposium, 6-7 November 2014*, Rome, Italy, 44.

Ferrazzi, P., Vercelli, M., Chakir, A., Romane, A., Mattana, M., Consonni, R. (2017). Pollination effects on antioxidant content of *Perilla frutescens* seeds analysed by NMR spectroscopy. *Natural Products Research*, *31*, 2705-2711. https://doi.org/10.1080/14786419.2017.1292267

Floris, I., Satta, A., Pusceddu, M., Francesconi, A.H.D., Ferrazzi, P. (2020). *Italian Apiculture. A Journey Through History and Honey Diversity Floris I*. Ed, Accademia Nazionale Italiana di Entomologia, Eva Crane Trust, Società Entomologica Italiana - Gruppo di lavoro Api, Università degli Studi di Sassari, pp 463.

Gallai, N., Salles, J.M., Settele, J., Vaissière, B.E. (2009). Economic valuation of the vulnerability of world agriculture con-

fronted with pollinator decline. *Ecological Economics*, *68*, 810-821. http://dx.doi.org/10.1016/j.ecolecon.2008.06.014

Goulson, D., Nicholls, E., Botías, C., Rotheray, E.L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, *347*, 1255957. http://dx.doi.org/10.1126/science.1255957

Guyot-Declerck, C., Le Berre, E., Bruneau, E., (2001). Analyse sensorielle: mode d'emploi. *Abeille & Cie*, 82, 22-26.

IPBES (2016). *The Assessment Report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on Pollinators, Pollination and Food Productio*n. Potts, S.G., Imperatriz-Fonseca, V.L., Ngo, H.T.(Eds). Secretariat of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services: Bonn, Germany, p. 552.

Klein, A.M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B Biological Science*, *274*, 3036 313. http://dx.doi.org/10.1098/rspb.2006.3721

Le Conte, Y., & Navajas, M. (2008). Climate change: Impact on honey bee populations and diseases. *Revue Scientifique et Techique (International Office of Epizootics), 27*(2), 499-510. https://doi.org/10.20506/rst.27.2.1819

Lippolis, V., De Angelis, E., Giuseppina, M.F., Di Gioia, A., Arlorio, M., Logrieco, A.F., and Monaci, L. (2020). Geographical origin discrimination of monofloral honeys by direct analysis in real time ionization-high resolution mass spectrometry (DART-HRMS). *Foods*, *9*(9), 1205. http://dx.doi.org/10.3390/foods9091205

Louveaux, J., Maurizio, A., Vorwohl, G. (1978). Methods of melissopalynology. *Bee World*, *59*(4), 139-157. https://doi.org/10.1080/0005772X.1978.11097714

Marcazzan, G.L., Mucignat-Caretta, C., Marchese, C.M., Piana, M.L. (2018). A review of methods for honey sensory analysis. *Journal of Apicultural Research*, *57*(1), 75-87. https://doi.org/10.1080/00218839.2017.1357940

Mekious, S., Houmani, Z., Bruneau, E., Masseaux, C., Guillet, A., Hance, T. (2015). Caractérisation des miels produits dans la région steppique de Djelfa en Algérie. *Journal Biotechnologie, Agronomie, Société et Environnement, 19*(3), 219-229.

Mekious, S., Masseaux, C., Daoud, N., Belhadj, S., Houman,i Z. (2020). Caractéristiques mélissopalynologiques et contenu phénolique du miel de *Ziziphus lotus* d'Algérie. *Agrobilogia*, *10(*2), 2220-2228.

Neff, J.L., &Simpson, B.B. (1993). *Hymenoptera and Biodiversity*. La Salle J., Gauld I.D., Eds. CAB International: Wallingford, UK, pp. 143-167.

Novelli, S., Vercelli, M., Ferracini, C. (2021). An Easy Mixed-Method Analysis Tool to Support Rural Development Strategy Decision-Making for Beekeeping. *Land*, *10*, 675.https://doi.org/10.3390/land10070675

Ouchemoukh, S., Louaileche, H., Schweizer, P. (2007). Physicochemical characteristics and pollen spectrum of some Algerian honeys. *Food Control*, *18*, 52-58. DOI: http://dx.doi.org/doi:10.1016/j.foodcont.2005.08.007.

Piana, M., PersanoOddo, L., Bentabol, A., Bruneau, E., Bogdanov, S., Guyot, Declerck C. (2004). Sensory analysis applied to honey: state of the art. *Apidologie, 35*(1), 26-S37. https://doi.org/10.1051/apido:2004048

Potts, S.G., Vulliamy, B., Dafni, A., Ne'eman, G., Willmer, P. (2003). Linking bees and flowers: How do floral communities structure pollinators communities? *Ecology*, *84*, 2628-2642. http://dx.doi.org/10.1890/02-0136

Prđun, S., Kremer, D., Bubalo, D., Svečnjak, L. (2020). Physicochemical, melissopalynological and sensory characteristics of *Satsuma mandarin* honey (*Citrus unshiu* Marc.), *Journal of Central European Agriculture*, *21*(2), 256-267. https://doi.org/10.5513/JCEA01/21.2.2787

Ricciardelli, d'Albore G. (1998). *Mediterranean melissopalynology*. Perugia, Italy, Instituto di Entomologia agrair, Università degli Studi. 466p.

Salami, M., Rahim, malek, M., Ehtemam, M.H., Sabzalian, M.R. (2016). Variation in bioactive compounds and anatomical characteristics of different fennel (*Foeniculum vulgare* Mill.) populations as affected by self-pollination. *Journal of Applied Botany and Food Quality*, *89*, 38-48. https://doi.org/10.5073/JABFQ.2016.089.0055

Song, X., Yao, Y., Yang, W. (2012). Pollen analysis of natural honeys from the central region of Shanxi, North China. *PLoS ONE*, *7*(11), e49545. https://doi.org/10.1371/journal.pone.0049545

Vercelli, M., Novelli, S., Ferrazzi, P., Lentini, G., Ferracini, C. (2021). A qualitative analysis of beekeepers' perceptions and farm management adaptations to the impact of climate change on honey bees. *Insects*, *12*, 228. https://doi.org/10.3390/insects12030228

Von der Ohe, W., Persano Oddo, L., Piana, L., Morlot, M., Martin, P. (2004). Harmonised methods of melissopalynological analysis. *Apidologie*, *35*, 18-25. http://dx.doi.org/10.1051/apido:2004050

Zawislak, J., Adamczyk, J., Johnson, D.R., Lorenz, G.; Black, J., Hornsby, Q., Stewart, S.D., Joshi, N. (2019). Comprehensive survey of area-wide agricultural pesticide use in southern United States row crops and potential impact on honeybee colonies. *Insects*, *10*, 280. http://dx.doi.org/10.3390/insects10090280

Zerrouk, S., Seijo, M. C., Escuredo, O., Rodríguez-Flores, M.S. (2017). Characterization of *Ziziphus lotus* (jujube) honey produced in Algeria. *Journal of Apicultural Research*, *54*,166-164. https://doi.org/10.1080/00 218839.2017.1399663

Zerrouk, S., Escuredo, O., Rodríguez-Flores, M. S., Seijo, M.C. (2021). Palynological characterisation of sedra honeys (*Ziziphus lotus*) produced In Algeria. *Grana*, *60*(1), 69-80. https://doi.org/10.1080/00173134.2020.1770853

Zhou, J., Suo, Z., Zhao, P., Cheng, N., Gao, H., Zhao, J., Cao, W. (2013). Jujube honey from China: Physicochemical characteristics and mineral contents. *Journal of Food Science*, *78*(3), C387–C394. https://doi.org/10.1111/1750-3841.12049

doi:10.14720/aas.2022.118.3.2419 Review article / pregledni znanstveni članek

Razvoj raziskovalnih metod za karakterizacijo združb arbuskularnih mikoriznih gliv in potencialni vpliv biodiverzitete glivnih endofitov na vegetacijo

Irena MAČEK [1, 2]

Received November 12, 2021; accepted July 13, 2022.
Delo je prispelo 12. novembra 2021, sprejeto 13. julija 2022

Development of research methods to characterise arbuscular mycorrhizal fungal communities and potential effects of fungal endophyte biodiversity on vegetation

Abstract: Characterization and quantification of the functional and taxonomic diversity of microbial communities is essential for understanding all aspects of microbial ecology and is closely related to ecosystem function. Arbuscular mycorrhiza is the most widespread symbiosis on Earth, with arbuscular mycorrhizal (AM) fungi present in more than 2/3 of all plant species. Just over a decade after the publication of the first review article on molecular approaches to study the ecology of AM fungi in Acta Agriculturae Slovenica (Maček, 2009), the rapid development of molecular tools, especially next generation sequencing (NGS) technology, has accelerated the study of the research field of plant root endophytes. In this paper, the current approach to study the ecology and taxonomy of AM fungi is presented, which also provides some insights into the study of other plant root endophytes. In addition, a widely used system for classifying AM fungi with so-called virtual taxa (VT) is presented, which is used for ecological studies and comparison between different studies. Finally, a brief overview of the importance of climate and soil properties for AM fungal community composition and taxa distribution in global ecosystems is presented.

Key words: arbuscular mycorrhiza; biodiversity; ecology; endophytes; rhizosphere; sequencing; soil

Razvoj raziskovalnih metod za karakterizacijo združb arbuskularnih mikoriznih gliv in potencialni vpliv biodiverzitete glivnih endofitov na vegetacijo

Izvleček: Karakterizacija in kvantifikacija funkcionalne in taksonomske raznolikosti mikrobnih združb je ključnega pomena za razumevanje vseh vidikov mikrobne ekologije in je povezana tudi širše z razumevanjem delovanja ekosistemov. Arbuskularna mikoriza predstavlja najbolj razširjeno in starodavno simbiozo na Zemlji, saj so arbuskularne mikorizne (AM) glive prisotne v koreninah več kot dveh tretjin vseh rastlinskih vrst. V dobrem desetletju od objave preglednega članka o uporabi molekulskih pristopov pri raziskavah arbuskularne mikorize v reviji Acta Agriculturae Slovenica (Maček, 2009) je razvoj metodologije, predvsem tehnologije določanja nukleotidnega zaporedja (sekvenciranja) naslednjih generacij (NGS), močno pospešil raziskave raznolikosti in ekologije združb AM gliv in drugih koreninskih endofitov. V tem članku so predstavljene novosti na področju raziskav endofitskih gliv v koreninah rastlin, s poudarkom na aktualnem pristopu k raziskavam v ekologiji in taksonomiji AM gliv, ter sistem njihove klasifikacije s tako imenovanimi virtualnimi taksoni (VT). Slednji je zelo uporaben za namen ekoloških raziskav in širše primerjave različnih študij med sabo. Na kratko je predstavljen tudi vpliv klimatskih in talnih lastnosti okolja na sestavo združb in pojavljanje posameznih taksonov AM gliv v različnih ekosistemih.

Ključne besede: arbuskularna mikoriza; biodiverziteta; ekologija; endofiti; rizosfera; sekvenciranje; tla

1 Oddelek za biologijo, Biotehniška fakulteta, Univerza v Ljubljani, Ljubljana, Slovenija
2 Korespondenčni avtor, e-naslov: irena.macek@bf.uni-lj.si

1 UVOD V RAZISKAVE DIVERZITETE AM IN DRUGIH SKUPIN ENDOFITSKIH GLIV V KORENINAH RASTLIN

Arbuskularne mikorizne (AM) glive (skupina Glomeromycotina ali tudi Glomeromycota) (Spatafora in sod., 2016, Tedersoo in sod., 2018) predstavljajo ključno skupino talnih mikroorganizmov v številnih kopenskih ekosistemih in najbolj široko razširjeno simbiozo na planetu (Brachmann in sod., 2006). Povezava med AM glivami in rastlinami je starodavna, saj so bile AM glive prisotne že ob prehodu rastlin iz morja na kopno v paleozoiku pred več kot 450 milijoni let. Poleg AM gliv (Glomeromycotina) pa so bile ob prehodu rastlin na kopno prisotne tudi endofitske glive iz starodavne in delno saprotrofne skupine Endogonales (Mucoromycotina, Mucoromycota), katerih predstavnike so dolgo uvrščali med AM glive zaradi podobnih morfoloških struktur (drobne hife in njihov razrastki podobni abuskulom), ki jih te glive tvorijo v koreninah rastlin. Na podlagi molekulskih označevalcev (markerjev) je bilo ugotovljeno, da predstavljajo t.i. drobni koreninski endofiti iz skupine Mucoromycotina (ang. ‚fine root endophytes' ali MFRE) filogenetsko ločeno skupino globalno razširjenih rastlinskih endosimbiontov (Orchard in sod. 2017). Slednji tudi danes še vedno tvorijo endosimbiozo z večino skupin kopenskih rastlin, pogosto tudi istočasno in funkcionalno komplementarno z AM glivami (Field on sod., 2015; Orchard in sod., 2017, Hoysted in sod., 2019, Besiana et al., 2021; Sinanaj in sod., 2021). Ker so ugotovitve o pomenu simbioze rastlin z glivami iz skupine Mucoromycotina še relativno nove, je podatkov o njihovi povezavi z rastlinami manj kot za arbuskularno mikorizo, tako da obstaja še kar nekaj odprtih vprašanj za boljše razumevanje te skupine gliv in njihove funkcije v ekosistemih (Sinanaj in sod., 2021). V tem preglednem članku se osredotočam predvsem na arbuskularno mikorizo, torej AM glive iz skupine Glomeromycotina, vsekakor pa bo v prihodnosti potrebno spremljati tudi razvoj raziskav drugih skupin rastlinskih endofitov, tako že dlje časa poznanih temnih septiranih endofitov (DSE) (Rodriguez in sod., 2009, Knapp in sod., 2018, Tonjer in sod., 2021), kot tudi drobnih koreninskih endofitov iz skupine Mucoromycotina (MFRE). Razvoj novih molekulskih metod v zadnjem desetletju vsekakor omogoča lažje odkrivanje in razumevanje celotnega spektra raznolikosti organizmov, ki živijo v območju rastlinskih korenin oz. rizosferi. V bližnji prihodnosti zato lahko pričakujemo veliko novih informacij o starodavnih simbiozah med glivami in rastlinami, njihovi biodiverziteti in pomenu za ekosisteme, vključno z agroekosistemi.

Že dolgo je znano, da arbuskularna mikoriza rastlinam prinaša številne koristi, od izboljšane mineralne prehrane in preskrbe z vodo ter obrambe pred patogeni, do posrednih koristi npr. izboljšane strukture tal in manjše erozije tal. AM glive so obvezni biotrofi, od rastlin dobivajo fotoasimilate, ki predstavljajo njihov edini vir organskega ogljika (Smith & Read, 2008). Simbioza vpliva tudi na sestavo združb kopenskih rastlin, s tem pa tudi na funkcioniranje ekosistemov in njihovo produktivnost (Fitter, 2005, Schnitzer & Klironomos, 2011, Wurzburger in sod., 2017). Taksonomsko lahko AM glive klasificiramo na več načinov, od tradicionalnih klasifikacij, ki temeljijo na morfologiji celične stene njihovih spor (npr. Oehl in sod., 2011, Blszkowski, 2012), kar je naslovljeno v prvem delu članka, do molekulskih pristopov, pri katerih uporabljamo različne molekulske označevalce, kar je obravnavano v drugem delu članka. Definicija vrste je pri mikroorganizmih, vključno z mikroglivami, na splošno zelo težavna in podvržena konsenzu na podlagi trenutnega poznavanja posamezne skupine organizmov. Prav zato lahko na tem področju v prihodnosti pričakujemo tudi nadaljnje spremembe klasifikacije, ki bodo temeljile na novo pridobljenih podatkih o AM glivah. V zadnjem delu članka so na kratko povzete tudi glavne omejitve različnih pristopov k raziskovanju raznolikosti AM gliv, s poudarki, kje je na mestu previdnost, z namenom čim bolj kvalitetne interpretacije rezultatov in pridobivanja novega znanja o tej zanimivi in pomembni skupini organizmov.

1.1 GOJENJE AM GLIV V ČISTIH KULTURAH IN KLASIČNA IDENTIFIKACIJA VRST AM GLIV

Veliko težavo pri morfološkem določanju vrst AM gliv predstavlja njihovo gojenje v čistih kulturah, saj AM glive brez primerne gostiteljske rastline ne uspevajo. V sklopu raziskav AM gliv čisto, enovrstno (monospecifično) kulturo predstavlja kultura, kjer je prisotna samo ena vrsta glive, ki je bila vzgojena iz ene same spore. Postopek vzpostavitve take kulture je zapleten in dolgotrajen, zahteva veliko ekspertnega znanja ter rastne razmere, ki preprečujejo navzkrižno kontaminacijo lončnih kultur rastlin z drugimi glivami iz okolja (npr. ob zalivanju rastlin, zaradi prenosa z živalskimi vektorji, kot so insekti itd.).

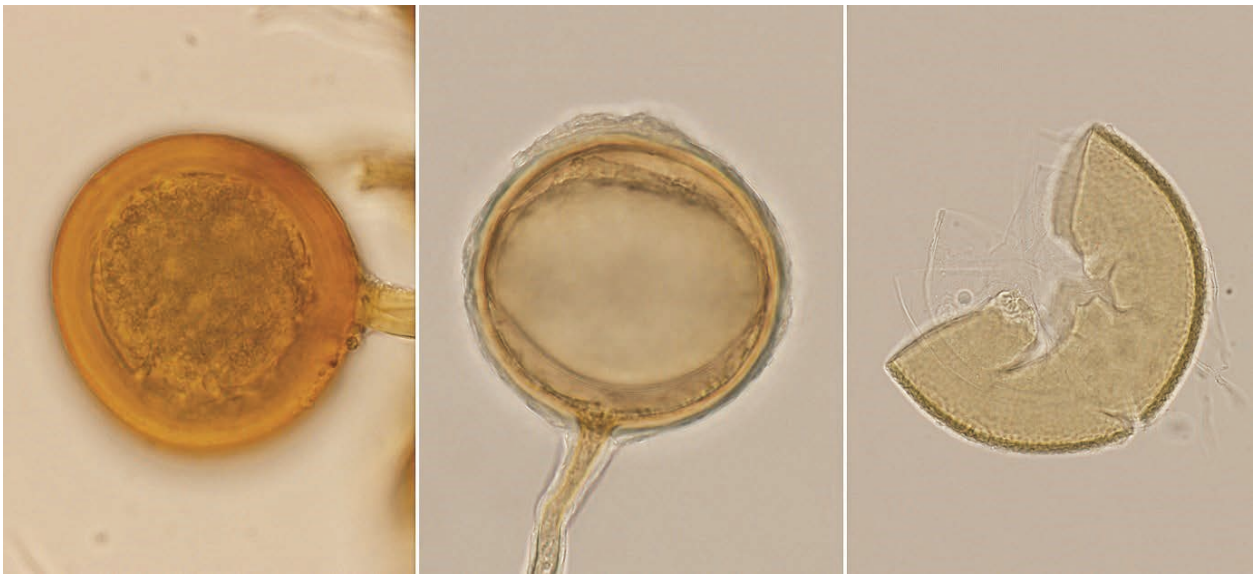
Spore nekaterih vrst AM gliv lahko izoliramo iz njihovega okolja (običajno iz talnih vzorcev ali pri nekaterih vrstah tudi iz korenin) s postopkom redčenja in koncentriranja v vodi in/ali raztopini saharoze ter iskanjem in prepoznavanjem spor s stereo mikroskopom. Novo, enovrstno lončno kulturo AM gliv vzpostavimo s prenosom posamezne spore v bližino korenin izbranih vrst rastlin. Rastline so običajno za namen tega postopka vzgojene iz semen, ki so bila predhodno površinsko sterilizirana. Da povečamo možnost neposrednega stika

Slika 1: Vzpostavitev enovrstnih (monospecifičnih) kultur AM gliv, vzgojenih iz ene same spore, v nastavkih za pipete (levo) in kasneje v lončnih kulturah (sredina, desno). Namen tega postopka je, da omogočimo čim boljši stik korenine rastline s posamezno izolirano sporo AM gliv in kasneje pomnoževanje AM gliv v kulturi. Rastline so vzgojene iz semena, ki je bilo predhodno površinsko sterilizirano (foto: I. Maček)
Figure 1: Monospecific cultures of AM fungi grown from a single AM fungal spore in pipette tips (left) and later in pot cultures (centre, right). The method allows good spatial contact between plant roots grown from a single surface-sterilised seed and a single AM fungal spore and further fungal development (photo: I. Maček)

med korenino rastline in viabilno sporo AM glive, vzpostavimo začetno interakcijo med obema organizmoma v omejenem prostoru. Za ta namen se je izkazala primerna uporaba nastavkov za pipete, napolnjenih z vlažnim, sterilnim substratom (Slika 1). Kasneje rastline skupaj z nastavkom prenesemo v večje lonce, napolnjene s sterilnim substratom (Slika 1). Da zagotovimo bolj raznolik življenjski prostor za glive (koreninska biomasa) lahko v kasnejši fazi rasti dosejemo še več gostiteljskih rastlin. Koristno je, če uporabimo več rastlinskih vrst, kar omogoči večji nabor potencialnih gostiteljev za AM glive, večjo raznolikost in biomaso korenin in s tem večjo možnost, da se simbioza (mikoriza) v rizosferi dejansko vzpostavi. Pomembno je, da tudi kasneje, tekom rasti rastlin, maksimalno zmanjšamo možnost navzkrižne kontaminacije s sporami ali delci infektivnih hif med posameznimi lončnimi kulturami. Taki preventivni postopki zajemajo gojenje rastlin v zaprtih prostorih, kontrolo škodljivcev, kontrolirano odtekanje vode iz loncev z mikorizo, ustrezno zalivanje, ki je lahko urejeno z avtomatiziranim kapljičnim sistemom, in omejeno ventilacijo zraka znotraj prostora. Rastline lahko gojimo tudi v posebnih vrečkah z vgrajenim filtrom, kjer se ustvari mikroklima in so izolirane od ostalih rastlin, obenem pa je poseganje v tak sistem (npr. zalivanje) omejeno na minimum.

Vzpostavitev enovrstne kulture AM gliv je dolgotrajni postopek, ki je lahko odvisen od številnih dejavnikov, med drugimi vrste oz. genotipa glive, rastne sezone, gostiteljskih rastlin in rastnih razmer. Tudi sporulacija (tvorba spor) je pri AM glivah zelo nepredvidljiva in odvisna od vrste glive. Namen vseh teh postopkov je zagotovitev zadostne količine materiala (nepoškodovanih, živih spor) za njihovo izolacijo in identifikacijo, saj za opis morfološke vrste AM gliv ne zadostuje ena sama spora, ampak jih potrebujemo vsaj nekaj deset za pripravo preparatov in še več za kasnejše shranjevanje glivnega materiala v banko gliv (Oehl in sod., 2011), kar je pogoj za opis nove vrste (Slika 2). Običajno se shranjuje posušen substrat s sporami v suhem, temnem in hladnem prostoru oz. se vzdržuje aktivno kulturo AM gliv, skupaj z živimi rastlinskimi simbionti v rastlinjaku, kar pa je časovno in finančno zelo zahtevno.

Taksonomske in ekološke raziskave AM gliv na podlagi morfoloh znakov so torej zelo težavne iz več razlogov. Če povzamemo, so glavne omejitve: (1) veliko taksonov AM gliv ne moremo gojiti v čistih (lončnih) kulturah, obenem je glive nemogoče identificirati na podlagi kolonizacije v koreninah (Slika 3), (2) zahteve za rast in sporulacijo različnih taksonov AM gliv so zelo raznolike in kompleksne, (3) pri številnih taksonih (še) nismo uspeli izolirati njihovih spor in tako morfološko določene vrste povezati s podatki, ki izvirajo iz molekulskih raziskav na osnovi DNA, (4) taksonomsko določanje AM gliv je zelo zapleteno in zahteva kompleksno ekspertno znanje, ki je



Slika 2: Spore dveh različnih vrst AM gliv iz rodu *Rhizophagus* (levo, sredina). Spora AM glive s strto celično steno iz rodu *Acaulospor*a (desno). Vidna je večplastna, strukturirana celična stena. Morfološke značilnosti celične stene, njenih plasti ter pritrjenih hif služijo kot taksonomski znaki v taksonomiji AM gliv (foto: I. Maček)
Figure 2: Spores of two different AM fungal species from the genus *Rhizophagus* (left, centre). Crushed AM fungal spore of the genus *Acaulospor*a with visible multi-layered structured cell wall (right). The morphological structures of the cell wall, its layers and attached hyphae serve as taxonomic features in the taxonomy of AM fungi (photo: I. Maček)

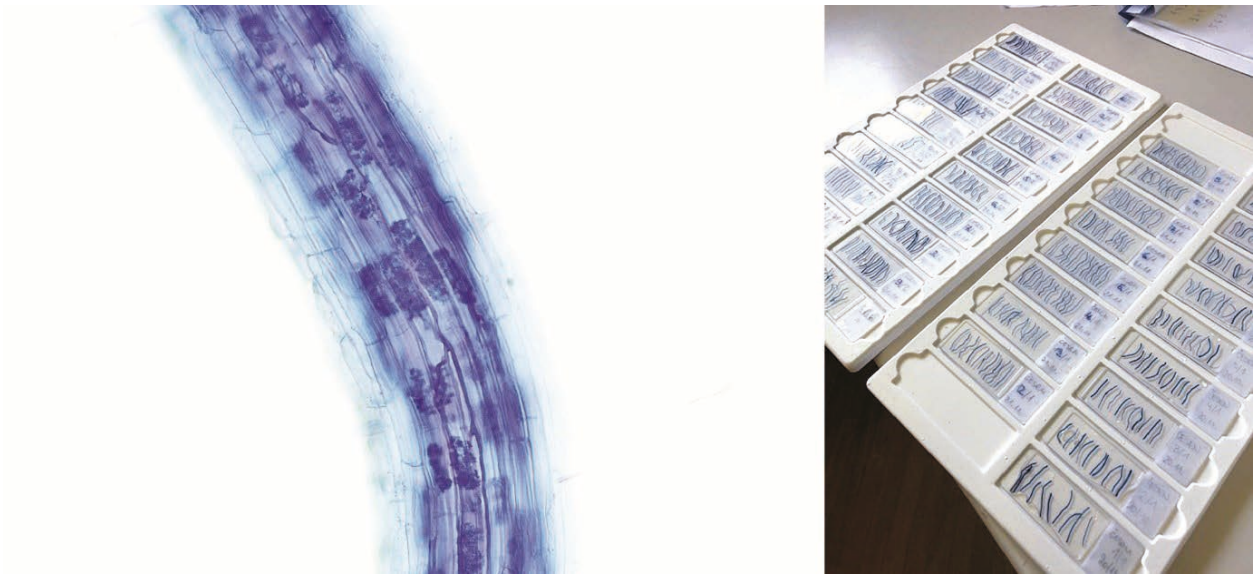
kljub temu, da na to temo obstaja kar nekaj strokovne literature (Oehl in sod., 2011, Blaszkowski, 2012), omejeno na samo nekaj strokovnjakov (Slika 2), (5) taksonomski znaki različnih skupin AM gliv se lahko tudi prekrivajo, oz. ni znano, če lahko npr. ista gliva v različnih okoljih tvori tudi več različnih morfoloških tipov spor.

Prav zato je razvoj molekulskih metod zelo pomemben za razumevanje ekologije in diverzitete AM in drugih endofitskih gliv. Ena izmed prednosti tega pristopa je tudi ta, da lahko DNA izoliramo direktno iz korenin (Slika 3), s tem pa dobimo podatke o združbi gliv, ki je fiziološko aktivna in v času vzorčenja v dejanski interakciji z rastlinami. Molekulske metode in njihov hitri razvoj tako predstavljajo pravo revolucijo v razumevanju teh organizmov in so še vedno med najbolj obetavnimi orodji za raziskave združb endofitskih gliv (Clapp in sod., 2003, Dickie & FitzJohn, 2007, Dumbrell in sod., 2017, Sinanaj in sod., 2021).

2 MOLEKULSKA IDENTIFIKACIJA AM GLIV V OKOLJSKIH VZORCIH IN GENSKI MARKERJI

Dejstvo je, da obstaja relativno malo vrst oz. taksonov AM gliv, ki jih lahko gojimo v lončnih kulturah. Pri številnih genotipih AM gliv, ki jih poznamo samo na osnovi molekulskih označevalcev izolirane DNA iz okoljskih študij, raziskovalci še nismo uspeli izolirati spor ali celo ugotoviti, če ti taksoni sploh sporulirajo v njihovem naravnem okolju. Taki genotipi so poznani samo na osnovi nukleotidnih zaporedij oz. sekvenc določenih genskih označevalcev. V okoljskih študijah se kot ustrezni označevalec za raziskave raznolikosti in ekologije združb AM gliv največkrat uporablja gen *18S rRNA* za malo podenoto ribosoma (SSU) (Simon in sod., 1992, Helgason in sod., 1998; Maček in sod., 2019), v zadnjem času pa v manjšem obsegu tudi regija notranjega prepisanega vmesnika – ITS (nuclear ribosomal internal transcribed spacer region) (npr. Alzarhani in sod., 2019) (Slika 4). Slednja se največkrat uporablja v primeru, da so v raziskavo vključene tudi druge skupine gliv (ne samo AM glive), za katere je regija ITS bolj primeren označevalec kot *18S rRNA*, obenem pa uporaba ene same regije za vse zmanjša stroške raziskave (uporaba enega označevalca, namesto dveh, čeprav regija ITS ni optimalno za identifikacijo AM gliv) (Alzarhani in sod., 2019).

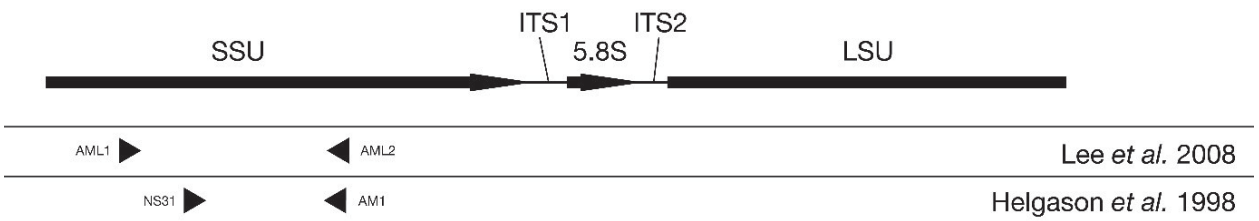
Molekulsko določanje omogoča številčno ovrednotenje (kvantifikacijo) taksonov AM gliv v tleh ali v koreninah rastlin. Geni, ki kodirajo to *18S rRNA* (ali *18S SSU*) genomsko regijo, so dostopni v velikem številu kopij in vsebujejo veliko ohranjenih kot tudi variabilnih regij, kar omogoča ločevanje taksonov na različnih ravneh. Sekvence male podenote ribosoma (*18S SSU*) se v ekologiji AM gliv uporabljajo nekje od začetka devetdesetih let prejšnjega stoletja (Simon in sod., 1992). Do



Slika 3: Kolonizacija korenin koruze (*Zea mays* L.) z AM glivami (levo) in mikroskopski preparati za oceno stopnje kolonizacije korenin z AM glivami (desno). Na sliki levo so vidne znotraj-koreninske hife AM gliv in arbuskuli (drevescom-podobne strukture), ki so pomembne za izmenjavo hranil med rastlinami in glivami (foto: I. Maček)
Figure 3:. Arbuscular mycorrhizal fungi colonising maize (*Zea mays* L.) roots (left), with visible intraradical hyphae and arbuscules (tree-like structures) important for nutrient exchange between plants and fungi. Slides for microscopy and estimation of AM fungal colonisation in plant roots (right) (photo: I. Maček)

danes je bilo objavljenih kar nekaj različnih začetnih oligonukleotidov za pomnoževanje odsekov sekvenc, specifičnih za različne skupine AM gliv, v postopku verižne reakcije s polimerazo (PCR). Najpogosteje uporabljen par začetnih oligonukleotidov male podenote ribosoma v okoljskih raziskavah je AM1 (Helgason in sod., 1998) in NS31 (Simon in sod., 1992), kjer je dolžina pomnoženega fragmenta DNA približno 550 baznih parov. Za ta par začetnih oligonukleotidov je značilno tudi za AM glive nespecifično pomnoževanje drugih fragmentov, sploh, kadar je glivne DNA v okolju oz. ekstraktu malo. To je bil tudi povod za izdelavo novih začetnih oligonukleotidov, kot je par AML1-AML2 (Lee in sod., 2008; dolžina pomnoženega fragmenta DNA je okrog 800 baznih parov) (Slika 4). Regija vsebuje tudi začetno regijo pomnoževanja oligonukleotidov AM1-NS31, in pomnožuje širok nabor taksonomskih skupin AM gliv, ne pa vseh – izključujeta na primer skupino Paraglomeraceae (Lee in sod., 2008).

Ribosomska DNA je v posamezni spori AM gliv zelo polimorfna, kar pomeni, da so sekvence rRNA gena med posameznimi taksoni AM gliv močno variabilne (Tisserant in sod., 2013). Dokazano je tudi, da hife in spore AM gliv vsebujejo na stotine jeder (Tisserant in



Slika 4: Shematski pregled najpogosteje uporabljenih parov začetnih oligonukleotidov, ki se uporabljajo v ekoloških raziskavah AM gliv (NS31-AM1 in AML1-AML2). Na vrhu slike so prikazani molekulski označevalci jedrne ribosomske RNA – mala podenota ribosoma (18S SSU), velika podenota ribosoma (28S LSU), 5.8S medgenski vmesnik (IGS) in notranji prepisani vmesnik (ITS). Trikotne puščice prikazujejo smer in mesto za prijemanje začetnih oligonukleotidov
Figure 4: Schematic of the most common primer pairs and DNA regions used in the community ecology of AM fungi (NS31--AM1 and AML1-AML2). At the top of the figure are shown molecular markers of nuclear ribosomal RNA (rRNA) – small ribosomal subunit (18S SSU), large ribosomal subunit (28S LSU), 5.8S intergenic spacer (IGS) and the internal transcribed spacer (ITS). The arrows indicate the direction and alignment range of the primers

sod., 2013). Zaradi polimorfizma trenutno razpoložljivih genskih označevalcev je težko določiti vrsto AM gliv z molekulskimi metodami. V večini študij združb AM gliv različne taksone identificiramo kot skupine sorodnih sekvenc (npr. operativne taksonomske enote – OTU), ki pa verjetno bolj kot nivoju posamezne vrste AM gliv ustrezajo posameznim rodovom (npr. Krüger in sod., 2009). Največ okoljskih raziskav raznolikosti AM gliv je bilo izvedenih s pomnoževanjem regije 18S rRNA (npr. Schwarzott & Schuβler, 2001; Helgason in sod., 2002; Vandenkoornhuyse in sod., 2002; Öpik in sod., 2006), najprej z uporabo pirosekvenciranja s platformo Roche 454 GS-FLX (npr. Öpik in sod., 2009, Dumbrell in sod., 2011), kasneje pa tudi s z določanjem nukleotidnega zaporedja (sekvenciranjem) s platformo Illumina (npr. Alzarhani in sod. 2019; Maček in sod., 2019, Davison in sod., 2021). Trenutno poznamo več kot 300 morfotipov AM gliv, določenih na podlagi morfologije spor (http://www.amf-phylogeny.com/). Na podlagi molekulskih analiz lahko ocenimo, da je število različnih taksonov AM gliv v okolju bistveno večje, kot kažejo taksonomske študije na podlagi morfoloških znakov. Virtualna taksonomija (virtualni taksoni – VT) AM gliv, ki je bila vzpostavljena v specializirani bazi podatkov s področja raziskav AM gliv, ki se imenuje MaarjAM (Öpik in sod., 2010), tudi bazira na uporabi genskega markerja 18S rRNA. Enote, definirane znotraj tega sistema, so t.i. virtualni taksoni (VT), ki po navedbah avtorjev baze MaarjAM verjetno predstavljajo monofiletske skupine AM gliv, pri katerih podobnost sekvenc znotraj skupine presega dogovorno določeno mejo 97 %. Resolucija VT naj bi približno ustrezala tisti, ki definira vrste AM gliv, določene na podlagi morfoloških znakov (Öpik & Davison, 2016). Baza MaarjAM (http://maarjam.botany.ut.ee) vsebuje več kot 450 virtualnih taksonov, identificiranih na podlagi sekvenc za malo podenoto ribosoma (18S rRNA) (Öpik in sod., 2014), vendar se številke spreminjajo oz. so večje, odvisno, katere genske označevalce uporabimo za identifikacijo taksonov. Klasifikacija sekvenc genov 18S rRNA z uporabo virtualnih taksonov (VT), ki so določeni na podlagi baze MaarjAM (Öpik in sod., 2009, 2010), nam omogoča poenoten sistem poimenovanja genotipov, ki jih lahko v ekoloških študijah uporabimo za najboljši približek identifikaciji do nivoja vrst oz. rodov (Öpik in sod., 2014).

Pogosta kritika uporabe molekulskih metod za številčno ovrednotenje mikrobnih združb so tudi napake posamezne metode, ki lahko vplivajo na končni rezultat analize. Ena izmed najpogosteje omenjenih napak je povezana z metodo PCR in neenakomernim pomnoževanjem DNA različnih taksonov znotraj združbe (t.i. PCR bias ali PCR pristranskost). PCR je ključni del praktično vseh molekulskih pristopov za analizo mikrobnih združb, vključno z združbami AM gliv. Znotraj izolata DNA iz vzorca korenin namreč glivna DNA predstavlja le majhen del celokupne ekstrahirane DNA, zato je potrebno specifično pomnoževanje (amplifikacija) regij DNA, ki so informativne za ločevanje različnih taksonov AM gliv. Odvisno od ciljev in namena raziskave se za to lahko uporablja različne genske označevalce, najpogosteje pa je v ekoloških raziskavah AM gliv uporabljena že omenjena regija 18S rRNA. Potencialne napake oz. pristranskost metode PCR za pomnoževanje specifičnih taksonov (genotipov) AM gliv ter primernost uporabe presejalne metode polimorfizma dolžine terminalnih restrikcijskih fragmentov (TRFLP) za kvantitativne raziskave je bila za regijo 18S rRNA AM gliv testirana v študiji Cotton in sod. (2014). V raziskavi so potrdili, da pri uporabi metod PCR za gene 18S rRNA ne prihaja do bistvenih razlik v pomnoževanju DNA med različnimi genotipi AM gliv in se zato te metode lahko uporablja tudi za kvantitativne analize združb AM gliv, zavedati pa se moramo določenih omejitev. Napake v pomnoževanju regij DNA z metodo PCR se lahko zgodijo, če se različni genotipi pomnožujejo različno hitro zaradi razlik v njihovih nukleotidnih zaporedjih ali zaradi same kinetike reakcije pomnoževanja (Kanagawa, 2003). Argument, da se to ne pojavlja pri AM glivah ob uporabi najpogosteje uporabljenih začetnih oligonukleotidov AM1 in NS31 je ta, da so regije DNA, na katere se ti oligonukleotidi vežejo zelo konzervativne (ohranjene med sorodstveno oddaljenimi taksoni) in imajo obenem zelo majhno variabilnost v vsebnosti baz gvanina (G) in citozina (C) v amplikonih (Dumbrell in sod., 2010). Odsotnost tovrstnih napak (pristranskosti pri pomnoževanju med različnimi genotipi) pri reakciji PCR, je eden izmed osnovnih pogojev, da je neka analiza lahko kvantitativna in obenem omogoča oceno različnih indeksov pestrosti oz. diverzitetnih indeksov (Cotton in sod., 2014).

V nadaljnjih testih so uporabili tudi analizo TRFLP za karakterizacijo umetno ustvarjene združbe z znanimi razmerji količin vhodne DNA različnih genotipov AM gliv. Podatki študije kažejo, da je protokol TRFLP v kombinaciji s PCR močno in konsistentno orodje za analizo združb AM gliv, medtem, ko na nivoju analiz populacij posameznih taksonov (genotipov) avtorji študije priporočajo več previdnosti (Cotton in sod., 2014). Ta eksperiment je torej potrdil hipotezo, da je možno z uporabo PCR in metodo TRFLP ustrezno ugotoviti relativne razlike med različnimi združbami AM gliv, v smislu njihove diverzitete in sestave. Študija je tudi pokazala, da pri pomnoževanju različnih genotipov AM gliv za gene 18S rRNA metoda PCR posameznih genotipov ne pomnožuje bolj, kot drugih, kar pomeni, da se ta metoda lahko uporablja tudi v sklopu drugih kvantitativnih analiz združb AM gliv, kjer predstavlja metoda PCR pomemb-

no komponento. Mednje vsekakor sodijo tudi vse metode sekvenciranja naslednjih generacij (NGS), ki so danes za raziskave ekologije združb AM gliv največ v uporabi in katerih sestavni del je tudi pomnoževanje sekvenc s PCR (npr. platforma Illumina).

2.1 UPORABA KLONIRANJA IN DOLOČANJA NUKLEOTIDNEGA ZAPOREDJA PO SANGERJU TER PRESEJALNIH METOD

V času nastanka prvega preglednega članka o uporabi molekulskih pristopov pri raziskavi AM gliv (Maček, 2009) se je za raziskave sestave mikrobnih združb, vključno z združbami rizosfernih AM gliv iz okoljskih vzorcev (tal ali korenin), v veliki meri uporabljalo molekulske pristope, ki so temeljili na postopkih kloniranja in določanja nukleotidnega zaporedja po Sangerju. Če povzamemo na kratko, tak pristop običajno vsebuje naslednje korake: (1) pomnoževanju glivne DNA z reakcijo PCR, (2) ‚*in vivo*' ločevanje pomnoženih fragmentov DNA s kloniranjem pomnožkov (amplikonov) v plazmide in uporabo kolonij bakterij vrste *Escherichia coli* za ločevanje sekvenc, (3) ponovna/sekundarna reakcija PCR za namen ločevanja sekvenc, potrebnega za uporabo (4) sekventorjev prve generacije (sekvenciranje po Sangerju) (glej opis postopka v Maček, 2009). Vsi našteti koraki, predvsem pa kloniranje in uporaba celičnih kultur, so predstavljali velik časovni in finančni zalogaj, zato je bilo število vzorcev (biološke ponovitve), kot tudi število sekvenc, ki so bile sekvencirane za posamezni vzorec (globina sekvenciranja), zelo omejeno. Za posamezno okoljsko študijo sestave združbe AM gliv smo na tak način tipično lahko pregledali nekje med 500 in 1000 sekvenc za posamezni genski marker (npr. fragment 18S rRNA). Posledica tega je bila, da so bile združbe opisane zelo pomanjkljivo. Lahko se namreč zgodi, da na ta način nevede izpustimo tiste taksone, ki so znotraj združbe manj pogosti (redki), združbo pa zaradi metodoloških omejitev opisujemo zgolj na podlagi bolj dominantnih in bolj številčnih predstavnikov skupine.

Vmesna faza med zgoraj opisanim postopkom in novimi postopki sekvenciranja naslednjih generacij (NGS) je bila uporaba različnih presejalnih metod, ki temeljijo na primerjavah t.i. prstnih odtisov združbe oz. ločevanju produktov PCR z dvojno vijačnico podobne dolžine, vendar z različno sekvenco. Te metode so omogočile zajem nekoliko večjega števila vzorcev in tudi večjo globino vzorčenja (število obravnavanih sekvenc). Mednje sodijo metode, kot so denaturacijska gradientna gelska elektroforeza (DGGE), temperaturna gradientna gelska elektroforeza (TGGE) in že omenjeni polimorfizem dolžine terminalnih restrikcijskih fragmentov (TRFLP). Med naštetimi metodami ima TRFLP največjo zmogljivost v smislu možnosti obdelave večjega števila bioloških vzorcev, medtem, ko so tehnike gelske elektroforeze (DGGE, TGGE) zamudne in omogočajo obdelavo le manjšega števila vzorcev. To pomeni, da so manj uporabne za ekološke študije, kjer je med vzorci običajno velika variabilnost in je zato potrebno v študijo vključiti veliko število vzorcev. Slaba stran vseh teh metod je tudi ta, da so primarno presejalne narave, zato v končni fazi običajno nimamo vpogleda v sekvence posameznega markerskega gena raziskovanih organizmov. Tako metodo lahko zato uporabljamo samo za relativne primerjave sestave združb in biodiverzitete med primerjanimi vzorci znotraj ene študije, ne omogočajo pa primerjave različnih študij med sabo. Tako je bila pred razvojem in širšo uporabo metod NGS relativno pogosto uporabljana analiza za relativne primerjave sestave združb organizmov iz okoljskih vzorcev preučevanje polimorfizma dolžine terminalnih restrikcijskih fragmentov (TRFLP) tarčnih genov (prokariontskih 16S ali evkariontskih 18S rRNA), ki v nekaterih primerih omogoča tudi kvantitativne študije. Metoda je bila pogosto uporabljana tudi na področju mikrobne ekologije in okoljske mikrobiologije, predvsem zaradi cenovne dostopnosti, filogenetske ločljivosti in enostavne analize večjega števila vzorcev.

Analiza TRFLP ima torej dovolj veliko zmogljivost (omogoča obdelavo zadostnega števila vzorcev), da lahko z njo preučimo tudi vplive različnih okoljskih dejavnikov na strukturo in dinamiko mikrobnih združb. Na tak način dobimo t.i. ‚prstni odtis' (finger-print) združbe raziskovane skupine organizmov za analizirane vzorce, zato tehniko TRFLP imenujemo tudi tehnika prstnih odtisov. Take podatke lahko uporabimo za analizo diverzitete (izračun različnih indeksov raznolikosti) in sestave združbe, pri čemer lahko izvajamo primerjave med vzorci, zajetimi znotraj ene študije, ne pa tudi med različnimi študijami, kar je pomanjkljivost tehnik, ki uporabljajo t. i. tehniko prstnih odtisov. Poznati moramo tudi specifike posamezne preučevane skupine organizmov in omejitve uporabe posamezne metode za to skupino. Primernost metode TRFLP so testirali tudi za kvantitativne analize združb AM gliv, pri tem pa avtorji študije opozarjajo, da tehnika TRFLP v vseh testiranih vzorcih precenjuje vrstno pestrost AM gliv znotraj raziskovane združbe, zato je pri kvantitativnem vrednotenju indeksov raznolikosti potrebna pozornost pri interpretaciji s to tehniko pridobljenih rezultatov (Cotton in sod., 2014). Sama metoda TRFLP ne producira celotnih sekvenc posameznih organizmov znotraj združbe, se pa lahko s TRFLP pridobljeni podatki primerjajo z obstoječo bazo sekvenc, če želimo pridobiti podatke o identiteti posmeznega vzorca (Dickie & FitzJohn, 2007, Roberts in sod., 2012). Danes so tehniko TRFLP v raziskavah ekologije združb mik-

roorganizmov v veliki meri nadomestile tehnike NGS, katerih velika prednost je, da končne izhodne podatke predstavljajo celotne sekvence posameznih amplikonov, kar omogoča lažjo in neposredno identifikacijo organizmov, ki pa je odvisna od informativnosti regije, ki jo sekvenciramo za posamezno skupino organizmov. Kot že rečeno, naj bi regija 18S rRNA pri AM glivah približno ustrezala nivoju morfološke vrste, se pa to področje še zelo intenzivno raziskuje in lahko v prihodnosti pričakujemo novosti.

2.2 DOLOČANJE NUKLEOTIDNEGA ZAPOREDJA (SEKVENCIRANJE NASLEDNJIH GENERACIJ – NGS)

Metode NGS so najprej uporabljali v medicini in pri poskusih sekvenciranja človeka in drugih primatov (Wheeler in sod., 2008). Relativno hitro so se te metode razširile tudi na področje ekoloških raziskav in raziskav mikrobnih združb (mikrobiomov) v različnih okoljih, saj so omogočale vključevanje bistveno večjega števila vzorcev v raziskavo in obenem bistveno večjo globino sekvenciranja znotraj posameznega vzorca (najprej od več sto tisoč pa vse do več sto milijonov sekvenc pri danes uporabljanih pristopih). Ti novi principi so močno vplivali na številna področja v mikrobni ekologiji, okoljski mikrobiologiji, ekologiji tal in raziskavah rizosfere, kot tudi pri raziskavah različnih interakcij med organizmi, vključno z interakcijami rastlin z drugimi organizmi (npr. mikoriza).

Danes je tudi v okoljskih raziskavah pogosta praksa pomnoževanja tarčnih filogenetskih in/ali funkcionalnih genskih označevalcev in obenem uporaba pristopov NGS za karakterizacijo njihove raznolikosti. Nova tehnologija omogoča vse številčnejše sete vzorcev, kar je bistvenega pomena za ekološke raziskave, ki se lahko izvajajo v različnih dimenzijah, tako prostora, kot tudi časa (vzorčenja v več prostorskih in časovnih točkah, raziskave dinamike procesov in sukcesije). Zavedati pa se moramo, da so prav vsi pristopi NGS podvrženi metodološkim napakam oz. so lahko pristranski, pri čemer gre na eni strani za produkcijo visoko kvalitetnih podatkov, ki jih zahtevajo raziskave, in na drugi napačnih sekvenc ter metodološkega šuma. Vsi pristopi z uporabo NGS zato zahtevajo natančno bioinformacijsko analizo in procesiranje podatkov, kar omogoča kvalitativno filtriranje in procesiranje sekvenc z namenom izogibanja zavajajočih interferenc iz metodoloških napak. Podobna previdnost je potrebna tudi pri vseh nadaljnjih statističnih analizah pridobljenih podatkov, saj lahko nepravilen izbor statističnega pristopa rezultira v napačnih zaključkih raziskave.

Prav s tem namenom je nastalo tudi kar nekaj preglednih objav oz. pregleda metodoloških pristopov, ki sistematično podajajo navodila za lažje spopadanje z izzivom obdelave podatkov, ki izhajajo iz NGS (npr. za osnovno analizo podatkov, ki izhajajo iz sekvenciranja amplikonov z namenom raziskav raznolikosti in ekologije združb AM gliv (Dumbrell in sod., 2017). Tipično bioinformacijski pristopi vključujejo metode za preverjanje kakovosti sekvenc in odstranjevanja šuma, formiranje operacijskih taksonomskih enot (Operational Taxonomic Unit – OTU), taksonomsko določanje posameznih sklopov sekvenc ter osnovne statistične analize za testiranje hipotez. Prikaz teh metod za dva pogosto uporabljena pristopa (QIIME in mothur), skupaj s samostojnimi orodji (vključno z odprtokodnim programskim okoljem ter jezikom R), so predstavljene v objavi Dumbrell in sod. (2017), kjer so predstavljeni pristopi za obdelavo podatkov sekvenciranja amplikonov, ki izhajajo iz dveh pogosto uporabljenih tehnologij NGS, v preteklosti več uporabljane tehnologije 454-pirosekvenciranja ter danes široko uporabljane platforme Illumina.

Zaradi hitrega napredka tehnologije in z njo povezane bioinformatike pa je nujno redno spremljanje tekočih objav in drugih virov na to temo. Zaradi obsežnosti in kompleksnosti podatkov, ki izhajajo iz tehnik sekvenciranja amplikonov z NGS je pogost izziv tudi njihova ustrezna predstavitev. Izziv, ki se skriva v novih tehnologijah je predvsem ta, da nas lahko zaslepi analitična moč novih metod, obenem pa je naše zavedanje o tem, da zaradi hitrega razvoja teh pristopov lahko pridemo tudi do napačnih zaključkov, pomanjkljivo. Napačni zaključki so lahko rezultat napak v sami tehnologiji, pomanjkljivega testiranja metod in/ali pomanjkanja izkušenj. Nujno je, da nek problem oz. študijo že v začetni fazi načrtovanja naslovimo z jasnimi vprašanji, posledica katerih so tudi jasno zastavljene raziskovalne hipoteze in ciljni metodološki pristopi, ki izhajajo iz teh hipotez. Vsekakor pa predstavljajo metode NGS ob pravilni uporabi močno analitično orodje, ki odpira povsem nove možnosti v raziskavah ekologije tako bolj vidnih in karizmatičnih nadzemnih organizmov, kot tudi bolj skritih, a pomembnih akterjev v podzemnem delu kopenskih ekosistemov.

3 VPLIV OKOLJSKIH IN GEOGRAFSKIH DEJAVNIKOV NA SESTAVO ZDRUŽB AM GLIV IN NJIHOVO RAZŠIRJENOST

Znano je, da sestavo združb AM gliv določajo različni dejavniki okolja, vključno s klimatskimi in talnimi specifikami, ki delujejo tako na lokalni, kot tudi na globalni ravni (npr. Dumbrell in sod., 2010, Kivlin in sod.,

2011, Lekberg in sod., 2011, Maček in sod., 2011, Hazard in sod., 2013, Davison in sod., 2015, Maček in sod., 2019, Vetrovsky in sod., 2019, Davison in sod., 2021). Na splošno je pri pojavljanju različnih taksonov AM gliv še vedno relativno malo dostopnih informacij na ravni odnosa posameznega organizma in okolja (Davison in sod., 2020). Po podatkih iz številnih študij, ki so zbrane v bazi MaarjAM (Öpik in sod., 2010) lahko vidimo, da so mnogi virtualni taksoni (VT) AM gliv široko geografsko razširjeni in se pojavljajo v številnih habitatnih tipih (Davison in sod., 2015, Savary in sod., 2018). Taka opažanja pa so nastala večinoma na podatkih o prisotnosti taksonov v določenem habitatu, ni pa podatkov v kolikšni meri številčnost (abundanca) posameznih VT variira vzdolž gradienta določenega abiotskega dejavnika. Taksone AM gliv so v preteklosti klasificirali v različne ekotipe (Alzarhani in sod., 2019) ter v ekološke skupine, kot so generalisti in specialisti na osnovi različnih rangov, ki vključujejo geografske dejavnike (Moora in sod., 2011, Bouffaud in sod., 2016), habitat (Sykorova in sod., 2007, Oehl in sod., 2010, Vályi in sod., 2015) ali rastlinske gostiteljske vrste (Helgason in sod., 2007). Poudariti pa je treba, da je tovrstno klasificiranje taksonov v posamezne ekološke niše omejeno na obseg okoljskih dejavnikov, ki jih pokriva posamezna študija in posplošenje tega pojava ni mogoče. V nekaterih novejših objavah poročajo, da so si določene funkcionalne lastnosti med sorodnimi morfološkimi vrstami AM gliv podobne (Powell in sod., 2009, Hoeksema in sod., 2018). Dejstvo pa je, da predstavljajo morfološko opisane vrste AM gliv, identificirane na podlagi morfologije celične stene njihovih spor, le majhen del raznolikosti AM gliv, ki so bile določene na osnovi molekulskih označevalcev (markerskih genov) (Öpik in sod., 2014). V zelo obsežni nedavni študiji, ki je vključevala več kot 300 talnih vzorcev iz različnih naravnih ekosistemov iz celega sveta so ugotovili, da so porazdelitev VT v različnih ekosistemih skupaj pojasnile tako okoljske, kot tudi prostorske (geografske) variable. Med njimi sta bili temperatura okolja in vrednost pH tal najpomembnejša okoljska določevalnika porazdelitve in lokalne relativne abundance (številčnosti) različnih taksonov AM gliv (Davison in sod., 2021). V študiji so ugotovili tudi različne vzorce ekoloških niš, ki so bili najbolj opazni na ravni družin AM gliv, kar kaže, da sorodni taksoni oz. VT zavzemajo podobne ekološke niše. Za predstavnike družine AM gliv Acaulosporaceae je tako značilen optimum pri manjši vrednosti pH tal, in nižji temperaturi, medtem, ko so predstavniki družine Gigaporaceae bolj številčni v bolj vlažnih območjih z več dežja (Davison in sod., 2021). Na to temo pa bo v prihodnjem desetletju sigurno še veliko novih podatkov, tudi v luči vpliva klimatskih sprememb na AM glive in njihovo simbiozo z rastlinami (Maček in sod., 2019).

4 ZAKLJUČEK

Razumevanje vpliva globalnih sprememb na terestrične ekosisteme zahteva povezovalen pristop med različnimi disciplinami, ki raziskuje odzive skozi vse ravni biološke organizacije in skozi različne prostorsko-časovne skale. Obenem mora ta pristop vključevati tako nadzemno, kot tudi podzemno raznolikost znotraj ekosistemov, saj sta obe neločljivo povezani in prepleteni. V večini primerov še vedno ni celostnega razumevanja odziva interakcij komponent nadzemne in podzemne raznolikosti na akutne kratkoročne (npr. suša, toplotni valovi) ter kronične dolgoročne globalne in klimatske spremembe (npr. segrevanje, povečana koncentracija CO_2, onesnaženje). Prav zato so nujno potrebni eksperimenti, ki naslavljajo te vrzeli v razumevanju delovanja ekosistemov. Nove metode sekvenciranja omogočajo veliko ponovljivost in s tem dokaj robusten pristop k raziskavam v ekologiji, predvsem v smislu velikega števila bioloških ponovitev (zadosti ponovitev za ustrezno analizo podatkov), obravnavo vseh ravni ekoloških združb (nadzemnega in podzemnega – rizosfernega), in v smislu primernega trajanja eksperimenta (zaželene so dolgoročne študije, ki zajemajo vzorčenje preko več sezon ali celo let). Slednje je nujno za razumevanje in ločevanje dolgoročnih in kratkoročnih odzivov na vseh ravneh biodiverzitete.

Najnovejša molekulska orodja za raziskave v ekologiji združb, kot so metode NGS, ki omogočajo pridobivanje podatkov o sekvencah organizmov iz različnih okolij, postavljajo vse cenejše in široko dostopne. Pri tem pa je nujno zavedanje, da kljub široki dostopnosti, kvalitetna biološka interpretacija molekulske karakterizacije združb posameznih skupin organizmov zahteva veliko specialističnega znanja o specifični skupini organizmov. Zavedati se moramo na primer, da tako tehnika TRFLP, kot tudi NGS, predstavljata analize relativne številčnosti (abundance) taksonov znotraj vzorcev, zato ju ne smemo uporabljati za ugotavljanje razlik v absolutni številčnosti med posameznimi vzorci. Na koncu se moramo zavedati tudi dejstva, da lahko podajajo ocene sestave združb, ki temeljijo na ekstraktih DNA, le podatke o genski raznolikosti in genski sestavi združb. Nemogoča je namreč ocena biomase posameznih vrst teh organizmov v nekem okolju na osnovi takih podatkov zaradi medvrstne in časovne variabilnosti števila kopij posameznih genov na enoto rasti, kar posebej velja za nitaste organizme, kot so AM glive (Corradi in sod., 2007, Jansa in sod., 2008). Bistveno je torej, da se obenem zavedamo tako moči tehnologije, da v kratkem času producira ogromno količino podatkov, kot tudi vseh omejitev in novosti, vključno s hitro spreminjajočimi se postopki bioinformatike, ki je neločljivo povezana z vsakim postopkom NGS. Tak pris-

top v kar največji možni meri zmanjša možnost napačne interpretacije podatkov, ki jih pridobimo z NGS, in obenem poveča trajno znanstveno vrednost in odmevnost našega dela.

5 ZAHVALA

Raziskovalno delo, povezano z vsebino tega članka, poteka v okviru programske skupine P4-0085, ARRS.

6 REFERENCE

Alzarhani, A.K., Clark, D.R., Underwood, G.J., Ford, H., Cotton, T.A., and Dumbrell, A.J. (2019). Are drivers of root-associated fungal community structure context specific? *ISME Journal, 13*, 1330–1344. https://doi.org/10.1038/s41396-019-0350-y

Besiana, S., Hoysted, G.A., Pressel, S., Bidartondo, M.I., and Field, K.J. (2021). Critical research challenges facing Mucoromycotina 'fine root endophytes. *New Phytologist, Forum*, 1–7. https://doi.org/10.1111/nph.17684

Bouffaud, M.L., Creamer, R.E., Stone, D., Plassart, P., van Tuinen, D., Lemanceau, P., Wipf, D., and Redecker, D. (2016). Indicator species and co-occurrence in communities of arbuscular mycorrhizal fungi at the European scale. *Soil Biology and Biochemistry*, *103*, 464–470. https://doi.org/10.1016/j.soilbio.2016.09.022

Błaszkowski J. (2012). *Glomeromycota*. W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków., 300 pp.

Brachmann, A. and Parniske, M. (2006). The most widespread symbiosis on earth. *PLoS Biol 4*, e239. DOI: 10.1371/journal.pbio.0040239. https://doi.org/10.1371/journal.pbio.0040239

Clapp, J.P., Helgason, T., Daniell, T.J., and Young, J.P.W. (2003). Chapter 8: Genetic studies of the structure and diversity of arbuscular mycorrhizal fungal communities. In: van der Heijden, M.G.A. and Sanders, I.R. (eds.). *Mycorrhizal Ecology (Ecological Studies 157)*. Germany: Springer., pp. 201–221. https://doi.org/10.1007/978-3-540-38364-2_8

Corradi, N., Croll, D., Colard, A., Kuhn, G., Ehinger, M., and Sanders, I.R. (2007). Gene copy number polymorphisms in an arbuscular mycorrhizal fungal population. *Applied an Environmental Microbiology, 73*(1), 366–369. https://doi.org/10.1128/AEM.01574-06

Cotton, T.E., Dumbrell, A.J., Helgason, T. (2014). What goes in must come out: testing for biases in molecular analysis of arbuscular mycorrhizal fungal communities. *PLoS One, 9*(10), *e109234*, doi: 10.1371/journal.pone.0109234. https://doi.org/10.1371/journal.pone.0109234

Davison, J., García de León, D., Zobel, M., Moora, M., Bueno, C.G., Barceló, M., Gerzm M., León, D., Meng, Y., Pillar, V.D., Sepp, S., Soudzilovaskaia, N.A., Tedersoo, L., Vaessen, S., Vahter, T., Winck, B., and Öpik, M. (2020). Plant functional groups associate with distinct arbuscular mycorrhizal fungal communities. *New Phytologist, 226*, 1117–1128. https://doi.org/10.1111/nph.16423

Davison, J., Moora, M., Öpik, M., Adholeya, A., Ainsaar, L., Bâ, A., Burla, S., Diedhiou, A.G., Hiiesalu, I., Jairus, T., Johnson, N.C., Kane, A., Koorem, K., Kochar, M., Ndiaye, C., Pärtel, M., Reier, Ü., Saks, Ü., Singh, R., Vasar, M., and Zobel, M. (2015). Global assessment of arbuscular mycorrhizal fungus diversity reveals very low endemism. *Science, 349*, 970–973. https://doi.org/10.1126/science.aab1161

Davison, J. Moora, M., Semchenko, M., Adenan, S.B., Ahmed, T., Akhmetzhanova, A.A., Alatalo, J.M., Al-Quraishy, S., Andriyanova, E., Anslan, S., Bahram, M., Batbaatar, A., Brown, C., Bueno, C.G., Cahill, J., Cantero, J.J., Casper, B.B., Cherosov, M., Chideh, S., Coelho, A.P., Coghill, M., Decocq, G., Dudov, S., Fabiano, E..C., Fedosov, V.E., Fraser, L., Glassman, S.I., Helm, A., Henry, H.A.L., Hérault, B., Hiiesalu, I., Hiiesalu, I., Hozzein, W.N., Kohout, P., Kõljalg, U., Koorem, K., Laanisto, L., Mander, U., Mucina, L., Munyampundu, J., Neuenkamp, L., Niinemets, U., Nyamukondiwa, C., Oja, J., Onipchenko, V., Pärtel, M., Phosri, C., Põlme, S., Püssa, K., Ronk, A., Saitta, A., Semboli, O., Sepp, S., Seregin, A., Sudheer, S., Peña-Venegas, C.P., Paz, C., Vahter, T., Vasar, M., Veraart, A.J., Tedersoo, L., Zobel, M., and Öpik, M. (2021). Temperature and pH define the realised niche space of arbuscular mycorrhizal fungi. *New Phytologist, 231*, 763–776. https://doi.org/10.1111/nph.17240

Dickie, I.A. and FitzJohn, R.G. (2007). Using terminal restriction fragment length polymorphism (T-RFLP) to identify mycorrhizal fungi: a methods review. *Mycorrhiza, 17*, 259–270. https://doi.org/10.1007/s00572-007-0129-2

Dumbrell, A.J., Ashton, P.D., Aziz, N., Feng, G., Nelson, M., Dytham, C., Fitter, A.H., and Helgason, T. (2011). Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. *New Phytologist, 190*, 794–804. https://doi.org/10.1111/j.1469-8137.2010.03636.x

Dumbrell, A.J., Ferguson, R.M.W., and Clark, D.R. (2017). Microbial Community Analysis by Single-Amplicon High-Throughput Next Generation Sequencing: Data Analysis - From Raw Output to Ecology. In: McGenity, T.J., Timmis, K.N., and Nogales, B. (eds) *Hydrocarbon and lipid microbiology protocols. Springer protocols handbooks.* Springer, Heidelberg, pp. 155–206. https://doi.org/10.1007/8623_2016_228

Dumbrell, A.J., Nelson, M., Helgason, T., Dytham, C., and Fitter, A.H. (2010). Relative roles of niche and neutral processes in structuring a soil microbial community. *ISME Journal, 4*, 337–345. https://doi.org/10.1038/ismej.2009.122

Field, K.J., Rimington, W.R., Bidartondo, M.I., Allinson, K.E., Beerling, D.J., Cameron, D.D., Duckett, J.G., Leake, J.R., and Pressel, S. (2015). First evidence of mutualism between ancient plant lineages (Haplomitriopsida liverworts) and Mucoromycotina fungi and its response to simulated Palaeozoic changes in atmospheric CO_2. *New Phytologist, 205*, 743–756. https://doi.org/10.1111/nph.13024

Fitter, A.H. (2005). Darkness visible: reflections on underground ecology. *Journal of Ecology, 93*, 231–243. https://doi.org/10.1111/j.0022-0477.2005.00990.x

Hazard, C., Gosling. P., Van Der Gast, C.J., Mitchell, D.T., Doohan, F.M., and Bending, G.D. (2013). The role of local environment and geographical distance in determining community composition of arbuscular mycorrhizal fungi at the landscape scale. *ISME Journal, 7*, 498–508. https://doi.org/10.1038/ismej.2012.127

Helgason T., Daniell T. J., Husband R., Fitter A. H., and Young J. P. W. (1998). Ploughing up the wood-wide web? *Nature, 394*, 431. https://doi.org/10.1038/28764

Helgason T., Merryweather J. W., Denison J., Wilson P., Young J. P. W., and Fitter A. H. (2002). Selectivity and functional diversity in arbuscular mycorrhizas of co-occurring fungi and plants from a temperate deciduous woodland. *Journal of Ecology, 90*, 371–384. https://doi.org/10.1046/j.1365-2745.2001.00674.x

Helgason, T., Merryweather, J.W., Youngm J.P.W., and Fitter A.H. (2007). Specificity and resilience in the arbuscular mycorrhizal fungi of a natural woodland community. *Journal of Ecology, 95*, 623–630. https://doi.org/10.1111/j.1365-2745.2007.01239.x

Hoeksema, J.D., Bever, J.D., Chakraborty, S., Chaudhary, V.B., Gardes, M., Gehring, C.A., Hart, M.M., Housworth, E.A., Kaonongbua, W., Klironomos, J.N., Lajeunesse, M.J., Meadow, J., Milligan, B.G., Piculell, B.J., Pringle, A., Rúa, M.A., Umbanhowar, J., Viechtbauer, W., Wang, Y., Wilson, G.W.T, and Zee, P.C. (2018). Evolutionary history of plant hosts and fungal symbionts predicts the strength of mycorrhizal mutualism. *Communications Biology, 1*, 116. https://doi.org/10.1038/s42003-018-0120-9

Hoysted, G.A., Jacob, A.S., Kowal, J., Giesemann, P., Bidartondo, M.I., Duckett, J.G., Gebauer, G., Rimington, W.R., Schornack, S., Pressel, S., and Field, K.J. (2019). Mucoromycotina fine root endophyte fungi form nutritional mutualisms with vascular plants. *Plant Physiology, 181*, 565–577. https://doi.org/10.1104/pp.19.00729

Jansa, J., Smith, F.A., and Smith, S.E. (2008). Are there benefits of simultaneous root colonization by different arbuscular mycorrhizal fungi? *New Phytologist, 177*, 779–789. https://doi.org/10.1111/j.1469-8137.2007.02294.x

Lee, J., Lee, S., and Young, J.P. (2008). Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. *FEMS Microbiology Ecology, 65*, 339–349. https://doi.org/10.1111/j.1574-6941.2008.00531.x

Lekberg, Y., Meadow, J., Rohr, J.R., Redecker, D., and Zabinski, C.A. (2011). Importance of dispersal and thermal environment for mycorrhizal communities: lessons from Yellowstone National Park. *Ecology, 92*, 1292–1302. https://doi.org/10.1890/10-1516.1

Kanagawa, T. (2003). Bias and artifacts in multitemplate polymerase chain reactions (PCR). *Journal of Bioscience and Bioengineering, 96*, 317–323. https://doi.org/10.1016/S1389-1723(03)90130-7

Kivlin, S.N., Hawkes, C.V., and Treseder, K.K. (2011). Global diversity and distribution of arbuscular mycorrhizal fungi. *Soil Biology & Biochemistry, 43*, 2294-2303. https://doi.org/10.1016/j.soilbio.2011.07.012

Knapp, D.G., Nemeth, J.B., Barry, K., Hainaut, M., Henrissat, B., Johnson, J., Kuo, A., Lim, J.H.P., Lipzen, A., Nolan, M., Ohm, R.A., Tamas, L., Grigoriev, I.V., Spatafora, J.W., Nagy, L.G., and Kovacs, G.M. (2018). Comparative genomics provides insights into the lifestyle and reveals functional heterogeneity of dark septate endophytic fungi. *Scientific Reports, 8*, 6321. https://doi.org/10.1038/s41598-018-24686-4

Krüger, M., Stockinger, H., Krüger, C., and Schüssler, A. (2009). DNA-based species level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. *New Phytologist, 183*, 212–223. https://doi.org/10.1111/j.1469-8137.2009.02835.x

Maček, I. (2009). Molekulski pristopi pri raziskavah arbuskularne mikorize. *Acta Agriculturae Slovenica, 93*(1), 77–85.

Maček, I., Clark, D.R., Šibanc, N., Moser, G., Vodnik, D., Müller, C., and Dumbrell, A.J. (2019). Impacts of long-term elevated atmospheric CO_2 concentrations on communities of arbuscular mycorrhizal fungi. *Molecular Ecology, 28*(14), 3445–3458. https://doi.org/10.1111/mec.15160

Maček, I., Dumbrell, A.J., Nelson, M., Fitter, A.H., Vodnik, D., and Helgason, T. (2011). Local adaptation to soil hypoxia determines the structure of an arbuscular mycorrhizal fungal community in roots from natural CO_2 springs. *Applied and Environmental Microbiology, 77*(14), 4770–4777. https://doi.org/10.1128/AEM.00139-11

Moora, M., Berger, S., Davison, J., Öpik, M., Bommarco, R., Bruelheide, H., Kühn I, Kunin WE, Metsis M, Rortais A., Vanatoa, A., Vanatoa, E., Stout, J.C., Truusa, M., Westphal, C., Zobel, M., and Walther G. (2011). Alien plants associate with widespread generalist arbuscular mycorrhizal fungal taxa: evidence from a continental-scale study using massively parallel 454 sequencing. *Journal of Biogeography, 38*, 1305–1317. https://doi.org/10.1111/j.1365-2699.2011.02478.x

Oehl, F., Laczko, E., Bogenrieder, A., Stahr, K., Bösch, R., van der Heijden, M., and Sieverding, E. (2010). Soil type and land use intensity determine the composition of arbuscular mycorrhizal fungal communities. *Soil Biology & Biochemistry, 42*, 724–738. https://doi.org/10.1016/j.soilbio.2010.01.006

Oehl, F., Sieverding, E., Palenzuela, J., Ineichen, K., and da Silva, G.A. (2011). Advances in Glomeromycota taxonomy and classification. *IMA Fungus, 2*, 191–199. https://doi.org/10.5598/imafungus.2011.02.02.10

Orchard, S., Standish, R.J., Dickie, I.A., Renton, M., Walker, C., Moot, D., and Ryan, M.H. (2017). Fine root endophytes under scrutiny: A review of the literature on arbuscule-producing fungi recently suggested to belong to the Mucoromycotina. *Mycorrhiza, 27*, 619–528. https://doi.org/10.1007/s00572-017-0782-z

Öpik, M. and Davison, J. (2016). Uniting species-and community-oriented approaches to understand arbuscular mycorrhizal fungal diversity. *Fungal Ecology, 24*, 106–113. https://doi.org/10.1016/j.funeco.2016.07.005

Öpik, M., Davison, J., Moora, M., and Zobel, M. (2014). DNA-based detection and identification of Glomeromycota: the virtual taxonomy of environmental sequences. *Botany-Botanique, 92*, 135–147. https://doi.org/10.1139/cjb-2013-0110

Öpik, M., Metsis, M., Daniell, T.J., Zobel, M., and Moora, M. (2009). Large-scale parallel 454 sequencing reveals eco-

logical group specificity of arbuscular mycorrhizal fungi in a boreonemoral forest. *New Phytologist, 184*, 424–437. https://doi.org/10.1111/j.1469-8137.2009.02920.x

Öpik, M., Moora, M., Liira, J., and Zobel, M. (2006). Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. *Journal of Ecology, 94*, 778–790. https://doi.org/10.1111/j.1365-2745.2006.01136.x

Öpik, M., Vanatoa, A., Vanatoa, E., Moora, M., Davison, J., Kalwij, J.M., Reier, U., and Zobel, M. (2010). The online database MaarjAM reveals global and ecosystem distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). *New Phytologist, 188*, 223–241. https://doi.org/10.1111/j.1469-8137.2010.03334.x

Öpik, M., Zobel, M., Cantero, J.J., Davison, J., Facelli, J.M., Hiiesalu, I., Jairus, T., Kalwij, J.M., Koorem, K., Leal, M.E., Liira, J., Metsis, M., Neshataeva, V., Paal, J., Phosri, C., Põlme, S., Reier, Ü., Saks, Ü., Orchard, S., Standish, R.J., Dickie, I.A., Renton, M., Walker, C., Moot, D., and Ryan, M.H. (2017). Fine root endophytes under scrutiny: a review of the literature on arbuscule-producing fungi recently suggested to belong to the Mucoromycotina. *Mycorrhiza, 27*, 619–638. https://doi.org/10.1007/s00572-017-0782-z

Powell, J.R., Parrent, J.L., Hart, M.M., Klironomos, J.N., Rillig, M.C., and Maherali, H. (2009). Phylogenetic trait conservatism and the evolution of functional trade-offs in arbuscular mycorrhizal fungi. *Proceedings of the Royal Society of London B, 276*, 4237–4245. https://doi.org/10.1098/rspb.2009.1015

Roberts, D.M., Schofield, P.G., Don, S., and Daniell, T.J. (2012). Directed terminal restriction analysis tool (DRAT): an aid to enzyme selection for directed terminal-restriction fragment length polymorphisms. *Methods in Ecology and Evolution, 3*(1), 24–28. https://doi.org/10.1111/j.2041-210X.2011.00139.x

Rodriguez, R.J., White, J.F., Arnold, A.E., and Redman, R.S. (2009). Fungal endophytes: diversity and functional roles. *New Phytologist, 182*, 314–330. https://doi.org/10.1111/j.1469-8137.2009.02773.x

Savary, R., Masclaux, F.G., Wyss, T., Droh. G., Corella, J.C., Machado, A.P., Morton, J.B., and Sanders, I.R. (2018). A population genomics approach shows widespread geographical distribution of cryptic genomic forms of the symbiotic fungus *Rhizophagus irregularis*. *ISME Journal, 12*, 17–30. https://doi.org/10.1038/ismej.2017.153

Schnitzer, S.A. and Klironomos, J. (2011). Soil microbes regulate ecosystem productivity and maintain species diversity. *Plant Signaling & Behavior, 6-8*, 1240–1243. https://doi.org/10.4161/psb.6.8.16455

Schwarzott, D. and Schüßler A. (2001). A simple and reliable method for SSU rRNA gene DNA extraction, amplification, and cloning from single AM fungal spores. *Mycorrhiza, 10*, 203–207. https://doi.org/10.1007/PL00009996

Simon, L., Lalonde, M., and Bruns, T.D. (1992). Specific amplification of 18S fungal ribosomal genes from vesicular arbuscular endomycorrhizal fungi colonising roots. *Applied and Environmental Microbiology, 58*, 291–295. https://doi.org/10.1128/aem.58.1.291-295.1992

Sinanaj, B., Hoysted, G.A., Pressel, S., Bidartondo, M.I., and Field, K.J. (2021). Critical research challenges facing Mucoromycotina 'fine root endophytes'. *New Phytologist*. https://doi.org/10.1111/nph.17684

Smith, S.E and Read, D.J. (2008). *Mycorrhizal Symbiosis*, 3rd Edition. Academic Press, 800 pg.

Spatafora, J.W., Chang, Y., Benny,G.L., Lazarus, K., Smith, M.E., Berbee, M.L., Bonito, G., Corradi, N., Grigoriev, I., Gryganskyi, A, James, T.Y., O'Donnell, K., Roberson, R.W., Taylor, T.N., Uehling, J., Vilgalys, R., White, M.M., and Stajich, J.E. (2016). A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. *Mycologia, 108*, 1028–1046. https://doi.org/10.3852/16-042

Sýkorová, Z., Ineichen., K., Wiemken, A., and Redecker, D. (2007). The cultivation bias: different communities of arbuscular mycorrhizal fungi detected in roots from the field, from bait plants transplanted to the field, and from a greenhouse trap experiment. *Mycorrhiza, 18*, 1–14. https://doi.org/10.1007/s00572-007-0147-0

Tedersoo, L., Sánchez-Ramírez, S., Kõljalg, U., Bahram, M., Döring, M., Schigel, D., May, T., Ryberg, M., and Abarenkov, K. (2018). High-level classification of the Fungi and a tool for evolutionary ecological analyses. *Fungal Diversity, 90*, 135–159. https://doi.org/10.1007/s13225-018-0401-0

Tisserant, E., Malbreil, M., Kuo, A., Kohler, A., Symeonidi, A., Balestrini, R., Charron, P., Duensing, N., Frei Dit Frey, N., Gianinazzi-Pearson, V., Gilbert, L.B., Handa, Y., Herr, J.R., Hijri, M., Koul, R., Kawaguchi, M., Krajinski, F., Lammers, P.J., Masclaux, F.G., Murat, C., Morin, E., Ndikumana, S., Pagni, M., Petitpierre, D., Requena, N., Rosikiewicz, P., Riley, R., Saito, K., San Clemente, H., Shapiro, H., van Tuinen, D., Bécard, G., Bonfante, P., Paszkowski, U., Shachar-Hill, Y.Y., Tuskan, G.A., Young, P.W., Sanders, I.R., Henrissat, B., Rensing, S.A., Grigoriev, I.V., Corradi, N., Roux, C., and Martin, F. (2013). Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *Proceedings of the National Academy of Sciences of the United States of America, 110*, 20117–20122. https://doi.org/10.1073/pnas.1313452110

Tonjer, L.R., Thoen, E., Morgado, L., Botnen, S., Mundra, S., Nybakken, L., Bryn, A., and Kauserud, H. (2021). Fungal community dynamics across a forest-alpine ecotone. *Molecular Ecology, 30*, 4926–4938. https://doi.org/10.1111/mec.16095

Vandenkoornhuyse, P., Husband, R., Daniell, T.J., Watson, I.J., Duck, J.M., Fitter, A.H., and Young, Y.P. (2002). Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. *Molecular Ecology, 11*, 1555–1564. https://doi.org/10.1046/j.1365-294X.2002.01538.x

Větrovský, T., Kohout, P., Kopecký, M., Machac, A., Man, M., Bahnmann, B.D., Brabcová, V., Choi, J., Meszárošová, L., Human, Z.R., Lepinay, C., Lladó, S., López-Mondéjar, R., Martinović, T., Mašínová, T., Morais, D., Navrátilová, D., Odriozola, I., Štursová, M., Švec, K., Tláskal, V., Urbanová, M., Wan, J., Žifčáková, L., Howe, A., Ladau, J., Peay, K.G., Storch, D., Wild, J., and Baldrian, P. (2019). A meta-analysis of global fungal distribution reveals climate-driven patterns. *Nature Communications, 10*, 5142. https://doi.org/10.1038/s41467-019-13164-8

Wurzburger, N., Brookshire, E.N.J., Mccormack, M.L. and Lankau, R. (2017). Mycorrhizal fungi as drivers and modulators of terrestrial ecosystem processes. *New Phytologist, 213*(3), 996–999. https://doi.org/10.1111/nph.14409

Wheeler, D.A., Srinivasan, M., Egholm, M., Shen, Y., Chen, L., McGuire, A., He, W., Chen, Y., Makhijani, V., Roth, G.T., Gomes, X., Tartaro, K., Niazi, F., Turcotte, C.L., Irzyk, G.P., Lupski, J.R., Chinault, Xing-zhi Song, Yue Liu, Ye Yuan, Lynne Nazareth, Xiang Qin, Donna M. Muzny, Marcel Margulies, C., Weinstock, G.M., Gibbs, R.A., and Rothberg, J.M. (2008). The complete genome of an individual by massively parallel DNA sequencing. *Nature, 452*, 872–876. https://doi.org/10.1038/nature06884

doi:10.14720/aas.2022.118.3.2488

Short scientific article / kratki znanstveni prispevek

Ultraviolet disinfection of water in recirculating aquaculture system: a case study at sturgeon caviar fish farm

Anatolii SEMENOV [1, 2], Kateryna SEMENOVA [3]

Received December 30, 2021; accepted July 04, 2022.
Delo je prispelo 30. decembra 2021, sprejeto 04. julija 2022

Ultraviolet disinfection of water in recirculating aquaculture system: a case study at sturgeon caviar fish farm

Abstract: In this report, we present a practical example of ultraviolet (UV) water disinfection in an aquaculture facility for sturgeon caviar production. Among the methods of water disinfection in recirculating aquaculture systems, the technical approaches using ozonation or ultraviolet radiation in combination with other methods are the most effective. However, improper use of ozonation can result in excessive ozone concentrations that can cause serious harm to fish and be harmful to the environment and personnel. Therefore, we describe an example of a reagent-free ultraviolet water disinfection system. Preliminary results show that filtration followed by ultraviolet irradiation inactivates microorganisms in fish tank water. Total microbial count, total coliform bacteria, and *E. coli* (CFU/m^3) did not exceed the permissible values. The described UV system provides an irradiance of 180 W/m^2. For a pool with a water volume of 300 m^3, bacteriological purity of the water was achieved with 480 W of UV-light.

Key words: aquaculture; fish; sturgeon; recirculation system; UV water disinfection

Razkuževanje vode z ultravijolično svetlobo v recirkulacijskem akvakulturnem sistemu reje: primer ribogojnice jesetrov za prirejo iker, namenjenih za proizvodnjo kaviarja

Izvleček: V članku predstavljamo primer razkuževanja vode z ultravijolično (UV) svetlobo v recirkulacijskem akvakulturnem sistemu reje jesetrov za prirejo iker, namenjenih za kaviar. Med metodami razkuževanja vode pri gojenju rib v zaprtih akvakulturnih sistemih reje so najučinkovitejši pristopi z uporabo ozoniranja ali UV sevanja v kombinaciji z drugimi metodami. Nepravilna uporaba ozoniranja za razkuževanje vode lahko povzroči nastanek prevelikih koncentracij ozona, ki lahko negativno vplivajo na zdravje rib in škodujejo okolju in osebju v ribogojnici. Predstavljamo primer razkuževanja vode, ki temelji na uporabi UV svetlobe. Preliminarni rezultati so pokazali, da razkuževanje vode z metodo filtracije in UV sterilizacije zagotavlja učinkovito inaktivacijo mikroorganizmov v bazenu za gojenje rib, saj skupno število mikrobov, skupno število koliformnih bakterij in *E. coli* (CFU/m^3) ni preseglo priporočenih vrednosti. Opisani sistem proizvaja jakost UV sevanja 180 W/m^2, kar ob uporabi 480 W kvarčnih UV žarnic zagotavlja bakteriološko čistost vode za bazen prostornine 300 m^3.

Ključne besede: akvakultura; ribogojstvo; ribe; jeseter; recirkulacijski sistem; razkuževanje vode; UV svetloba

1 Poltava University of Economics and Trade, Faculty of Commodity science, Trade and Marketing, Department of Commodity research, Biotechnology, Expertise and Customs, Poltava, Ukraine
2 Corresponding author, e-mail: asemen2015@gmail.com
3 Poltava University of Economics and Trade, BA in International Economics, Master`s degree student

1 INTRODUCTION

In recent years, in Ukraine, as in other countries, industrial farming methods in aquaculture facilities have become increasingly important. These include fish farming in recirculating aquaculture systems (RAS) (Martins et al., 2010; Bulc et al., 2011). This approach can achieve high growth rates with minimal energy costs (Zainal et al., 2021) and is economically advantageous because of the reuse of water resources and the possibility of optimizing the hydrochemical regime without depending on environmental conditions.

The development of aquaculture farms and the associated increase in production volume has led to problems with effective disinfection of water in RAS. The choice of the method and means of purification of recycled water is of crucial importance for the technological cycle of fish farming. New technologies offer alternatives to classical water treatment methods (e.g., particle filtration, biofiltration, and gas exchange) (Huyben et al., 2018). Gundula et al. (2019) consider recirculation systems as systems that incorporate a number of water purification stages, which consists of: 1) devices for removing solid particles from water, 2) biofilters for ammonia reduction, and 3) gas exchange devices for carbon dioxide removal and oxygen addition. Rearing fish in RAS may be beneficial for increased fish survival rates, when compared to standard cage systems, mostly due to stable microbial environment that prevents opportunistic microbe multiplication (Dahle et al., 2020).

UV irradiation and ozonation are the most common methods of water disinfection in aquaculture. Studies have shown that it is possible to achieve optimal conditions for the microbiological composition of water also with the combined effects of filtration, UV radiation, and ozonation (Gregersen et al., 2020; Middlemiss et al., 2015). The effectiveness of these methods, as well as their combination depends on the presence of dissolved and suspended organic compounds in the water (Semenov et al., 2021a). Overexposure to ozone can cause serious damage to fish and can be harmful to the environment (Sharrer et al., 2005). During ozone treatment, microparticles are broken down into molecular structures and then removed at different stages of filtration. This method of water purification is suitable for fish incubators that are sensitive to microparticles and bacteria in the water. However, there are arguments against the use of ozonation in RAS (Attramadal et al., 2012) as such systems require a large amount of ozone, which is mostly consumed in the reactions with organic substances, however residual ozone and reaction by-products can be toxic for fish and live feed. On the other hand, UV irradiation of water is considered a safe alternative to ozonation. When using ultraviolet radiation, the number of microorganisms is significantly reduced (Moriarty et al., 2018) as it inactivates microorganisms through photochemical reactions of nucleic acids, which occurs in a special ultraviolet chamber (Semenov et al., 2018) with no harmful effects on fish, environment, and personnel.

According to Runia (1995), different irradiances are required for different types of microorganisms: for inactivation of bacteria and fungi from 100 mJ/cm^2 and for viruses from 250 mJ/cm^2. These relatively high doses compensate for the possible change in turbidity of the water and the change in transmittance of UV radiation energy. For example, Sharrer et al. (2005) used UV doses ranging from 75 to 1800 mW/cm^2 to achieve inactivation of coliform bacteria in rearing salmonids. However, the inactivation process is not guaranteed if suspended solids are present in the water stream. In practice, radiation intensity of at least 400 mJ/m^2 is required for the operation of fish incubators and RAS. In Ukraine, ozonation is the predominant method of water disinfection in aquaculture (Semenov et al., 2021a; Semenov et al., 2021b). In this report we present an example of an alternative solution – UV disinfection of water in aquaculture facility for caviar production.

2 MATERIALS AND METHODS

Experimental work was carried out in an aquaculture farm (Zhashkov, Cherkasy region) when growing sturgeon (*Acipenser gueldenstaedtii*) for caviar production. Fish farming was carried out in an insulated hangar. Bioload of the system was 53–55 kg of live fish per 1 m^2 of pool area. Fish were fed four times a day using commercial diet. All experimental work was carried out in a closed water supply system with a volume of 300 m^3, water temperature of 21–22 °C, pH level of 7.3–7.7, and dissolved oxygen content of 5.6–5.8 mg/l. Water purification was carried out continuously through the water recirculation channel with width of 200 mm and water flow height of 840-860 mm. The recirculation channel provides a water flow of 75 m^3/h. For UV disinfection of water, quartz lamps (type ZW80D19W) were used with the power of 80 W, lamp current of 800–1200 mA, and UV (254 nm) irradiance (d = 1 m) of 240–270 μW/cm^2. Presence of bacteria was determined with bacteriological cultures on dense nutrient media, followed by identification of phenotypic or serological properties of the studied strains.

3 RESULTS AND DISCUSSION

Among the types of UV disinfection units considered, there are two types – surface and submersible. A surface sterilizer consists of a battery of UV lamps set-up above the water. Submersible sterilizers, in which water disinfection takes place in the irradiation chamber are more efficient and reliable (Semenov et al., 2018). In order to obtain satisfactory results in terms of water quality it is necessary to continuously treat the water. For this, filtration and bacterial disinfection are used together. For

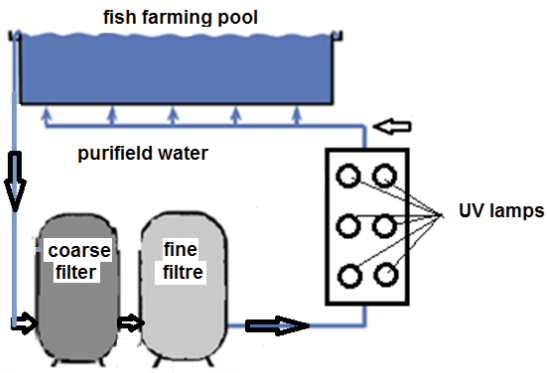


Figure 1: Scheme of the water purification and disinfection installation

the purification and disinfection of water for sturgeon fish farming in RAS we assembled a UV irradiation system schematically presented in Figure 1.

The system includes the following: 1) installation for removing coarse dirt, 2) installation for removing highly dispersed impurities (fine cleaning), 3) installation for ultraviolet water disinfection, and 4) equipment for pH correction, water saturation with oxygen, water heating and electronic control system. For the inactivation of microorganisms, a 480 W UV installation has been developed, which consists of six low-pressure ultraviolet lamps with the previously described characteristics. Lamps are placed after the filtration units and fitted vertically within the water flow. They are inserted in quartz glass covers to maximize the irradiation area. The total bactericidal flow is 180 W/m^2.

The obtained results of bacteriological studies of water when growing fish are presented in Table 1.

Bacteriological studies of the water in the pool showed that ultraviolet disinfection combined with filtration provides the necessary bacteriological purity of water in pools with a volume of up to 300 m^3. With the proposed system, bacteria causing fish diseases such as *Flexibacter Cytophaga*, *Aeromonas* and mycoses (*Saprolegniales*) were not detected within first three months and after six months.

4 CONCLUSIONS

A reagent-free system for disinfecting water in fish breeding pools based on UV irradiation was assembled and tested. In the case of RAS with a water volume of 300 m^3, the proper bacteriological quality of the water was ensured for six monitored months by installing UV quartz lamps with a power of 480 W and a UV irradiation intensity of 180 W/m^2.

5 REFERENCES

Attramadal, K. J. K., Øie G., Størseth, T. R., Alver, M.O., Vadstein, O., Olsen, Y. (2012). The effects of moderate ozonation or high intensity UV-irradiation on the microbial environment in RAS for marine larvae. *Aquaculture*, *330*, 121–129. https://doi.org/10.1016/j.aquaculture.2011.11.042

Bulc, T. G., Istenic, D., Klemenci, A. K. (2011). The efficiency of a closed-loop chemical-free water treatment system for cyprinid fish farms. *Ecological Engineering, 37*(6), 873–882. https://doi.org/10.1016/j.ecoleng.2011.01.004

Dahle, S. W., Bakke, I., Birkeland, M., Nordøy, K., Dalum, A. S., Attramadal, K. J. K. (2020). Production of lumpfish (Cyclopterus lumpus L.) in RAS with distinct water treatments: Effects on fish survival, growth, gill health and microbial communities in rearing water and biofilm. *Aquaculture*, *522*, 735097. https://doi.org/10.1016/j.aquaculture.2020.735097

Gregersen, K. J., Pedersen, P. B., Pedersen, L.-F., Liu, D., Dalsgaard, J. (2020). UV irradiation and micro filtration effects on micro particle development and microbial water quality in recirculation aquaculture systems. *Aquaculture, 518,* 734785. https://doi.org/10.1016/j.aquaculture.2019.734785

Table 1: The results of bacteriological studies of water in the pool when growing fish

Indicator name	Requirements (CFU/cm^3)	Research results (days)					
		0	7	30	60	90	120
Total microbial count (CFU/cm^3 at 37 °C)	< 100	17	40	79	52	60	89
Total coliforms (CFU/100 cm^3)	-	-	-	-	-	-	-
E. coli (CFU/100 cm^3)	-	-	-	-	-	-	-

Gundula, P., Ianchenko, A., Kotzen, B. (2019). Aquaponics in the Built Environment. In: Goddek, S., Joyce, A., Kotzen, B., Burnell, G. M. (eds.) Aquaponics Food Production Systems: Combined Aquaculture and Hydroponic Production Technologies for the Future. *Springer Nature*, Switzerland, P. 619. https://doi.org/10.1007/978-3-030-15943-6

Huyben, D., Bevan, D., Stevenson, R., Zhou, H., Moccia, R. (2018). Evaluation of membrane filtration and UV irradiation to control bacterial loads in recirculation aquaculture systems. *Aquaculture International, 26*(2). https://doi.org/10.1007/s10499-018-0301-z

Martins, C. I. M., Eding, E. H., Verdegem, M. C. J., Heinsbroek, L. T. N., Schneider, O., Blancheton J. P., ... Verreth, J. A. J. (2010). New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. *Aquacultural Engineering*, *43*(3), 83–93. https://doi.org/10.1016/j.aquaeng.2010.09.002

Middlemiss, K., Daniels, C., Urbina, M., Wilson, R. (2015). Combined effects of UV irradiation, ozonation, and the probiotic Bacillus spp. on growth, survival, and general fitness in European lobster (Homarus gammarus). *Aquaculture*, *444,* 99–107. https://doi.org/10.1016/j.aquaculture.2015.03.028

Moriarty, M. J., Semmens, K., Bissonnette G. K., Jaczynski J. (2017). Inactivation with UV-radiation and internalization assessment of coliforms and Escherichia coli in aquaponically grown lettuce. *Lebensmittel-Wissenschaft und-Technologie, 89*(2), 624–630. https://doi.org/10.1016/j.lwt.2017.11.038

Runia, W. T. (1995). A review of possibilities for disinfection of recirculation water from soilless culture. *Acta Horticulturae, 382,* 221–229. https://doi.org/10.17660/ActaHortic.1995.382.25

Semenov, A. (2018). Device for disinfection of water by using ultraviolet radiation. *8th International Conference Physics of Liquid Matter: Modern Problems (PLMMP 2018),* 18–22 May 2018, Kyiv, Ukraine, P.1-20. http://dspace.puet.edu.ua/handle/123456789/9287

Semenov, A., Vyzhva S., Sakhno T., Semenova N. (2021a). Combined method of UV treatment and ozonation during water disinfection in swimming pools. *XV International Scientific Conference* «Monitoring *of Geological Processes and Ecological Condition of the Environment*», 17–19 November 2021, Kyiv, Ukraine, Mon-21-095. http://dspace.puet.edu.ua/handle/123456789/11527

Semenov, A., Sakhno, T., Sakhno, Y. (2021b). Photobiological safety of lamps and lamp systems in agriculture. *Journal of Achievements in Materials and Manufacturing Engineering*, *106*(1), 34–41. https://doi.org/10.5604/01.3001.0015.0527

Sharrer, M. J., Summerfelt, S. T., Bullock, G. L, Gleason, L. E., Taeuber J. (2005). Inactivation of bacteria using ultraviolet irradiation in a recirculating salmonid culture system. *Aquacultural Engineering, 33*(2), 135–149. https://doi.org/10.1016/j.aquaeng.2004.12.001

Zainal, A. G., Yulianto, H., Rudy, Yanfika, H. (2021). Financial benefits of the environmentally friendly aquaponic media system. *IOP Conference Series: Earth and Environmental Science, 739,* 012204. https://doi.org/10.1088/1755-1315/739/1/012024