120 • 2
2024

ACTA AGRICULTURAE SLOVENICA

Acta agriculturae Slovenica • eISSN 1854-1941 • 120 – 2 • Ljubljana, julij 2024

UNIVERZA V LJUBLJANI
BF Biotehniška fakulteta

Acta agriculturae Slovenica **eISSN 1854-1941**
Letnik / Volume 120 · Številka / Number 2 · julij / July 2024

Acta agriculturae Slovenica izhaja s finančno pomočjo / *is published with the financial support:* Javne agencije za znanstvenoraziskovalno in inovacijsko dejavnost Republike Slovenije / *Slovenian Research and Innovation Agency*.

Acta agriculturae Slovenica je vključena v / *is included into*: Scopus (SJR, SNIP), DOAJ, WOS Zoological Records, CrossRef, CAB Abstracts, FSTA, Google Scholar, dLib, COBISS.

Ovitek: Sladki krompir z oranžnim in belim mesom (Foto: Chikezie Onuora ENE, 1–17)
Cover: Sweet potato with orange and white flesh (Photo: Chikezie Onuora ENE, 1–17)

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doi:10.14720/aas.2024.120.2.12559 Original research article / izvirni znanstveni članek

Identification of metal tolerance proteins (MTP) and their gene expression under drought stress in potato (*Solanum tuberosum* L.)

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Received March 02, 2023; accepted June 10, 2024.
Delo je prispelo 2. marca 2023, sprejeto 10. junija 2024.

Identification of metal tolerance proteins (MTP) and their gene expression under drought stress in potato (*Solanum tuberosum* L.)

Abstract: Metal tolerance proteins (MTPs) are as metal efflux transporters, existing extensively at all plant sections and play significant roles in regulation of the metal levels in biological processes. In the current study, phylogenetic relationships, gene structures, conserved motifs, and StMTP domains were analyzed. Here, 12 *MTP* genes in *S. tuberosum* were detected and categorized in three major clusters namely *Fe/Zn-MTP*, *Zn-MTP*, and *Mn-MTP* and seven groups (1, 5, 6, 7, 8, 9, and 11) according to phylogenetic relationships. Based on *in silico* and qPCR analysis, all of *StMTPs* included a cation diffusion facilitator (CDF) domains and the putative *Mn-MTP* harbored the ZT-dimer. An evolutionary analysis indicated that *StMTP* genes had undergone gene duplication leading to gene loss and gene expansion events. Analysis of transcription factor binding sites (TFBS) and microRNA in promoter region and coding sequence of *StMTP* genes revealed the presence of 5312 putative TFBS and 13 StmiRNAs. The analysis of promoter regions of *StMTP* genes possess various frequencies of TFBS, illustrating various responses in different growth and developmental stages as well as under abiotic stress. Expression profile analysis revealed that the *StMTP9* were up-regulated in leaves and stem, while, *StMTP8* up-regulated in leaves. Both genes down-regulated in tubers, roots as well as under drought stress. These results will provide a better insight for functional characterization of StMTP genes and can be helpful to elucidate the biological structure of their genes in potato.

Key words: biological processes, transporter, metal tolerance proteins, *S. tuberosum,* gene expression

Določanje na kovine tolerantnih beljakovin (MTP) in izražanje njihovih genov v razmerah sušnega stresa pri krompirju (*Solanum tuberosum* L.)

Izvleček: Na kovine tolerantni proteini (MTPs) so transporterji kovin iz celice, ki so prisotni v velikem številu pri vseh rastlinah in igrajo pomembno vlogo pri uravnavanju količine kovin v bioloških procesih. V raziskavi so bila analizirana filogenetska razmerja, zgradbe genov, ohranjena zaporedja in *StMTP* domene. V krompirju je bilo ugotovljeno 12 *MTP* genov, ki so bili razporejeni v tri glavne skupine in sicer *Fe/Zn-MTP*, *Zn-MTP* ter sedem skupin *Mn-MTP* genov (1, 5, 6, 7, 8, 9, in 11) glede na filogenetska razmerja. Na osnovi *in silico* in qPCR analize so se vsi *StMTPs* geni vključevali domene za olajšanje difuzije kationov (CDF) in gene z domnevno isto funkcijo (*Mn-MTP*), ki so vsebovali ZT-dimer. Evolucijska analiza je pokazala, da so *StMTP* geni prešli podvojevanje, kar je vodilo do izgube genov in njihovega povečevanja. Analiza mest vezave transkripcijskega faktorja (TFBS) in mikroRNK v promotorski regiji in kodirajočih sekvencah *StMTP* genov je odkrila prisotnost 5312 možnih TFBS in 13 StmiRNAs. Analiza promotorske regije *StMTP* genov je pokazala, da ti vsebujejo različne frekvence TFBS, kar kaže na različne odzive v različnih rastnih in razvojnih fazah kot tudi učinke abiotičnega stresa. Analiza izražanja profila je odkrila, da so geni *StMTP9* bolj aktivni v listih in steblu med tem, ko so geni *StMTP8* bolj aktivni v listih. Obe skupini genov sta manj aktivni v gomoljih in koreninah kot tudi v razmerah sušnega stresa. Ti rezultati prispevajo boljši vpogled v funkcionalno opredeljevanje *StMTP* genov in bi lahko bili koristni za razjasnitev biološke zgradbe genov v krompirju.

Ključne besede: biološki procesi, transporter, proteini tolerance na kovine, *S. tuberosum,* izražanje genov

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1 INTRODUCTION

Transition metals participate in many biological and physiological processes. Since they act as essential cofactors for many enzymes, they are components of transcription factors and other proteins and are important for both mitochondrial and chloroplast functions. However, high concentration together with non-essential metals can lead to extremely toxicity and can cause oxidative damages or compete with other essential ions. The physiological range of transition metals from deficiency to toxicity is extremely narrow and therefore a network to control the micronutrient fluctuations is required for all organisms. Since transmission metals are also essential components in reaction centers of enzymes, deficiency will also cause stress symptoms (Ducic and Polle, 2005). To regulate toxic effects of high and low concentration metals, it is necessary that plants maintain metal homeostasis at cellular levels (Hall and Williams, 2003). Special transporters were encoded by multigenic families which are responsible for the uptake and secretion of metal cations in different organelles (Montanini et al., 2007).

Transporters of the cation diffusion facilitator (CDF) family are namely Zn^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+}, Cd^{2+}, and Mn^{2+}, first detected by Nies and Silver (1995). CDFs are divided into three substrate-specific clades Zn-CDF, Fe/Zn-CDF, and Mn-CDF. These transporters are ubiquitous and spanning in all three kingdoms of organisms: Archaea, Eubacteria, and Eukaryotes. Expressed fundamentally in both root sand shoots, *AtMTP1* is over-expressed conferring Zn tolerance in rice (Zhang and Liu, 2017). *OsMTP1*, a cation transporter localized in tonoplast, possesses low affinity to Co, Fe, and Cd, controlling ion hemostasis in rice (Menguer et al., 2013). AtMTP5 and AtMTP12, other functional complex members of Zn-CDF proteins, were found to transport Zn into the Golgi apparatus (Fujiwara et al., 2015). In Mn-CDF groups, there were four AtMTP proteins (AtMTP8-11), which both AtMTP9 and AtMTP11 functioned as a Mn transporter. AtMTP11 was involved in maintaining Mn hemostasis and localized in pre-vacuolar compartment and/or trans-Golgi. Mutation of *atmtp11* display Mn sensitivity and higher levels of Mn in shoots and roots than the wild-type plants were accumulated. Five Mn-CDF members (OsMTP8.1/8.2/9/11/11.1) with known functions are classified into groups 8 and 9. ShMTP8, another member of Mn-CDF, is isolated from the Mn-tolerant legume *Stylosanthes hamata* (L.) Taub.. ShMTP8 is localized in tonoplast, exhibiting Mn-tolerance when expressed in *A.thaliana* (L.) Heynh. (Delhaize et al., 2003). In *Arabidopsis* and cucumber, AtMTP7, CsMTP7 act as Fe transporter member and are localized in plant mitochondrial (Migocka et al., 2018). CsMTP8 was found in vacuolar membrane and participated in the maintenance of Mn hemostasis (Migocka et al., 2014). CsMTP9 is involved in the efflux of Mn^{+2} and Cd^{+2} from cucumber root cells using H+-coupled with manganese and cadmium antiporter (Migocka et al., 2015).

Potato (*S. tuberosum* L.) is one of the largest non-cereal food crop worldwide and sequencing of its entire genome is completed (Zhang et al., 2017). Potato can be utilized for molecular plant biological research and to facilitate gene discovery and comparative genetics (Jaillon et al. 2007). There has been few relevant research on the *StMTP* genes in potato. The present genome-wide survey was conducted to identify the *MTP* gene family in *S. tuberosum* and systematically analyzed their sequence and structural characteristics as well as evolutionary relationships. Besides, the transcription factor binding sites distributions, and the potential microRNA target sites in *StMTP* genes were predicted. In addition, the expression profiles of *StMTP* genes in different potato tissues and in response to abiotic and biotic stresses were analyzed using a microarray data approach. Results in this study could provide a better insight into the biological functions of StMTP proteins and the molecular mechanisms underlying these metal transporters and the homeostasis maintained by them in potato.

2 MATERIAL AND METHODS

2.1 IDENTIFICATION OF *MTP* GENES IN *S. tuberosum*

The *MTP* genes of *A.thaliana* and *O.sativa* L. were taken from TAIR and RAP-DB databases, respectively.

To detect the potential *StMTP* genes in potato, the HMM file of the MTP domain (PF01545) was taken from the Pfam database and utilized to perform the HMMER search. Then, the resulting MTP sequences were adopted for tBLASTn. Finally, following the removal of redundant predicted sequences, the sequences all putative MTPs were further confirmed using InterProScan.

2.2 SEQUENCE ALIGNMENT AND PHYLOGENETIC

Sequence similarity analysis of MTPs proteins between *S. tuberosum* and *A. thaliana* were performed in blastp at NCBI. Each protein sequence of MTPs in *Arabidopsis* and *O.sativa* was used at the query, and all 12 StMTP protein sequences were used as the subject sequence.

For phylogenetic analysis, multiple sequence align-

ments at protein levels were performed by ClustalX, and MEGA 6.0. Phylogenetic tree construction was established by the Maximum likelihood method (Tamura et al., 2013). The MTPs sequence from *S. tuberosum, A. thaliana*, and *O. sativa* were downloaded from the above databases, as described by Liu et al (2019).

2.3 AMINO ACID PROPERTIES AND STRUCTURE CHARACTERISTICS OF MTP PROTEINS

The molecular weight, Pi, and peptide length were evaluated using the ProParam software and prediction of protein transmembrane helices was examined using protter. Sub-cellular localization was predicated using Plant-Mploc server (Hall 2002). MEME program was utilized to detect the conserved motifs (Bailey et al., 2009; Finn et al., 2016). Motifs functions were determined using the hmmscan tool. Then, detected MTP sequences were aligned using Muscle, and identity residue was calculated. The exon-intron structures of StMTP genes were characterized using GSDS program.

2.4 TFBS ANALYSIS AND MIRNA TARGET SITES PREDICTION

The promoter regions (up-stream 1000 bp) of *StMTP* genes were extracted to predict the TFBS using PlantPAN. The miRNA target sites of *StMTPs* were examined using small RNA target analysis server.

2.5 PLANT GROWTH AND qRT-PCR OF *StMTP* GENES

To analyze specific expression in root, stem and leaf tissue, samples were taken from two-week-old seedlings. Three tubers were planted in the pot. To analyze the expression under drought stress, two treatments of drought and irrigation were used. Each treatment was in a completely randomized block design with three blocks. In the first six weeks, all the plants in each two treatments were watered equally. After 2 weeks of stress, leaf and tuber samples were taken under the mentioned conditions. Then the leaves and tubers were immersed in liquid nitrogen and kept at -80 temperature until RNA extraction. RNA extraction was performed using the Synaclone kit. Then cDNA synthesis was performed as follows. Potato *EF-1α* gene was used as internal control. All primers used in gene expression analysis are listed in Table S1. Real time was done using SYBR Green Supermix. Relative expression was determined via $2^{-\Delta\Delta Ct}$.

3 RESULT

3.1 IDENTIFICATION AND CLASSIFICATION OF *MTP* GENES IN POTATO

Using 12 and 10 AtMTP and OsMTP protein sequences as the queries, a total of 12 *MTP* genes were detected in *S. tuberosum*. Subsequently, HMM verification was performed in 12 MTP sequences, including the cation efflux domain in the potato genome. According to sequence identity, cover value, and orthologous relationship, the 12 StMTP proteins were designated as StMTP1 to StMTP11. For each AtMTP protein, there was at least one MTP homolog in *S.tuberosum* except for AtMTP2, AtMTP10, and AtMTP12, where no corresponding StMTP was found. To understand the evolutionary relationships of MTP gene family members among potato, *Arabidopsis*, and rice 35 MTP protein sequences from three species were comprehensively analyzed and a phylogenetic tress was constructed. According to the classification of previous surveys (Montanini et al., 2007; Shirazi et al., 2019), 35 MTP proteins were divided into three substrate-specific groups (Zn-CDFs, Zn/Fe-CDFs, and Mn-CDFs) and seven primary groups (1, 5, 6, 7, 8, 9, and 11) that were similar to the AtMTPs and OsMTPs. Of the seven groups, group 1 had the maximum StMTP with 12 members, whereas groups 5, 6, and 7 contained the minimum StMTP with three members each. There are four, five, and five StMTP members in groups 11, 8, and 9, respectively. StMTP1, StMTP3, StMTP4, and StMTP5 belonged to Zn-CDF family; StMTP6 and StMTP7 to Fe/Zn-CDF family and StMTP8, StMTP8.1, StMTP9, StMTP9.1, StMTP9.2 and StMTP11 to Mn-CDF family (Fig 1).

3.2 STRUCTURE AND CHARACTERISTIC ANALYSIS OF *STMTP* GENES

The characteristics of the *StMTP* genes were analyzed in detail. The length of protein sequences of *StMTP* genes ranged from 86 to 503 amino acids. The molecular weights and pIs of these potato proteins ranged from 9781.63 to 55006.92 KDa and 4.98-10.45, respectively (Table 1). Most of the StMTP proteins included five to six

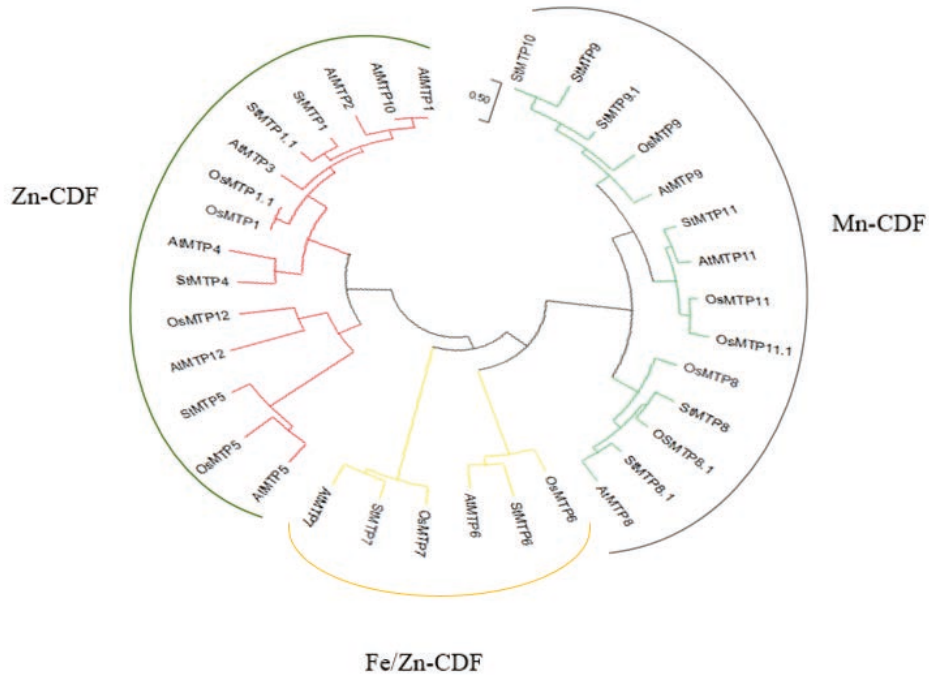

Figure 1: Phylogenetic relationship of MTP proteins in three main plants of *Arabidopsis*, rice, and potato. The tree was constructed using the MEGA 6.0 software by the Maximum likelihood method. The identical proteins were categorized into three sub-families (Mn-MTPs, Zn-MTPs, and Zn/Fe-MTPs). The Zn-MTP sub-family (red line) contains 1: (MTP1:4), 5: MTP5 (red); Zn/Fe-MTPs (yellow line) includes (MTP6-7); and Mn-MTP (green line) contains (MTP8-11)

putative transmembrane domains (TMDs), StMTP8 and StMTP11 had only four TMDs, StMTP7 contained three TMDs, and StMTP6 carried twelve TMDs. Particularly, StMTP5 and StMTP8.1 proteins lacked any of the TMDs.

To examine differences in *StMTP* genes, the exon and intron structures of 12 potato MTP genes were compared. As illustrated in Figure 2, the number of introns in the *StMTP* genes ranged from 1 to 12. Further, the results showed that most of the *StMTP* in same groups exhibited similar exon-intron compositions. Most members of Mn-CDF had six exons, all of StMTP in Fe/Zn included two exons, and more members in ZN-CDF possessed variable number of exons (Fig 2a).

To obtain more insight into the structure characteristics of the StMTP proteins and conserved motif analysis, their amino acid sequences were submitted to MEME program. As shown in Figure 3 and Table 2, ten motifs were in total identified in StMTP family members, while only four of them were explored to encode functional domains when subjected to Pfam. Motif 1, 4, and 8 were annotated as cation_efflux (PF01545), motif 3 as ZT-dimer (PF16916) while motifs 2, 5, 6, 7, 9, and 10 were not assigned by the Pfam. Highly similarity motifs are expected to have similar functions. StMTPs belonged to Mn-CDF group included three motifs sequences namely, motif 1 and 4. StMTPs relevant to Zn-CDF contained three motif (4, 8, and 9). StMTPs (1/3/4/5) included both motifs 4 and 8 cation-efflux. Whereas, StMTP 6 and StMTP7 had only one of 4 motif which belonged to Zn/Fe-CDF groups (Fig 2b). As explained earlier, the cation efflux domain is a typical feature of the MTP transporters. Hence, the domain architectures in StMTP proteins were analyzed. Results showed that all the StMTP proteins included the cation efflux domain. However, the members of groups 8, 9, and 11 (except to 9.2) possessed a ZT.dimer which is a significant zinc transporter dimerization domain.

3.3 MULTIPLE SEQUENCE ALIGNMENT, CONSERVED MOTIFS, AND DOMAIN ARCHITECTURES IN STMTPS PROTEINS

To evaluate the sequence of the StMTP proteins, the amino acid sequences of the AtMTPs, OsMTPs, and StMTPs from the three substrate-specific groups were multiple aligned by ClustalX, respectively. Results revealed that total of the AtMTPs, OsMTPs, and StMTPs proteins has one and two conserved HxxxD residues in Zn/Fe-

Table 1: MTP proteins information for potato

Gene	Accession number	Peptide lenght	MW (kDa)	pI	No. of TMDs N to C	Subcellular localization
StMTP9	PGSC0003DMG400004287	317	36293.33	6.41	5/into in	Cell memebrane. Vaculoe.
StMTP9.1	PGSC0003DMG400009656	86	9781.63	10.45	5/into in	Cell membrane. Vacuole.
StMTP9.2	PGSC0003DMG400011247	413	47080.09	6.22	5/into in	Vacuole.
StMTP8.1	PGSC0003DMG400001111	373	42328.02	4.98	0	Cell membrane. Vacuole.
StMTP8	PGSC0003DMG400032189	405	45429.20	5.07	4/into out	Vacuole.
StMTP7	PGSC0003DMG400026506	463	50752.05	6.36	3/into out	Vacuole.
StMTP6	PGSC0003DMG402011364	503	55006.92	6.19	12/out to out	Vacuole.
StMTP5	PGSC0003DMG400014975	387	43337.68	6.67	0	Vacuole.
StMTP4	PGSC0003DMG400030333	380	42495.88	5.85	6/into in	Vacuole.
StMTP3	PGSC0003DMG400030740	385	42861.52	5.86	6/into in	Vacuole.
StMTP1	PGSC0003DMG400030701	415	45972.68	6.05	6/into in	Vacuole.
StMTP11	PGSC0003DMG400023516	401	45240.14	5.03	4/ into out	Vacuole.

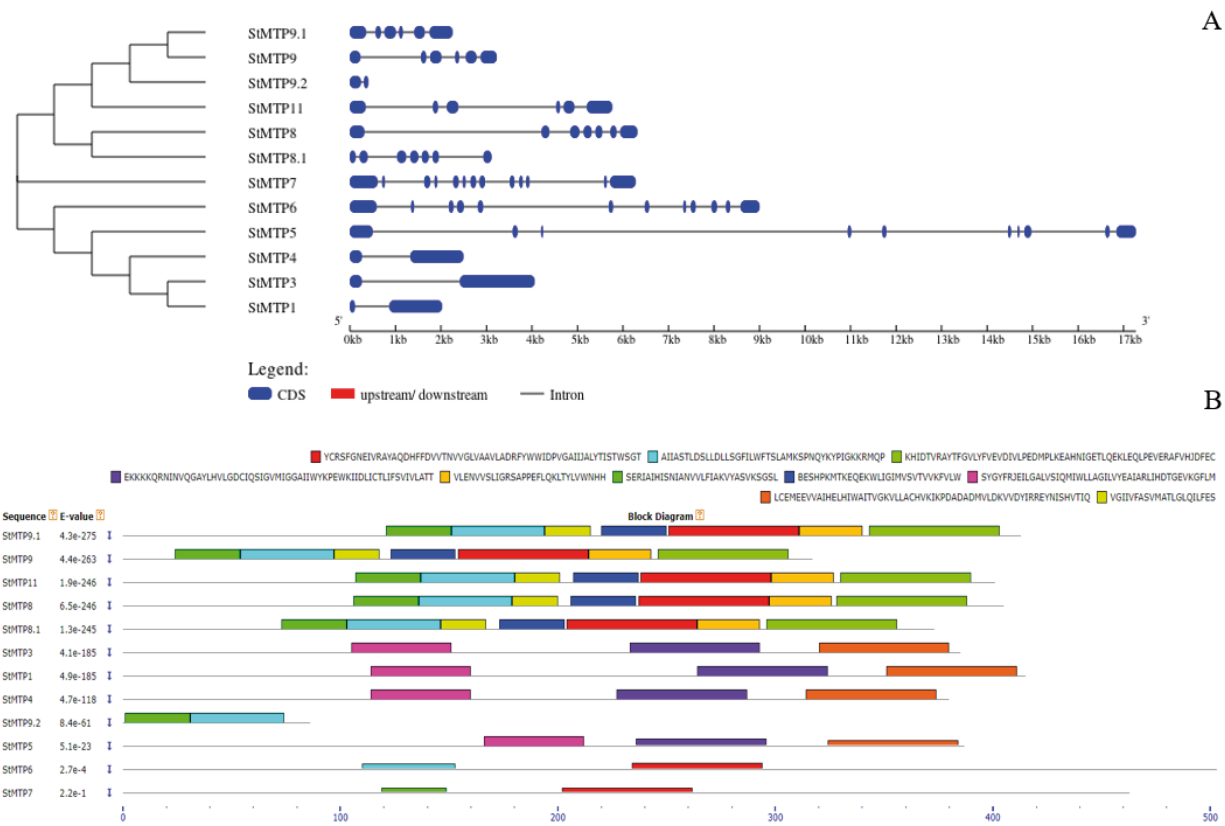

Figure 2: A) Distributions of the conserved domains in StMTP proteins. B) Conserved motifs detected by MEME and displayed in different colored boxes

Table 2: The sequences and the Pfam annotations of conserved motifs in StMTP proteins

Motif ID	Motif sequence	Length	Pfam
Motif 1	YCRSFGNEIVRAYAQDHFFDVVTNVVGLVAAVLADRFYWWIDPVGAIIJALYTISTWSGT	60	Cation-efflux, Pfam, PF01545
Motif 2	AIIASTLDSLLDLLSGFILWFTSLAMKSPNQYKYPIGKKRMQP	43	No motif was found in Pfam
Motif 3	KHIDTVRAYTFGVLYFVEVDIVLPEDMPLKEAHNIGETLQEKLEQLPEVERAFVHJDFEC	60	ZT_dimer, PF16916
Motif 4	EKKKKQRNINVQGAYLHVLGDCIQSIGVMIGGAIIWYKPEWKIIDLICTLIFSVIVLATT	60	Cation-efflux, Pfam, PF01545
Motif 5	VLENVVSLIGRSAPPEFLQKLTYLVWNHH	29	No motif was found in Pfam
Motif 6	SERIAIHISNIANVVLFIAKVYASVKSGSL	30	No motif was found in Pfam
Motif 7	BESHPKMTKEQEKWLIGIMVSVTVVKFVLW	30	No motif was found in Pfam
Motif 8	SYGYFRJEILGALVSIQMIWLLAGILVYEAIARLIHDTGEVKGFLM	46	Cation-efflux, Pfam, PF01545
Motif 9	LCEMEEVVAIHELHIWAITVGKVLLACHVKIKPDADADMVLDKVVDYIRREYNISHVTIQ	60	No motif was found in Pfam
Motif 10	VGIIVFASVMATLGLQILFES	21	No motif was found in Pfam

CDFs and Zn-CDFs, respectively, and two DxxxD residues were explored in the Mn-CDF subgroups (Fig 3).

3.4 POTENTIAL MicroRNA TARGET SITES IN *StMTP* GENES

MicroRNA (miRNAs) are small non-coding RNA molecules that can play key roles in gene expression (Zhang and Chen, 2013). With the expectation score lower than 3.0, a total of 13 StmiRNAs comprising target sites in three *StMTP* genes were detected (Table 3). Two members of group 1 can be targeted by stu-miR7992-3p. Moreover, StMTP5 was targeted by stu-miR5303g, stu-miR5303i, stu-miR5303h, stu-miR5303j, stu-miR156e, stu-miR156f-5p/g-5p/h-5p/i-5p/j-5p/k-5p, and stu-miR5303f. All identified miRNAs-targeted *StMTP* genes were predicted to be silenced by cleavage inhibition. Given that miRNA regulate a large section of mRNA transcripts, resulting nearly all biological events are affected by miRNAs (Bartel, 2009). The findings showed that the UPE ranged from 18.379 (stu-miR7992-3p/ StMTP3) to 23.914 (stu-miR5303f/ StMTP5) (Table 3).

3.5 ANALYSIS OF THE TFBS IN THE PROMOTER REGIONS OF *STMTP* GENES

TF binding sites (TFBS), regions of DNA binding sites in promoter, are important in transcription initiation of its target genes (Yu et al., 2016). As shown in Table 4, 7 TFBS groups, containing elements associated to biotic and abiotic stresses, light response, developmental response, cell cycle, basic transcription, phytohormonal response, and other binding sites were annotated. Among the more common TFBS, MYB and bZIP appeared to be the most frequent elements (with 1046 and 606 numbers, respectively), and were commonly established by all *StMTP* genes. Notably, elements involved in light control was distributed in the promoter regions of all *StMTP* genes. While, elements involved in hormone responsiveness were less abundant than the others (Table 4), it appears that the presence of these elements are an indication that *StMTP* genes could be transcriptionally regulated by different hormones (Table 4).

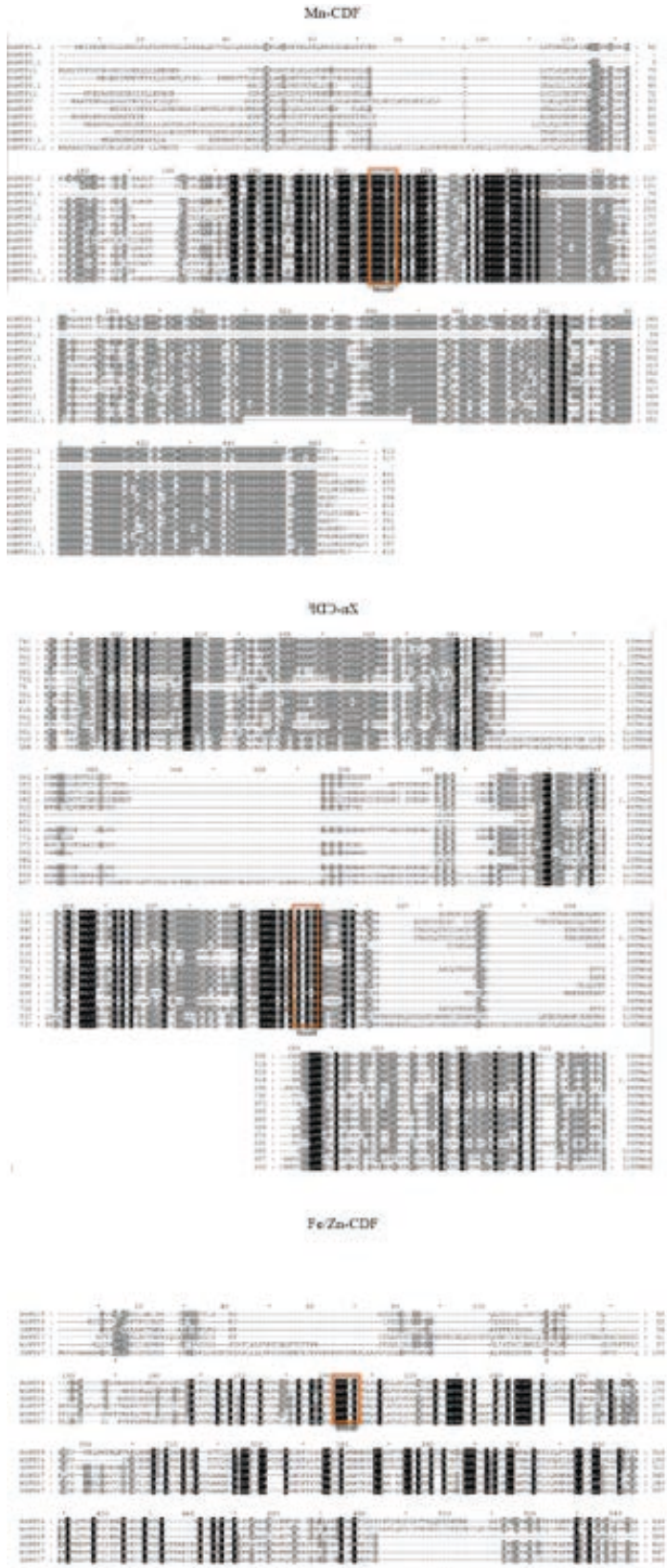

Figure 3: Multiple sequence alignment of StMTP, AtMTP, and OsMTP proteins. The signature sequences and the consensus sequence HXXXD or DXXXD (X = any amino acid) are indicated with black line and open boxes, respectively

Table 3: The potential miRNA target sites in *StMTP* genes

	miRNA_Acc.	Target_Acc.	Expectation	UPE	miRNA-length	Target Start-End	miRNA aligned fragment	Target aligned fragment	Inhibition
StMTP3	stu-miR7992-3p	StMTP3	3	18.379	22	1342-1364	UGUCUAGAUGUGCAUUUCAAAGU	UCCAUGAAUUGCACAUUUGGGCG	Cleavage
StMTP1	stu-miR7992-3p	StMTP1	3	20.777	22	1101-1123	UGUCUAGAUGUGCAUUUCAAAGU	UCCAUGAAUUGCACAUUUGGGCG	Cleavage
StMTP5	stu-miR5303g	StMTP5	1	20.855	23	13278-13301	AUAUUUUUGAAGAGUCUGAG-CAAC	GUUGCUCGGACUCUUCAAAAAU-GU	Cleavage
StMTP5	stu-miR5303i	StMTP5	1	20.855	23	13278-13301	AUAUUUUUGAAGAGUCUGAG-CAAC	GUUGCUCGGACUCUUCAAAAAU-GU	Cleavage
StMTP5	stu-miR5303h	StMTP5	1.5	19.962	23	13279-13302	AACAUUUUUGAAGAGUCUGAG-CAA	UUGCUCGGACUCUUCAAAAAU-GUC	Cleavage
StMTP5	stu-miR5303j	StMTP5	2	19.962	23	13279-13302	AAUAUUUUUGAAGAGUCUGAG-CAA	UUGCUCGGACUCUUCAAAAAU-GUC	Cleavage
StMTP5	stu-miR156e	StMTP5	3	19.533	19	16071-16090	UGACAGAAGAGAGUGAGCAC	AAGCCUACUCUUUUCUGUCA	Cleavage
StMTP5	stu-miR156f-5p	StMTP5	3	18.572	19	16072-16091	CUGACAGAAGAGAGUGAGCA	AGCCUACUCUUUUCUGUCAC	Cleavage
StMTP5	stu-miR156g-5p	StMTP5	3	19.533	19	16071-16090	UGACAGAAGAGAGUGAGCAC	AAGCCUACUCUUUUCUGUCA	Cleavage
StMTP5	stu-miR156h-5p	StMTP5	3	19.533	19	16071-16090	UGACAGAAGAGAGUGAGCAC	AAGCCUACUCUUUUCUGUCA	Cleavage
StMTP5	stu-miR156i-5p	StMTP5	3	19.533	19	16071-16090	UGACAGAAGAGAGUGAGCAC	AAGCCUACUCUUUUCUGUCA	Cleavage
StMTP5	stu-miR156j-5p	StMTP5	3	19.533	19	16071-16090	UGACAGAAGAGAGUGAGCAC	AAGCCUACUCUUUUCUGUCA	Cleavage
StMTP5	stu-miR156k-5p	StMTP5	3	19.533	19	16071-16090	UGACAGAAGAGAGUGAGCAC	AAGCCUACUCUUUUCUGUCA	Cleavage
StMTP5	stu-miR5303f	StMTP5	3	23.914	23	13308-13331	AUUUUUGGAGAAUCU-GACACGGGU	GUGCAUGGCGAAUUCUCCAAAA-GU	Cleavage

Table 4: Summary of the transcription factor binding sites (TFBS) detected in the promoter regions of *StMTP* genes

TFBS related to hormone/ tissue-specific/ stress response/binding site	Name of TFBS	StMTP5	StMTP3	StMTP1	StMTP6	StMTP8	StMTP8.1	StMTP9.1	StMTP9.2	StMTP9	StMTP11	StMTP4	StMTP7	Expected function
Tfs related to hormone response	AP2	87	92	78	51	53	14	5	19	15	39	28	37	Ethylene -responsive element
Tfs related to hormone response	BBR-BPC	6	2	0	0	0	0	0	0	0	0	0	0	Cytokinin -responsive element
Tfs related to hormone response	BES1	4	3	0	2	0	0	0	1	2	0	0	2	Strigolactone and Brassinosteroids -responsive element
Tfs related to hormone response	ARF	2	1	0	0	0	0	0	0	0	0	0	0	Auxin -responsive element
Tfs related to hormone response	EIN3 ; EIL	8	3	7	5	4	2	0	0	0	0	3	0	Involved in ethylene and JA signaling
Tfs related to hormone response	VOZ	9	3	0	0	0	0	0	0	0	0	1	0	Gibberellin -responsive element
TFs related to light response	bHLH	28	17	8	36	36	14	5	37	38	7	34	34	Light-responsive element
TFs related to light response	Dof	36	54	51	16	11	12	9	13	15	13	11	15	Light-responsive element
TFs related to light response	GATA	30	26	26	17	18	13	4	7	15	13	13	11	Light-responsive element
TFs related to tissue-specific localisation	AT-Hook	30	18	18	29	19	21	3	13	27	23	11	26	Vasculature-specific expression
TFs related to tissue-specific localisation	SBP	26	5	5	21	19	2	2	18	13	16	3	17	Involved in flower and fruit development
TFs related to tissue-specific localisation	LOB	1	0	0	0	0	0	0	0	0	0	0	0	Involved in lateral organ development
TFs related to tissue-specific localisation	MADS box	8	3	0	0	0	0	0	0	3	0	0	8	Involved in flowering development
TFs related to tissue-specific localisation	MADF	16	0	0	0	0	0	0	3	1	0	1	1	Involved in flower and fruit development

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TFs related to tissue-specific localisation	TCR	14	0	6	4	3	4	0	4	0	0	4	0	Involved in development male and female reproductive tissues/ tissue-specific expressions
TFs related to tissue-specific localisation	WOX	9	0	2	0	0	1	0	1	0	0	1	1	Tissue-specific expressions
TFs related to cell cycle	E2F/DP	3	1	0	1	0	0	0	0	0	2	0	0	Involved in cell polification
TFs related to stress response	MYB	259	214	165	70	58	37	9	47	46	49	32	60	responsive to environmental stress
TFs related to stress response	WRKY	106	55	3	33	27	34	2	13	20	23	33	27	involved in developmental and physiological processes
TFs related to stress response	HSF	20	2	2	2	0	3	0	0	0	0	0	20	Involved in cell differentiation, and proliferation
TFs related to stress response	C2H2	50	37	37	14	11	9	4	9	7	9	11	9	responsive to stress and the hormone signal transduction
Tfs related to basic transcription	NF-Y	7	2	3	2	1	1	4	1	2	2	3	3	Involved in transcription by recognizing and binding to a CCAAT motif in promoters
other tfs binding sites	WRC;GRF	4	0	0	0	0	1	1	1	1	0	0	0	Involved in stem and leaf development
other tfs binding sites	Sox	7	2	4	4	0	2	0	2	3	3	0	3	Involved in cell cycle regulation
other tfs binding sites	FAR1	1	0	0	0	1	0	0	1	0	0	0	0	Light-responsive element
other tfs binding sites	SRS	4	0	2	4	0	0	0	0	0	2	2	2	Involved in style and stigma development
other tfs binding sites	NAC	175	75	16	7	18	6	0	6	6	12	2	11	Involved in developmental process and stress responses
other tfs binding sites	bZIP	121	87	66	51	53	26	5	34	48	56	22	37	Developmental and physiological processes

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other tfs binding sites	Homeodomain	69	42	56	30	30	31	1	27	26	36	29	27	Involved in cell fate and differentiation
other tfs binding sites	Storekeeper	4	0	0	1	0	0	0	0	0	2	0	1	Involved in sucrose inducible expression of patatin gene
other tfs binding sites	B3	37	28	13	21	18	12	9	13	13	21	7	20	Involved in developmental process
other tfs binding sites	Trihelix	24	6	0	0	0	0	0	0	0	0	3	1	Tissue-specific expressions
other tfs binding sites	TCP	22	5	0	14	8	2	6	9	4	23	9	5	Involved in plant morphology
other tfs binding sites	ZF-HD	13	13	12	0	0	0	1	0	0	0	0	0	Involved in spike development

3.6 EXPRESSION PATTERNS OF *STMTP* GENES UNDER DROUGHT STRESS AND TISSUE-SPECIFIC ANALYSIS

3.6.1 The expression patterns of *StMTP* under drought stress

To better understand the expression of *StMTP* genes under the influence of drought stress, two *StMTP* genes were selected and their expression levels were checked by qPCR in leaves and tubers under stress. The expression levels of drought and normal treatments are given in Figure 1. The analysis results showed that *StMTP8* and *StMTP9* showed the highest level of expression in leaf and tuber (natural) under normal treatment, while both genes decreased under drought conditions. In leaves and tubers, the expression level of *StMTP9* was higher than *StMTP8* in both leaves and tubers (normal) (Fig 4a).

3.6.2 The expression patterns of *StMTP* in tissue-specific

The tissue expression patterns of *StMTPs* were investigated based on the qPCR data. As shown in Fig 1B and C, both genes (*StMTP8* and *StMTP9*) were expressed in the four determined tissues. The results of qPCR analysis revealed that the *StMTP9* gene significantly had higher expression levels as compared with *StMTP8* in all tissues such as root, stem, leaf, and tuber. *StMTP9* and *StMTP8* genes exhibited maximum levels of gene expression in leaf whereas, the minimum levels had in tuber. Moreover, the high expression of *StMTP9* gene was observed in the stem (Fig 4b,c).

4 DISCUSSION

In the present study, a total of 12 *StMTPs* were detected in potato. The *MTPs* were named based on the sequence similarities and orthologous relationships between them and the *AtMTPs*. First, the phylogenetic relationships of the MTP proteins between *S.tuberosum, A. thaliana,* and *O.sativa* were assessed. Based on previous studies, *A.thaliana* included 12 MTPs (AtMTP1-12). Contrasted with *Arabidopsis*, *S.tuberosum* genome carried multiple MTP homologs for each AtMTP, but the homologs for AtMTP2 and AtMTP3 were absent. There were two, four, and six *StMTP* genes belonging to Fe/Zn-CDFs, Zn-CDFs, and Mn-CDFs, respectively. It is established that phylogenetic relationships can be utilized to infer structure and functional roles among species (Vatansever et al. 2017). This finding could provide clues to

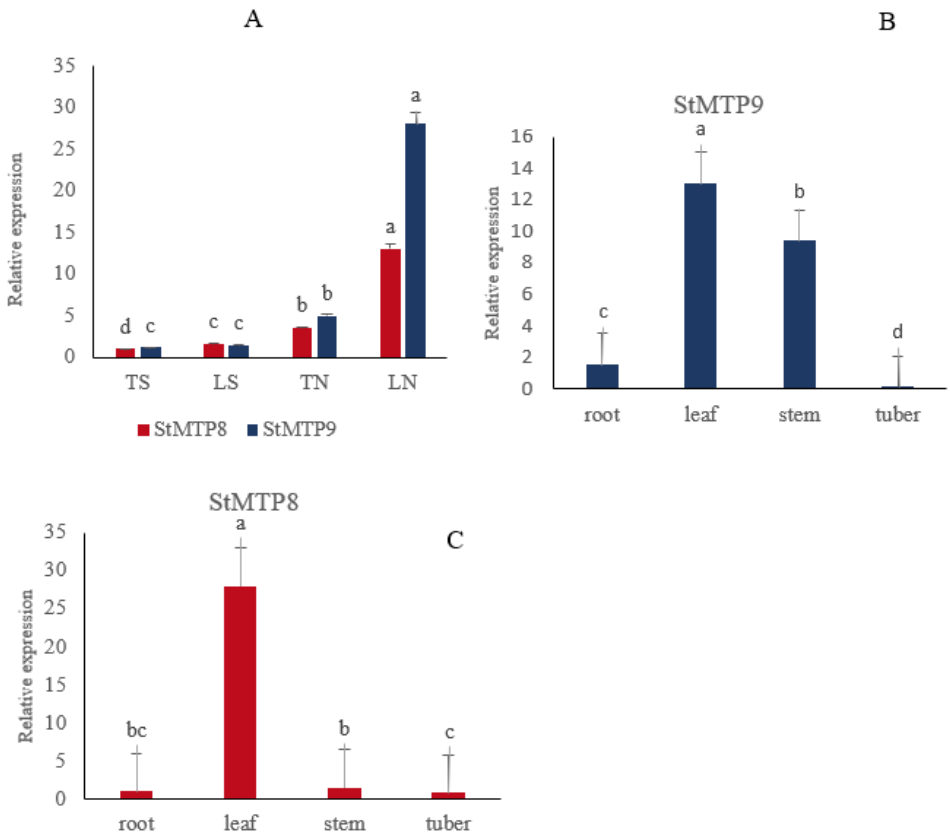

Figure 4: A) The qPCR expression of the potato *StMTP8* and *StMTP9* genes from tuber and leaf samples under drought stress. B, C) The expression of the potato *StMTP8* and *StMTP9* genes among different tissues in organs such as root, leaf, stem, tuber under control condition. Tuber normal, (TN), Tuber stress (TS), Leaf normal, (LN), Leaf stress (LS)

discover the functional characteristics, particularly the substrate-specificities of StMTP proteins. Montanini et al. (2007) identified a modified signature available in the trans-membrane regions of the metal tolerance proteins, and proposed a functional role the conserved group-residues in metal selectivity (Montanini et al., 2007). Further, the signature sequences HxxxD (x = any amino acid) and DxxxD were detected to illustrate the sequence characteristics of the both Zn-CDFs and Fe/Zn-CDFs, and Mn-CDFs, respectivey.

Features of the *StMTP* genes including peptide length, MW, Pi, sub-cellular, and TMD localization were analyzed. Our results agree with previous studies in wheat and tobacco (Vatansever et al., 2017; Liu et al., 2019), StMTP proteins were mainly predicted to be localized to vacuole, whereas some others are localized in cellular membrane and nucleus. It is suggested that StMTPs could function as the vacuole-localized cation transporters. Other studies in *Arabidopsis* revealed that AtMTP1 and AtMTP3 are involved in the transport of excess Zn into vacuoles, regulating cellular Zn hemostasis (Kobae et al. 2004; Arrivault et al. 2006). Although all of the StMTPs were identified the cation efflux domain and the modified features, however, some other motifs were not present in some StMTP members. StMTP6 and StMTP8.1 do not possess any TMD, a common signature of membrane proteins, which may have distinct biological functions and novel roles except other transporters.

Besides the transmembrane region, the modified signature sequence between TMDs I and II, and the characteristics C-terminal cation-efflux domain are two structural features of MTP proteins. Our findings revealed that all the StMTP proteins included two typical structural characteristics. Further, the signature sequences HxxxD and DxxxD were also detected in associated members of three main substrate-specific groups, which were in accordance with consensus residues. Also, these results provided a precious support for our phylogeny tree. Moreover, ZT-dimer was as molecule of zinc transporter that formed a homodimer during activity (Lu and Fu, 2007). The existence of ZT-dimer in specific StMTPs suggested that these proteins could require to organize heterodimers and homodimers when ministering as metal ion transporters. In this study, the ZT-dimer was iden-

tified in members of groups 8, 9 (except for StMTP9.2), and 11. Overall, these structure features of StMTP proteins were consistant with the structure characteristics of MTP transporters. These results revealed that there are structural similarity of StMTPs within the same groups.

The regulatory mechanisms controlling *StMTP* gene expression were evaluated at two levels, transcriptional and post-transcriptional using TFBS and the microRNA target sites in the promoter regions and the coding sequences of *StMTP* genes, respectively. A total of 5312 putative TFBS involved in multiple biological processes and thirteen StmiRNAs were detected. Former studies have revealed that some of these detected miRNAs were implicated in abiotic and biotic stress response. For example, the expression of stu-miR156e, stu-miR5303f, stu-miR5303g, stu-miR5303h, and stu-miR5303j would be up-regulated under late blight infection in potato (Kumar et al., 2018). In addition, mir156 possesses various functional roles in response to heat, cold, drought, and hypoxia (Stief et al., 2014). Stu-miR7992-3p was up-regulated in defense-related miRNAs to virus (Kondhare et al., 2018). Stu-miR5303g might also respond to Li^+ stress through regulating their target genes (Kwenda et al., 2016). Thus, it would be of interest to discover the functions of *StMTP* genes in this biological and physiological processes in latter studies.

The importance of *StMTP* function in potato growth and developmental stages could be identified through tissue expression profile analysis. For example, StMTP8 is highly expressed in leaf, an indication that it might be vital for potato leaf development. On the other hand, the expression levels of StMTP8 was most abundant in all three types of leaf structures during leaf formation, indicating it might be involved in regulating leaf development. StMTP9 was exclusively expressed in leaf and stem, indicating they have important roles in leaf and stem growth and development stages in potato. StMTP9, was slightly expressed in root and tuber, demonstrated a non-significant role in root growth and development. Also, StMTP8 was not or rarely induced in the evaluated root and tubers. In *Populus trichocarpa,* PtrMTP9 is expressed in roots and is sharply up-regulated by excess Fe (Gao et al., 2020). Earlier study has shown that OsMTP9 knockout significantly decreased Mn uptake and root-to-shoot translocation (Sasaki et al., 2016).

Drought treatment expression analysis showed that the *StMTP8* and *StMTP9* genes were down-regulated. Previous studies suggested that both AtMTP9 and VvMTP9 share two identical orthologues in potato, which StMTP9 is expected to be down-regulated in response to drought stress (Shirazi et al., 2019). However, both genes (AtMTP9 and VvMTP9) were upregulated in response to drought, salinity, osmotic shock, and hormonal stresses. AtMTP8 is expressed in root while it is orthologues with StMTP8 in potato. *StMTP8* gene is expressed in leaves whereas, it is slightly expressed in roots, stem, and tubers. *AtMTP 1* and *AtMTP3* were up-regulated in response to ABA, but were down-regulated in response to biotic and abiotic stresses. Additionally, *AtMTP2* was down-regulated in response to all stresses. Other *AtMTPs* varied with respect to expression, up and down regulation. This result showed that *MTP* genes have diverse roles in adaptation of plants under various stresses.

Using analysis of each StMTP genes promoter regions, different elements were identified that may regulate gene expression in developmental stages and drought stress in potato. In cucumber, MTP8 is a Mn transporter which maintain Mn homeostasis in root. CsMTP7 is constitutively induced in all cucumber tissues during plant development, a putative Fe/Zn transporter (Migocka et al., 2014). OsMTP1 was widely expressed in mature leaves and stems. Analysis of expression profiles revealed that StMTP might be involved in several aspects of potato development, and also be significant in leaf and shoot development. Further, *StMTP* genes may play significant roles drought and abiotic stress.

The expression profiles of the *MTP* genes under drought stress could reflect differences in the type and number of TFBS in the promoter region of the genes. As a result, different genes can respond to various stresses (Vatansever et al. 2017; Saidi et al., 2020a, b). The MYB and bZIP were two common TFBS found in the upstream regions of *StMTP* genes at a high frequency.

MYB plays a key role in plants under metal stress. In *Arabidopsis*, MYB4 is induced following exposure to Cd and Zn while MYB43, MYB48, and MYB124, member of MYB family were found to be particularly expressed in roots in response to Cd stress. MYB28 is as another member of MYB family which is induced after Cd-stress. Moreover, MYB, bZIP, AP2 play vital role in regulating the specific response of plants under Cd stress through modulating the particular responsive genes (Wu et al., 2012; Hajibarat and Saidi, 2022 a, Hajibarat et al., 2022b). BZIP has been identified as one of the most TFBS in *Arabdiopsis*, bean, sesame, and wheat, involved in adapting to zinc deficiency through inducing the expression of members of membrane transporters (ZIPs) (Wang et al., 2018; Saidi et al., 2020a, b).

In the current study, diverse MTPs revealed drought responses to stress conditions, the lowest level expression was observed for StMTP9 in response to drought stress. These results did not agree with our findings in *Arabiopsis*. Previous studies showed that some genes were up/down-regulated with similar stress with corresponding genes to same group (Li et al., 2018; Shirazi et al., 2019; Saidi and Hajibarat, 2019). AtMTP9, 10, 11 were up-reg-

ulated in plants exposed to drought, cold and salt stresses, regulating gene expression and functional proteins to enhance stress tolerance. In addition, drought stress lead to changes in plant metals concentration in *Brachypodium* (Chen et al., 2018). In general, our findings could provide significant evidence for highlighting the metal transport mechanism mediated by StMTP proteins in growth and developmental stages and drought stress.

5 CONCLUSION

Twelve *MTP*s in *S. tuberosum* were identified in the current study. Using bioinformatics tools, comprehensive analysis of *StMTP* genes were performed including protein properties, analysis of TFBS and structure, MicroRNA analysis, and analysis of gene expression in developmental and growth and drought stress. Based on phylogenic study, StMTPs were clustered into three subfamilies and seven groups (1, 5, 6, 7, 8, 9, and 11), similar to the MTP genes in *Arabidopsis*, rice, and tobacco. The MTP genes may have apparently been underwent gene loss and expansion via tandem duplication after polyploidization. All StMTPs contained cation-efflux and signature sequence, while, few of them also possess the ZT-dimer. The expression profiles of *StMTP* genes in tissue-specific and in response to drought stress predicted that *StMTP* genes had the necessary roles in potato developmental stage, particularly in drought stress. This study provides valuable resources for better insight into the biological roles of *StMTP* genes in potato.

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doi:10.14720/aas.2024.120.2.13294 Original research article / izvirni znanstveni članek

Combined and single osmopriming effects on wheat (*Triticum aestivum* L.) performance

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Received March 23, 2023; accepted May 13, 2024.
Delo je prispelo 13. marca 2023, sprejeto 13. maja 2024.

Combined and single osmopriming effects on wheat (*Triticum aestivum* L.) performance

Abstract: Osmopriming has been shown to improve the germination and growth of bread wheat (*Triticum aestivum* L.). This study explores the impact of various priming agent NaCl (3g l^{-1}), proline (1 mM), ZnSO4 (1 mM), and their combination on wheat performance during the summer season (Jul-Aug 2022) at the greenhouse of Payame Noor University, Tabriz. Wheat seeds treated with a combination of priming agent demonstrated significantly enhanced performance compared to untreated seeds. Chlorophyll fluorescence measurements taken 35 days post-cultivation revealed a higher Photosystem Performance Index (PIabs) in osmoprimed seeds, particularly those treated with combined priming agent. Furthermore, primed plants demonstrated elevated concentrations of chlorophyll a, b, and carotenoids. Osmopriming also modulated the oxidative status of enzymes such as glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD). Genetic analysis showed that osmopriming could influence the expression of *NHX2*, a gene linked to improving plant growth, water uptake, and yield in stress conditions.

Key words: priming, antioxidant capacity, phenolic compounds, gene expression, fluorescence, wheat (*Triticum aestivum* L.)

Rastni učinki kombiniranega in enovrstnega tretiranja semen krušne pšenice (*Triticum aestivum* L.) z ozmotiki

Izvleček: Tretiranje semen krušne pšenice (*Triticum aestivum* L.) z ozmotiki izboljša kalitev in rast. V raziskavi so bili preučevani učinki različnih obravnavanj z ozmotiki kot so NaCl (3 g l^{-1}), prolin (1 mM), ZnSO4 (1 mM) in njihovih kombinacij na rast pšenice v poletni rastni sezoni (julij-avgust 2022) v rastlinjaku na Payame Noor University, Tabriz. Zrna pšenice, tretirana s kombinacijami osmotikov so pokazala značilno boljšo rast kot netretirana. Meritve fluorescence klorofila, opravljene 35 dni po gojenju v loncih so pokazale večje vrednosti indeksa učinkovitosti fotosinteze (PIabs) v primeru z ozmotiki tretiranih semen, še posebej tistih tretiranih s kombinacijo osmotikov. Z ozmotiki pred kalitvijo tretirane rastline so imele povečane vsebnosti klorofila a, b in karotenoidov. Predobravnava z ozmotiki je vzpodbudila aktivnost antioksidacijskih encimov kot so glutation peroksidaza (GPX), katalaza (CAT) in superoksid dizmutaza (SOD). Geneteske analize nakazujejo, da ima predobravnavanje semen z ozmotiki pred kalitvijo pozitivni učinek na parametre uspešne rasti krušne pšenice.

Ključne besede: predobravnavanje, antioksidacijska sposobnost, fenolne spojine, ekspresija genov, fluorescenca, krušna pšenica (*Triticum aestivum* L.)

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1 INTRODUCTION

Bread wheat (*Triticum aestivum* L.), a vital cereal crop, is extensively cultivated and accounts for over 20 % of the global population's daily protein intake (Rai-Kalal & Jajoo, 2021; Singhal, Pandey, & Bose, 2021). However, challenges such as poor seed properties, suboptimal soil conditions, and biotic and abiotic stresses can severely impact wheat productivity (Adnan et al., 2020; Amoah et al., 2019; da Costa et al., 2011; Dalil, 2014).

Expanding the range of wheat production is crucial, and developing stress-tolerant wheat cultivars through selective breeding is highly recommended (Amoah et al., 2019; Lobato et al., 2009). Seed priming techniques such as hydropriming, osmopriming, nanopriming, and mix priming offer cost-effective and efficient methods to enhance crop speed and stand in the field (Adnan et al., 2020). Osmopriming wheat seeds with osmotic components can improve stand establishment and reduce the time between seed sowing and seedling emergence (Farooq et al., 2019). Various priming agent, such as PEG, KNO_3, $K3PO_4$, $CaCl_2$, and NaCl, have been utilized for wheat seed priming (Amin, Khan, & Khalil, 2012). The properties and effectiveness of priming solutions differ based on the crop species. (Rai-Kalal & Jajoo, 2021). However, the study on the impact of combined priming agent on plant performance during seed germination and growth is limited.

The seed industry is actively seeks potent priming agents that can enhance plant resilience in challenging field conditions (Srivastava et al., 2010). Nonetheless, seed osmopriming, a chemical treatment for seeds, raises environmental and health concerns due to its detrimental effects on the environment and human health (Hasan et al., 2016). The adaptability of seed priming relies on the selection of suitable priming agents and understanding their mechanisms (Islam, Mukherjee, & Hossin, 2012). Factors such as economic costs, the nature of pretreatment agents, priming exposure duration, and crop species influence the effectiveness of pretreatment (Bisen et al., 2015). Despite its constraints, seed priming has demonstrated promise in improving seed germination, growth, and resilience to abiotic stresses such as salinity, drought, and heat within agriculture (Siyar et al., 2020). In previous research, we refined the osmopriming technique for wheat seeds. This study encompassed experiments conducted in Petri dishes, where different concentrations of NaCl, $ZnSO_4$, proline, and trehalose were used. The findings indicated a significant increase in wheat seed germination when exposed to 3 and 10 g l^{-1} NaCl concentrations, 1 and 20 mM ZnSO4, 1 and 10 mM proline, and 0 and 1 mM trehalose. These concentrations were validated using the surface-response method and experiments. Utilizing three priming reagents, individually or in combination, resulted in a marked increase in seed germination. Notably, the most efficacious treatments included NaCl (3 g l^{-1}), Proline (1 mM), and $ZnSO_4$ (1 mM) for 12 hours (Yavari et al., 2022). Therefore, the present investigation aimed to examine optimized concentrations and evaluate their potential in improving the germination, emergence, and early stand establishment, as well as specific physiological attributes of wheat in soil.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL AND EXPERIMENTAL CONDITIONS

We utilized NaCl (3 g l^{-1}), proline (1 mM), $ZnSO_4$ (1 mM), and combinations thereof (NaCl (3 g l^{-1}) + proline (1 mM) + $ZnSO_4$ (1 mM)) as osmopriming agents, which were optimized in our previous Petri dish experiments (Yavari et al., 2022). Seeds were soaked in osmopriming agents for 12 hours and subsequently sown at a depth of 0.5 cm in soil-filled vases. Both non-primed and primed seeds were sown in three replicates of 50 seeds each in the greenhouse of Payame Noor University, Tabriz, during the summer season (July–August 2022). Each vase received daily irrigation of 10 ml of water. The performance indexes of the seeds were measured on the 35^{th} day.

2.2 FLUORESCENCE ANALYSIS AND PHOTOSYNTHETIC PIGMENTS

An analysis of leaf fluorescence was performed at room temperature using a plant efficiency analyzer (PEA, Packet-PEA, Hansatech Instruments Ltd., England). The efficiency of the oxygen-evolving complex on the donor side of PSII (*Fv/Fo*) and the maximum quantum yield of photosystem II (*Fv/Fm*) were determined. In this context, Fm represents the maximal intensity of chlorophyll fluorescence, *Fv* stands for variable chlorophyll fluorescence, and *F*o indicates minimal fluorescence. Spectrophotometry was employed to determine the content of photosynthetic pigments, including chlorophyll a/b and carotenoids. Samples were homogenized with methanol, centrifuged at 1000 rpm, and the resulting supernatants were used for analysis. Calculation was performed based on the method described by Lichtenthaler and Wellburn (1983) (Lichtenthaler & Wellburn, 1983).

2.3 COMPATIBLE SOLUTE CONTENT MEASUREMENTS

The process of extracting leaf samples was carried out using a sodium phosphate buffer solution. (PBS, 50 mM, pH = 6.8). Following centrifugation (15000 g, 20 min), protein content was quantified using an auto-analyzer device (Abbott Alcyon 300). To determine sugar content, 200 µl of the supernatant was mixed with anthrone-sulfuric reagent (1 ml), boiled in a hot water bath (10 min, 100 °C). After cooling, the solution was measured for absorbance at 650 nm. Total soluble sugars were calculated using a glucose standard curve (Sigma). Starch analysis followed the method described by Magné et al. (2006) (Magné, Saladin, & Clément, 2006). Starch was dissolved in a 4 : 1 (v/v) mixture of 8 N HCl/dimethylsulfoxide and the solution was then mixed with an iodine HCl solution and absorbance was measured at 600 nm. In order to measure the amount of starch present, a standard curve of starch obtained from Merck was utilized. Proline content was assessed using the method outlined by Bates et al. (1973) (Bates, Waldren, & Teare, 1973). Leaf samples were homogenized in sulfosalicylic acid (3 % w/v, 4 °C) and centrifuged (3000 g, 20 min). The supernatant was mixed with acid ninhydrin and glacial acetic acid for 1 hour in a hot water bath, and proline content was calculated at 520 nm using a proline (Sigma) standard curve.

2.4 PHENYLALANINE AMMONIA-LYASE (PAL) ACTIVITY AND RELATED METABOLITES

The activity of PAL was measured using the modified method of Zucker (1965). Briefly, leaf samples were homogenized in PBS (50 mM, pH 7.0) supplemented with polyvinyl polypyrrolidon (PVPP) (2 % w/v), EDTA (2 mM), β-mercaptoethanol (18 mM) and Triton X-100 (1 % v/v). The cinnamic acid formation was monitored by spectrophotometry at 290 nm, representing PAL activity (one unit (U) activity equals one nmol cinnamic acid per hour produced by the enzyme). The Velioglu et al. (1998) method was used to measure total phenolic content. (Velioglu et al., 1998). A standard curve was created using gallic acid, and the results were expressed as milligrams per gram of fresh mass. Total flavonoid content was determined using the method outlined by Meda et al. (2005) (Medaet al., 2005). In brief, 5 ml of aluminum chloride (2 %) in methanol was mixed with 5 ml of leaf extracts (0.02 mg ml^{-1}). The total flavonoid content of the extract was determined using a standard curve of quercetin and expressed as mg quercetin equivalent (QE) 100 g^{-1} extract after 10 minutes.

2.5 ASSAY OF ANTIOXIDANT ENZYMES AND RELATED METABOLITES

SOD and CAT activity were determined using the method previously reported by Habibi and Hajiboland (2012) (Habibi and Hajiboland, 2012). Glutathione peroxidase (GSH-Px) activity was assessed using the modified method by Flohé and Günzler (1984). (Flohé & Günzler, 1984). To determine the extent of lipid peroxidation in membranes, the concentration of malondialdehyde (MDA) was measured. Leaf samples were homogenized in thiobarbituric acid (1 ml, 0.1 %) and centrifuged at 12,000 × g for 10 min. A 1, 1 , 3, 3-tetra ethoxy propane-based standard curve was used to quantify MDA, and the absorbance was measured at 525 nm.. The hydrogen peroxide (H_2O_2) content was assayed according to the procedures described by Velikova et al. (2000) (Velikova, Yordanov, & Edreva, 2000). H_2O_2 content was determined using a standard curve.

2.6 RNA EXTRACTION, CDNA SYNTHESIS AND RT-PCR ANALYSIS

The Trizol reagent was used to isolate total RNA from both primed and non-primed plant leaves.cDNA synthesis was carried out using the cDNA Reverse Transcription Kit (Applied BiosystemsTM) according to the protocol. The quality of the synthesized cDNA was verified using a 1 % agarose gel. Forward (F-ATTTTGCTCGGGTTGGTTCTGGTT) and reverse (R-GTGCAGGGACTTCGGTGACGC) primers targeting the NHX2 gene were employed. The actin gene of wheat served as an internal standard.

2.7 STATISTICAL ANALYSIS

The results were derived from three independent series of experiments. Chlorophyll fluorescence parameters were analyzed using the PEA Plus V1.10 software. GraphPad Prism (version 9.4.1) was used to perform statistical analysis, and differences among treatments were assessed by one-way ANOVA at a significance level of $p < 0.05$. (Refer to Table 1).

Table 1: Results of variance analysis of parameters

Variables	Nonprime	Combination	NaCl (3 g l^{-1})	Proline (1 nM)	ZnSO4 (1 mM)
Germination (%)	76 ± 7.21	98 ± 2	89.33 ± 1.15	93.33 ± 7.02	82 ± 12.16
Chl*a* content (mg g FM^{-1})	22.43 ± 0.11	23.51 ± 1.51	30.96 ± 1.66	27.22 ± 1.51	25.75 ± 2.07
Chl*b* content (mg g FM^{-1})	11.25 ± 0.96	22.93 ± 2.66	28.01 ± 0.39	27.73 ± 1.76	12.05 ± 1.31
Carotenoid content (mg g FM^{-1})	4.15± 0.87	5.33± 0.32	3.006± 0.83	5.89± 0.22	5.1 ± 0.32
Seedling length (mm)	421.2 ± 24.69	456.86 ± 3.28	431.93 ± 20.51	451.8 ± 8.74	384.4 ± 5.92
RWC (%)	59.11 ± 1.23	85.87 ± 7.21	92.24 ± 5.51	84.91 ± 10.32	101.02 ± 4.86
PI abs	1.39 ±0.04	2.207 ± 0.01	1.129 ± 0.07	0.43 ± 0.08	2.25 ± 0.05
Fv/Fm	0.67 ± 0.11	.08 ± 0.02	0.77 ± 0.06	0.68 ± 0.09	0.79 ± 0.009
Fv/Fo	2.28 ± 1.13	4.16 ± 0.75	3.58 ± 1.25	2.35 ± 0.97	3.89 ± 0.2
Starch content (mg g FM^{-1})	124.25 ± 3.5	138.79 ± 0.66	144.18 ± 1.95	140.77 ± 6.40	131.04 ± 0.40
Soluble sugars content (mg g FM^{-1})	7.74 ± 0.86	20.2 ± 0.22	13.51 ± 1.08	7.77 ± 0.95	16.60 ± 3.46
Protein content (mg dl^{-1})	34 ± 1	47.5 ± 1.5	71 ± 3	49.5 ± 1.5	36.5 ± 5.5
Proline content (µM g FM^{-1})	3.66 ± 0.33	9.25 ± 0.21	14.77 ± 0.67	9.33 ± 0.10	4.69 ± 0.40
SOD activity (U ml^{-1} protein)	0.038 ± 0.0005	0.017 ± 0.001	0.026 ± 0.001	0.023 ± 0.002	0.021 ± 0.0002
GPX activity (U g^{-1} protein)	1.81 ± 0.08	1.88 ± 0.08	1.26 ± 0.05	2.64 ± 0.18	2.52 ± 0.47
Flavonoid content (mg QE g FM^{-1})	1.56 ± 0.16	21.63 ± 0.08	16.75 ± 0.17	16.53 ± 0.12	17.18 ± 0.16
Phenol content (mg GEA g FM^{-1})	22.33 ± 0.83	25.06 ± 2.85	25.16 ± 0.95	25.2 ±.5	25.73 ± 0.35
PAL activity (µmol cinamic cid g^{-1} protein min^{-1})	0.49 ± 0.04	0.57 ± 0.02	0.71 ± 0.02	0.71 ± 0.018	0.51 ± 0.108
CATactivity (µmol H_2O_2 mg^{-1} protein min^{-1})	17.55 ± 1.95	33.15 ± 1.95	17.55 ± 1.95	6.85 ± 0.95	25.35 ± 1.95
H_2O_2 content (µM g FM^{-1})	61.77 ± 0.76	42.03 ± 2.28	44.31 ± 3.04	25.31 ± 0.76	42.79 ± 1.52
MDA content (nM g FM^{-1})	32.5 ± 2.5	16.5 ± 1.5	30.5 ± 4.5	24.5 ± 1.5	27.5 ± 1.5

3 RESULTS AND DISCUSSION

3.1 COMBINED AND INDIVIDUAL OSMOPRIMING SIGNIFICANTLY ENHANCE SEED GERMINATION INDICES AND PHOTOSYNTHETIC FUNCTION

The efficacy of priming agent as seed osmopriming agents on germination depends on concentration and priming duration. Wheat seeds primed with NaCl (3 g l^{-1}), proline (1 mM), $ZnSO_4$ (1 mM) and their combination exhibited improved germination rates. A combined priming treatment resulted in a significant increase in seed germination compared to untreated seeds. (Figure 1A). Previous studies have highlighted the beneficial effects of NaCl (Mirza, 2021), proline (Ambreen et al., 2021) and $ZnSO_4$ (Rehman et al., 2022) in wheat. For instance, seed priming with $ZnSO_4$ (0.1 and 0.5 M) enhanced plant water relations, grain yield, seedling growth, and stand establishment in wheat compared to non-primed seeds (Rehman et al., 2022). Similarly, proline priming improved germination rate (GR) and relative germination energy (RGE) under salinity stress in rice seeds (Hua-long et al., 2014) with the most effective concentration being 25 mM (Feghhenabi et al., 2020). Our findings align with these results, showing maximum germination in wheat seeds primed with different concentrations of NaCl, proline and $ZnSO_4$. Additionally, combined osmopriming exhibited significant improvement in germination compared to individual priming. This observation is consistent with the synergistic effect of combined $Mg(NO_3)_2$ and $ZnSO_4$ under drought stress compared to individual and non-priming treatments (Singhal et al., 2021). Relative water content (RWC), a crucial physiological parameter, reflects plant water status and stress tolerance (Singhal et al., 2021). Non-priming treatments exhibited the lowest RWC values, while combined priming resulted in the highest RWC values (Figure 1B). The effectiveness of combined osmopriming likely arises from the synergistic interaction of NaCl, proline and $ZnSO_4$, which collectively contribute to seed germination and early growth. NaCl facilitates water up-

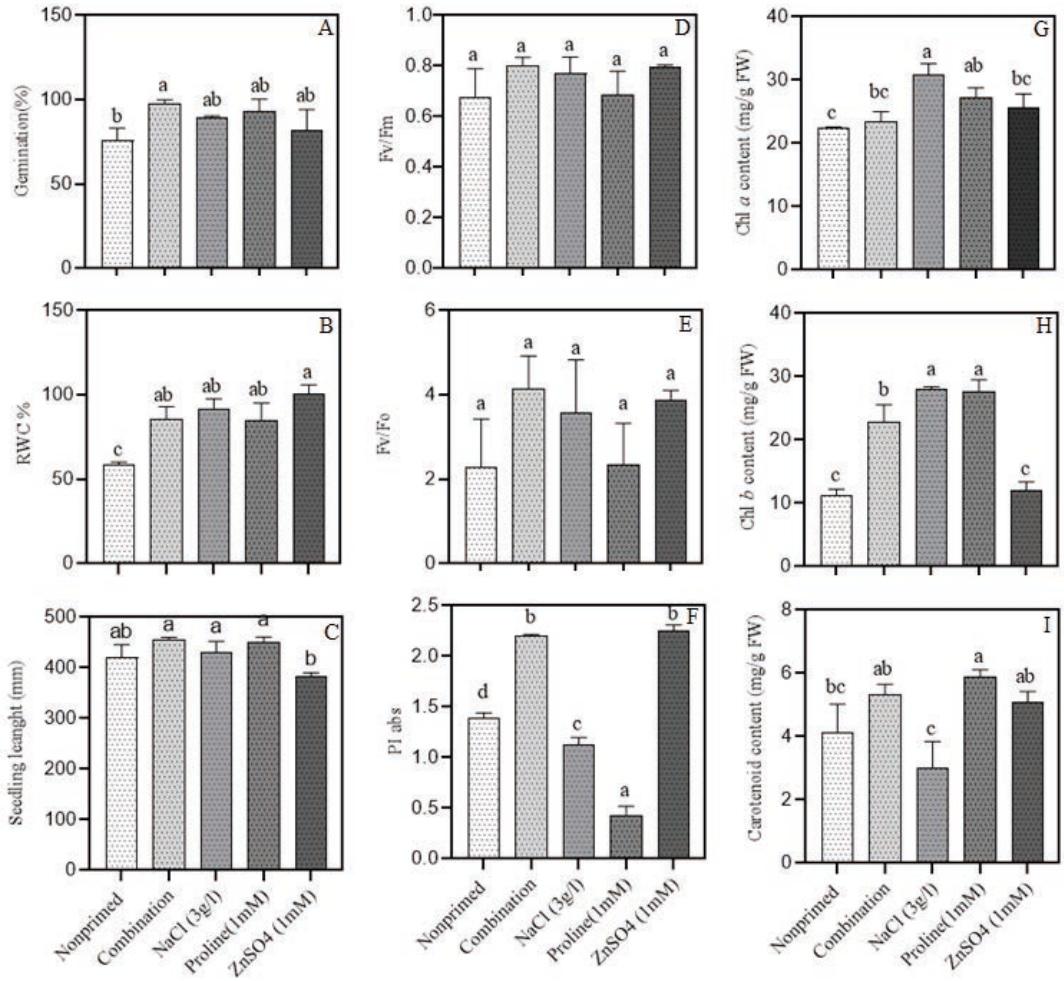

Figure 1: Screening the effect of different priming treatments on A) germination percentage, B) Relative water content (RWC) percentage, C) seedling length, D) The efficiency of oxygen-evolving complex on the donor side of PSII (Fv/Fo) (E) the maximum quantum yield of photosystem II (Fv/Fm), F) The Performance Index (PIabs), G) chlorophyll a, H) chlorophyll b, I) content of carotenoids . The error bars represent the standard deviation, and groups with the same letter are not significantly different from each other

take (Biswas et al., 2023), proline protects against osmotic stress and reactive oxygen species (ROS) (Kavi Kishor et al., 2022) and $ZnSO_4$ aids in enzyme activation and DNA synthesis, thereby enhancing seed performance. Bibi et al. (2017) reported a significant increase in plant growth and RWC of wheat under sodium nitroprusside priming (Bibi et al., 2017). However, our results suggest that applying $ZnSO_4$ hurt seedling length (Figure 1C) compared to other priming agents. Similar observations have been reported who noted retardation in the growth of seedlings treated with $ZnSO_4$ (Pavani et al., 2014; Rai-Kalal & Jajoo, 2021). This may be due to the high solubility of ZnSO4, which has minimal retention within the plant and results in inefficient Zn bioavailability over a prolonged period. (García-López et al., 2019; Prasad et al., 2012). Seed germination and seedling growth in wheat were negatively affected by seed priming with CuSO4 and ZnSO4, according to the results (Mim et al., 2021).

Figure 1D-F depicts the typical polyphasic rise (O–J–I–P) of fluorescence transients for NaCl, proline, $ZnSO_4$ and their combination after 30 days of cultivation. A non-significant increase in the ratio of *Fv/Fo* was observed in primed plants (Figure 1D). The *Fv/Fm*, representing the maximum quantum yield of photosystem II, remained unchanged under seed priming conditions (Figure 1E). However, a significant increase in the photosystem performance index (PIabs) was observed in treated plants (Figure 1F), suggesting that osmopriming can mitigate damage to the electron transport chain of PSII. Interestingly, the efficiency of the water-splitting complex increased in primed plants with combinational treatment and $ZnSO_4$ compared to unprimed plants. This

finding aligns with previous research on seeds primed with zinc oxide nanoparticles (Rai-Kalal & Jajoo, 2021).

It was found out that the osmopriming treatments resulted in the mildest increase in chlorophyll content. Notably, NaCl and proline treatments significantly influenced the content of photosynthetic pigment chlorophyll a compared to the control (Figure 1G). Furthermore, the largest increase in chlorophyll b was observed in treatments with NaCl, proline, and combination treatment (Figure 1H). Carotenoids content, which play a crucial role in plant photoprotection mechanisms, also significantly increased in the proline treatment compared to non-primed plants (Figure 1I). Additionally, osmopriming increased chlorophyll fluorescence in the I–P phase from the OJIP transient, possibly due to reduced availability of ferredoxin and NADP (Kalaji et al., 2016).

Osmopriming is a method that enhances plant tolerance to stress by improving photosynthesis. Studies have demonstrated that applying 0.9 MPa polyethylene glycol (PEG) as a priming agent at 18 °C for 30 hours can confer drought resistance in wheat reproductive stages. This effect is achieved by increasing the net photosynthetic rate and enhancing photo-protective and antioxidative mechanisms (Sherin, Aswathi, & Puthur, 2022). Primed plants under stress conditions exhibit higher levels of carotenoids, which are crucial for optimal growth and yield (Abid et al., 2018). Osmopriming preserves the structure and function of photosynthetic pigments and the photosynthetic apparatus (Sherin et al., 2022). In comparison, untreated plants may have a higher relative growth rate and yield of grains in barley during drought conditions (Kaczmarek egt al., 2017). Investigations into the effects of osmopriming, specifically with 30 % PEG 6000, on sunflower plants under water stress conditions have shown that it increases the net assimilation of CO_2 and improves photosynthesis. Priming also influences the accumulation of soluble sugars in sunflower plants, leading to higher yields even under water stress. This improved performance is correlated with a 40 % increase in chlorophyll levels in primed leaves (Bourioug et al., 2020; Sherin et al., 2022). Osmopriming with PEG 6000 has also improved drought tolerance in *Medicago sativa* L. by increasing PSII efficiency, enhancing plant height, leaf area and growth (Mouradi et al., 2016). Additionally, it improves growth and biomass in *Lens cullinaris* Medik by reducing oxidative damage through improved sugar and calcium accumulation (Farooq et al., 2020). Overall, osmopriming boosts seedling growth and germination by improving photosynthesis, carbohydrate production, energy, light absorption, CO_2 uptake, biomass, stress tolerance and antioxidant protection. (Sherin et al., 2022).

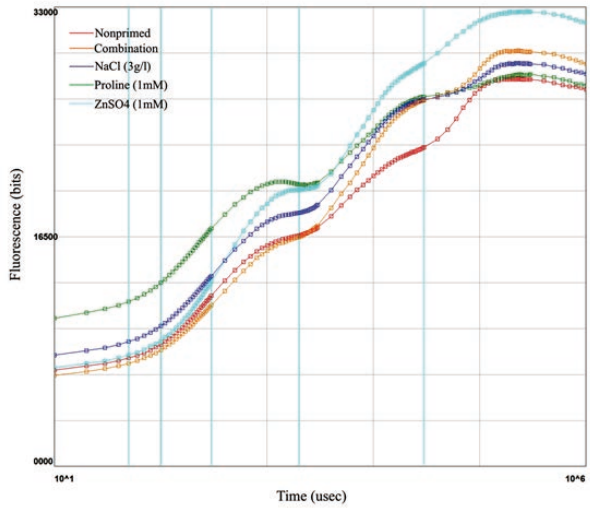

Figure 2: Effects of different priming treatments on the chlorophyll a fluorescence induction curve of wheat

3.2 COMBINED AND SINGLE OSMOPRIMING HAVE SHOWN SIGNIFICANT EFFECTS ON COMPATIBLE SOLUTES

When seeds are osmoprimed under low external water potential, they release organic solutes such as proline, glycine- free amino acids, and betaine (Ibrahim, 2016; Lemmens et al., 2019). Studies have demonstrated a significant correlation between starch content and germination index, seedling vigor index, shoot length, root length, and total seedling length (Salleh, Nordin & Puteh, 2020). Osmopriming can enhance the activities of acid invertase, alkaline invertase, and sucrose synthase (cleavage), as well as the contents of reducing sugars and starch in the grains of stressed plants (Kawatra, Kaur & Kaur, 2019). According to statistical data, the process of osmopriming using proline and NaCl resulted in the highest starch content (Figure 3A). Seed priming leads to enhanced accumulation of soluble sugars compared to non-primed seeds. The breakdown of starch into soluble sugars fuels seedling growth and germination. (Savvides et al., 2016). The highest soluble sugar content was found in NaCl, $ZnSO_4$, and combined-treated seeds, while the lowest was recorded in proline-treated seeds (Figure 3B). Khaing et al. (2020) indicated that 1 % K_2SO_4 significantly increased proline content in two wheat cultivars, Keumkang and Backjung (Khainget al., 2020). Consistent with these results, our data also showed that proline content increased in primed seeds compared with unprimed seeds (Figure 3C). There was a significant increase in soluble proteins (TSP) in treatments, except $ZnSO_4$,

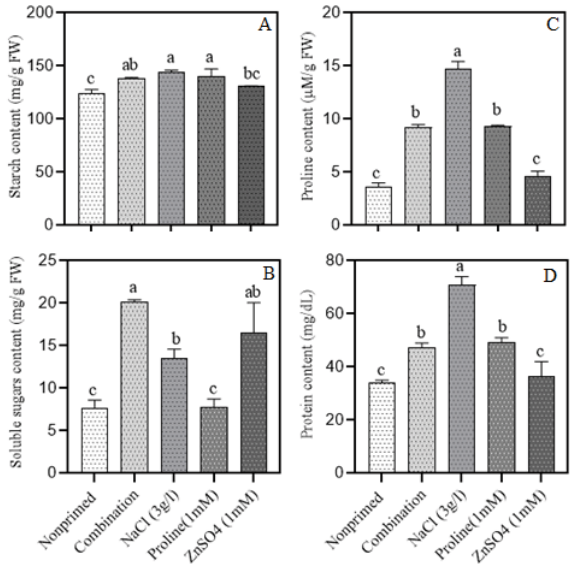

Figure 3: Effects of different priming treatments on A) starch content, B) soluble sugars, C) proline, D) protein. The error bars represent the standard deviation, and groups with the same letter are not significantly different from each other

compared to non-primed seeds (Figure 3D). Seed priming with combinational concentrations of Fe and Zn (4 and 8 mg l^{-1}) significantly increased soluble proteins in bread wheat compared to the control (Carvalho et al., 2019). Studies have shown that priming with $Mg(NO_3)_2$, $ZnSO_4$, and their combination can significantly improve the protein content of seeds (Choudhary et al., 2021). Priming also increased the total protein in amaranth seeds (Moosavi et al., 2009).

It is well established that secondary metabolites such as flavonoids and phenolic acids play a central role in defense mechanisms, signaling, and scavenging of free radicals, ultimately leading to increased nutritional values of crops (Kanjevac et al., 2022; Mousavi et al., 2021; Tohidi, Rahimmalek, & Arzani, 2017). In this study, the applied osmopriming treatments had a significant effect on PAL activity, phenolic and flavonoid content (Figure 4A-C). Previous studies have demonstrated increased flavonoid concentration in radish seedlings after priming with $MgSO_4$, IAA, and H_2O_2 (Kanjevac et al., 2022).

3.3 COMBINED AND INDIVIDUAL OSMOPRIMING EXERTED SIGNIFICANT EFFECTS ON THE ANTIOXIDANT DEFENSE SYSTEMS

Enzyme activity of GPX, CAT and SOD was assessed, revealing a prominent decrease in the activity of these enzymes in primed seeds, likely attributable to low levels of reactive oxygen species (ROS). CAT enzyme activity notably increased with combined and $ZnSO_4$ treatments but decreased with proline compared to non-primed seeds (Figure 4D). These findings align with Weisany et al. (2012), who suggested that the elevated CAT activity may result from the indirect requirement of zinc for H_2O_2 detoxification (Rai-Kalal & Jajoo, 2021; Weisany et al., 2012). Additionally, a decrease in SOD enzyme level was observed in treated seeds (Figure 4E), consistent with nanoparticle $ZnSO_4$-based seed priming effects (Rai-Kalal & Jajoo, 2021). However, in contrast to our results, Rai-Kalal (2021) showed enhanced SOD activity in $ZnSO_4$-primed plants compared to non-primed ones (Rai-Kalal & Jajoo, 2021). The activity of GPX increased in seed osmopriming with proline and $ZnSO_4$ (Figure 4F). Plants primed with $ZnSO_4$ exhibit higher GPX activity, which may be due to increased ROS generation from the greater solubility of toxic Zn^{2+} ions. (Rai-Kalal & Jajoo, 2021). Osmopriming-based high GPX/CAT activity likely contributes to restoring the antioxidant defense system for early seedling establishment. For instance, CAT activity upregulation during early germination and higher overall antioxidant activity in germinated seeds/seedlings than non-germinated ones have been reported (Chen & Arora, 2011).

Plant photosynthesis is linked to antioxidant defense mechanisms and osmolyte accumulation, particularly in response to various environmental stresses. Drought (water or moisture stress) increases photosynthetic pigment and proline content, indicating a responsive mechanism (Binodh et al., 2023). Similarly, under drought stress conditions, antioxidant enzymes such as superoxide dismutase, peroxidase and catalase are upregulated, suggesting an enhanced antioxidant defense system (Wang et al., 2019). Priming agent like proline, glycine betaine, and trehalose accumulate under salinity stress, playing a vital role in osmotic adjustment (Forough et al., 2018). This osmotic adjustment is crucial for plants to adapt to saline environments, as evidenced by changes in photosynthesis observed in halophyte plants (Nikalje et al., 2018). Furthermore, in maize plants subjected to water stress, the application of a combination of 24-epibrassinolide, spermine, and silicon enhances photosynthetic metabolites and antioxidant enzyme activity, leading to improved drought resistance and reduced accumulation of reactive oxygen species (Ghasemi et al., 2022). Similarly, alterations in photosynthesis in pigeon pea seedlings exposed to copper stress contribute to increased antioxidant defense mechanisms and osmolyte accumulation (Sharma et al., 2017). These changes are manifested through increased catalase and peroxidase enzyme activity, along with the production of priming agent like proline, glycine betaine, and trehalose (For-

ough et al., 2018). High antioxidant capacity is advantageous for plants as it helps desensitize photosynthesis to over-reduction in the photosynthetic electron transport (PET) chain and can alleviate over-reduction in water-water cycle activity. However, the precise influence of antioxidant capacity on retrograde signaling pathways is not fully understood. Exploring redox signaling pathways could provide valuable molecular insights for upregulating plant protective genes (Foyer & Shigeoka, 2011).

Malondialdehyde (MDA) is a reliable marker for assessing plant injury caused by stress, as it correlates with the degree of plant damage (Fayez & Bazaid, 2014). Under stress conditions, plants produce reactive oxygen species (ROS) that inhibit biomolecule production, leading to increased levels of MDA and cellular leakage. Monitoring MDA levels provides valuable insights into plant growth dynamics, enabling real-time assessment of stress conditions and facilitating preemptive measures against drought (Zhang et al., 2021). In our experimental setup, MDA levels exhibited a significant decrease in plants subjected to combine and proline priming compared to non-primed plants (Figure 4G). This reduction in MDA content in primed plants aligns with findings reported by Prabha Rai-Kalal et al. (Rai-Kalal & Jajoo, 2021). Regardless of the priming agent and stress imposition, hydrogen peroxide (H_2O_2) levels decreased under primed conditions. (Ellouzi, Sghayar, & Abdelly, 2017). Primed seeds demonstrated lower tissue H_2O_2 contents than the control (Figure 4H). Additionally, seeds subjected to osmopriming with melatonin OMel50 and OMel500 exhibited the lowest H_2O_2 accumulation during the experiment (Marta, Szafrańska, & Posmyk, 2016).

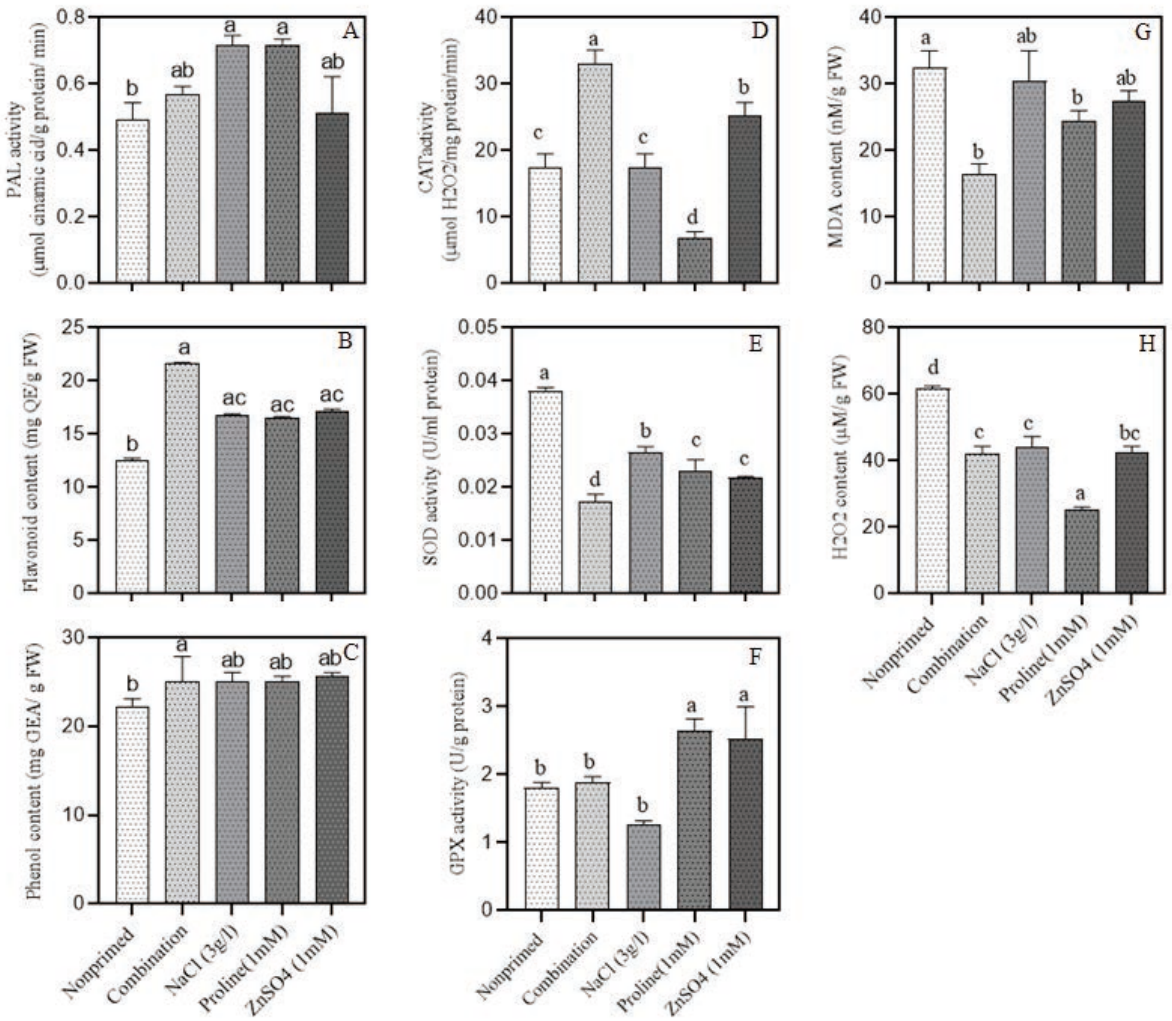

Figure 4: Effects of different priming treatments on A) PAL activity, B) flavonoid, C) phenol content, D) CAT E) SOD F) GPX activity, G) malondialdehyde (MDA), H) H2O2 content The error bars represent the standard deviation, and groups with the same letter are not significantly different from each other

3.4 OSMOPRIMING HAD AN IMPACT ON THE EXPRESSION OF THE *NHX2* ANTIPORTER GENE

Intracellular Na^+/H^+ (*NHX*) antiporters are crucial for maintaining cellular pH and the homeostasis of Na^+ and K^+ ions (Bassil et al., 2011; Xu et al., 2013). *NHX1* and *NHX2* are pivotal in regulating K^+ levels and intravacuolar pH, essential for cell expansion and flower growth (Xu et al., 2013). They enhance salt stress resistance by facilitating intracellular potassium partitioning, thereby regulating cellular pH and K+ homeostasis (Bassil et al., 2011). *NHX* genes in the wheat genome, particularly *NHX2*, are crucial for salinity tolerance across various plant species (Yarra, 2019). Transgenic plants expressing *NHX2* exhibit elevated levels of chlorophyll, relative water content, superoxide dismutase, ascorbate peroxidase, reduced hydrogen peroxide levels, and malondialdehyde content compared to wild-type plants (Bulle et al., 2016; Yarra, 2019). In this study, the potential role of the *NHX2* gene in wheat germination under optimized priming concentrations was investigated. The study revealed that priming seeds with NaCl and proline, as well as combinations of treatments, led to an increase in the expression of the *NHX2* gene in primed seeds. This increase may be attributed to the rise in sodium content under non-stressed conditions (Figure 5A-D). Recent research has shown that *NHX1* and *NHX2* are transporters located in the vacuole that play a key role in regulating the pH and potassium levels within the vacuole. These proteins are essential for facilitating the uptake of potassium at the tonoplast, maintaining osmotic balance and turgor pressure, and have a notable impact on stomatal function (Barragan et al., 2012). In response to 500 mM NaCl, the *NHX2* gene exhibited a similar pattern of expression, showing a significant increase in leaves of both non-primed and

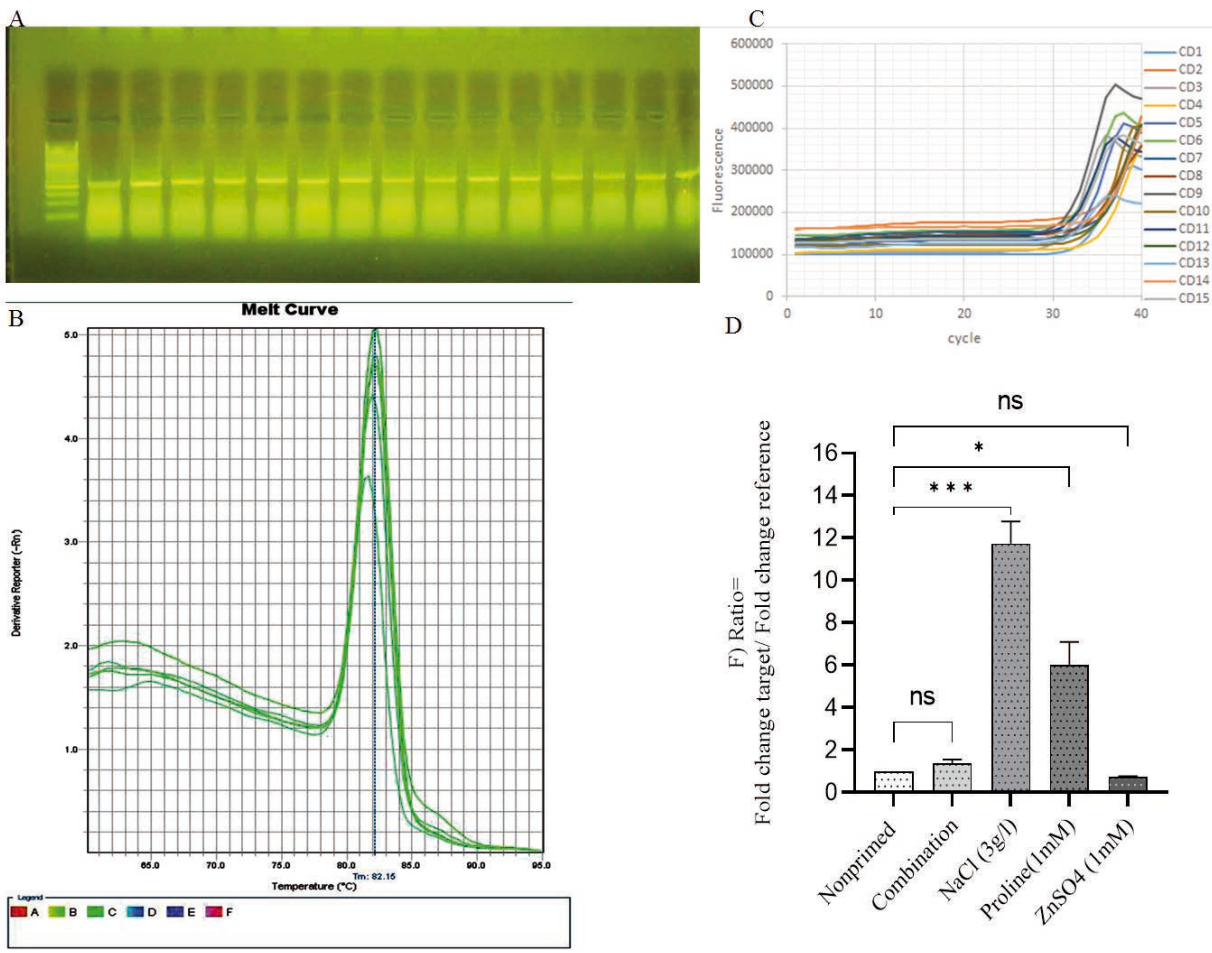

Figure 5: The effect of wheat seed osmopriming on NHX2 gene expression. A) Gel electrophoresis B) Melting curve. C) Real-time curve nonprime (CD1-CD3), combination (CD4-CD6), NaCl (3 g l-1) (CD7-CD9), proline (1 mM) (CD10-CD12), ZnSO4 (1 mM) (CD13-CD15). D) Gene expression graph compared to the group's average without prime (values are the average of 3 repetitions and the same letters indicate no significant difference between the averages at the $p < 0.05$ level)

primed plants (Janda et al., 2016). It was demonstrated that priming with jasmonic acid had a positive impact on the expression of *NHX2* gene in wheat plants under both saline and non-saline conditions (Sheteiwy et al., 2022).

4 CONCLUSION

Several indicators were selected to evaluate the impact of priming agents on wheat because stress responses are multifaceted and require a thorough assessment of plant physiological, biochemical, and molecular alterations. These indicators encompass photosynthetic pigments, protein levels, sugar and starch content, PAL activity, antioxidant enzymes, associated metabolites, RNA, and specific genes such as *NHX2* (E Sobhy et al., 2023; Faisal et al., 2023; Hosen et al., 2023). Photosynthetic pigments indicate the health and stress tolerance of plants, while protein levels show growth and stress reactions. Sugar and starch levels reveal energy availability, PAL activity is linked to stress defense mechanisms, and antioxidant enzymes indicate responses to oxidative stress. RNA and *NHX2* gene expression offer insights into molecular responses to stress. By examining these various indicators, researchers can obtain a thorough understanding of how priming agents affect wheat growth, stress tolerance, and overall productivity. This study evaluates the impact of combined and individually optimized osmopriming on wheat plant growth and development. Priming resulted in a significant increase in antioxidant enzymes, soluble sugars, and proteins, while reducing endogenous levels of H_2O_2 and MDA. It has been demonstrated that osmopriming treatments, such as hydro- and osmopriming with PEG solutions, improve germination attributes and seedling performance in various plant species (Debta et al., 2023; Mehboob et al., 2022). The results indicate that combinational osmopriming has the highest positive effect on wheat seed performance, enhancing the efficiency of PSII functioning, primary photochemistry, and biochemistry. Combined osmopriming treatments are an effective method for enhancing seed germination and seedling growth in crop production, especially in mitigating stress effects (Singhal et al., 2021). Combined osmopriming with Ca^{2+} and K^+ enhances salt tolerance in quinoa seeds and seedlings, improves growth, nodulation, chlorophyll fluorescence, and nutrient uptake in alfalfa under drought conditions, and significantly enhances seedling length and dry weights (Mamedi et al., 2022; Mirmazloum et al., 2020; Mouradi et al., 2016). Combined osmopriming with melatonin is more effective than treating with fungicides due to its enhanced germination capacity, reduced fungal incidence, and improved seed quality (Rosińska, Andrzejak, & Kakkerla, 2023). The effectiveness of this method is attributed to the synergistic interaction of NaCl, Proline, and $ZnSO_4$, which contribute to different aspects of seed germination and early seedling growth. NaCl improves water uptake, Proline protects against osmotic stress and reactive oxygen species, and $ZnSO_4$ aids enzyme activation and DNA synthesis.

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doi:10.14720/aas.2024.120.2.13447 Original research article / izvirni znanstveni članek

Screening and identification of IAA-capable and cellulose-degrading bacteria with the potential for plant growth-promoting traits

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Received April 19, 2023; accepted May 06, 2024.
Delo je prispelo 19. aprila 2023, sprejeto 6. maja 2024.

Screening and identification of IAA-capable and cellulose-degrading bacteria with the potential for plant growth-promoting traits

Abstract: Strains with both straw degradation and plant growth promotion ability were selected from the cultivated soil in Bac Kan, Vietnam to solve the problems of poor soil microbial diversity status, weak corrosion promotion effect, and poor crop growth caused by fungal rot diseases. Among seventeen bacteria isolated, strain NR1 presented the highest value for cellulase enzyme activity (Hydrolysis index = 24.8 mm), and IAA production (20.15 mg l^{-1}), and was identified as *Bacillus amyloliquefaciens* Priest et al., 1987. Inoculation with NR1 significantly increased the rot promotion rate of straw under liquid fermentation by 54.71 % compared with the control and increased the root length and average diameter, and SPAD value of maize under soil culture by 18.3 %, 22.0 %, and 5.24 % respectively ($p < 0.05$). In addition, fertilizing 8 or 9 tons of NR1-degraded compost fertilizer per hectare had the best effect on the growth, development, and productivity of the L14 peanut variety. These results suggest strain NR1 could be used to produce multi-functional humus, accelerate the decomposition of straw in the cultivated soil, and promote crop growth.

Key words: *Bacillus* sp., cellulose, organic matter, peanut plant, PGPR

Iskanje in določanje bakterij, ki so sposobne razgraditi celulozo s pomočjo IAA kot potencialnih pospeševalcev rasti rastlin

Izvleček: Sevi bakterij sposobni razgradnje slame in sposobnostjo pospeševanja rasti rastlin so bili izolirani iz kmetijskih tal na območju Bac Kan v Vietnamu z namenom razrešiti problem majhne mikrobne raznolikosti tal, šibke sposobnosti razgradnje in slabe rasti poljščin, ki jo povzročajo glive, povzročiteljice gnilobe korenin. Med sedemnajstimi izoliranimi sevi je sev NR1 pokazal največjo vrednost aktivnosti celulaze (Indeks hidrolize = 24,8 mm), in tvorbe IAA (20,15 mg l^{-1}). Sev je bil določen kot vrsta *Bacillus amyloliquefaciens* Priest et al., 1987. Inokulacija s sevom NR1 je značilno povečala rast korenin v slami pri tekočinski fermentaciji za 54,71 % v primerjavi s kontrolo in povečala dolžino, poprečni premer korenin in SPAD vrednost pri koruzi pri gojenju v tleh za 18,3 %, 22,0 % in 5,24 % ($p < 0.05$). Dodatno je imelo gnojenje z 8 ali 9 t ha^{-1} od NR1-razgrajenega komposta najboljši učinek na rast, razvoj in produktivnost L14 sorte graha. Rezultati nakazujejo, da bi sev NR1 lahko uporabili za pripravo multifunkcionalnega humusa pri pospeševanju razgradnje slame v obdelovalnih tleh in s tem pospešili rast gojenih rastlin.

Ključne besede: *Bacillus* sp., celuloza, organska snov v tleh, arašidi, PGPR

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1 INTRODUCTION

Soil organic matter (SOM) decomposed from plant or animal biomass is returned to the soil. In soil, the SOM is an important source of nutrients for plants, especially in sloping soils. It was reported that the turnover of SOM was strongly affected by several factors such as moisture, temperature, clay content, soil porosity, soil cover, and the structure of the soil microbial communities (Don et al., 2017). So, the SOM accumulation in the soil depends on the biomass of the crop when it is returned to the soil and the soil microbial communities responsible for the organic matter decomposition. In addition, the shift of structures and activities of the microbial community in soil has been shown important roles in SOM accumulation due to their ability to secrete different types of enzymes in soil, which are involved in C cycling in soil (Sardans et al., 2008). Hence, the reaction of microbial communities to plant inputs plays significant implications for nutrient cycling and ecosystem functioning.

Moreover, the significant role of plant inputs to the soil microbial processes relies on C availability, the main factor inhibiting microbial growth and activity (Fierer et al., 2009). Notably, the main component of plant biomass is cellulose, which was the dominant waste material from the agricultural industry in the form of stalks, stems, and husks (Shankar et al., 2011). Using these agricultural byproducts as a source to produce biofertilizers is attracting scientific interest because it both reduces waste and utilizes it to make compost to provide nutrition for crops. Furthermore, the application of microbiological technology, especially applying cellulase-producing bacteria, to compost agricultural byproducts is an emerging solution for sustainable agriculture. For example, previous studies have demonstrated that soil added cellulose presented a strong stimulation of cellulose-degrading enzymes (Fontaine et al. 2004) and lignin-degrading enzymes (Talbot & Treseder 2012). These microorganisms are all available in the wild and belong to the group of mycelial fungi, bacteria, actinobacteria, and yeast (Fontaine et al., 2004). Therefore, there has been great interest in screening microorganisms with strong cellulose-degrading capabilities from soil that could be applied in compositing agroforestry byproducts into compost, which reclaimed fertility for the soil.

It was reported that plant-stimulating bacteria produce plant hormones that directly stimulate seedling growth (Do et al., 2023). Therefore, if the straw-degrading bacteria screened from the soil have both plant-promoting functions, then their potential application will be broader. To meet the multifaceted needs faced in agricultural production, more and more researchers are trying to breed strains with multiple functions. For example, Luo et al. (2018) screened multi-functional strains that degraded cellulose, starch, protein, and oil from forest soil, which played a significant role in improving soil fertility and improving crop quality on agricultural farmland. From the above, when the straw is returned to the soil field, the straw-degrading bacteria with the ability to produce IAA is applied as the core of the saprophytic agent or can solve the two major problems faced by the direct return of straw to the field.

It was reported that perennial cropping systems present extensive root networks and high allocation of belowground C, which may enhance the plant–microbial linkages. For example, a significant source of C inputs to soils from perennial root systems and a change in microbial community composition were observed during grassland restoration (Bach et al., 2010) or during crop cultivation (Dodor & Tabatabai, 2003). Especially, a similar observation was reported in annual agroecosystems that applied organic residues, cover crops (Bandick & Dick, 1999), or rotated diverse crops (Dodor & Tabatabai, 2003). These suggest the root rhizosphere is a source of microorganisms that could be exploited to support the development of sustainable agriculture.

Thus, the study was conducted to screen cellulose-degrading bacteria from soil samples grown in perennial and annual crops; and also to investigate their abilities in composting agricultural byproducts, and finally study their effects on the growth of maize.

2 MATERIAL AND METHODS

2.1 ISOLATION AND SCREENING OF CELLULOSE-DEGRADING BACTERIA FROM RHIZOSPHERE

A total of 15 soil samples were collected in cultivated fields in Na Ri district, Bac Kan, Vietnam in June 2021. In each sample plot, three sub-plots were randomly selected to collect five soil cores (2.5 cm diameter × 15 cm in length) at depths of 0–15 cm. Then these five soil cores were homogenized and bulked into one composite sample and kept in a ziplock bag. The soil samples were stored on ice until they could be transferred to the laboratory refrigerator.

Mass 1g of rice soil sample and dilute with 100 ml of sterile distilled water, shake for 15 minutes, then pipetted 20 µl and spread it on a carboxymethyl cellulose (CMC) medium (Ulrich et al., 2008). The inoculated plates were incubated at 30 °C for 2-3 days. After incubation, each colony was transferred to a new plate. Then the medium plate was stained with Congo Red solution (1 g l^{-1}) for 15 min and finally washed with 1M NaCl saline. Bacterial

isolates that hydrolyzed CMC would produce a colorless zone around the colony (halo). The hydrolysis index (HI) was calculated as the following formula:

Hydrolysis index (HI) = D – d

In which D: Halo Diameter (mm); d: Colony Diameter (mm).

CMC medium included 1 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.001 g NaCl, 10 g CMC, 15 g agar, water was added to a volume of 1 liter, and adjusted pH 7.

2.2 CHARACTERIZE THE ISOLATED STRAINS

2.2.1 Cellulase enzyme activity assay

Inoculate the cultures in a liquid medium with corn stover powder as the only carbon source. The culture was incubated in a liquid flask at 37°C for 60 h, and the fermentation broth was centrifugated at 4 °C, 5000 rpm for 10 min, and the collected supernatant is crude enzyme solution. Cellulase enzyme activity was measured by determining the reducing sugar content in enzyme solutions by DNS (3,5-dinitrosalicylic acid) method (Chen et al., 2014). One unit (U) of enzyme activity was defined as the amount of enzyme equivalent to 1 μmol of reducing sugars released per minute in a 1 ml enzyme solution at a temperature of 50 °C and a pH of 4.8.

2.2.2 IAA production

Inoculation of cellulose-degrading bacteria in Luria-Bertani (LB; 10 g l^{-1} Peptone, 5 g l^{-1} Yeast Extract, 5 g l^{-1} NaCl) liquid medium containing L-tryptophan (100 mg l^{-1}). The inoculated media were incubated at 30 °C for 1 day on the shaker at 180 rpm, then centrifuged for 10 min at 5000 rpm. Then 2 ml supernatant was mixed with an equal volume of Salkowski colorimetric solution, and kept at room temperature for 30 min, and the IAA content was calculated based on spectral absorbance measurements of the standard curve at 530 nm (Liu et al., 2017).

2.2.3 Molecular identification of selected cellulose-degrading bacteria

The total DNA of selected microorganisms 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 27 F (5′-AGA GTT TGA TCC TGG CTC AG-3′), and 1492 R (5′-TAC GGT TAC CTT GTT ACG ACT T-3′). The amplification was done in a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, CA, USA) using the following program: 95 ˚C for 5 min; 30 cycles at 95 ˚C for 30 s, 55 ˚C for 30 s, and 72 ˚C for 90 s; and 72 ˚C for 7 min. The fragment of 16S rDNA sequences (1.5 kb) was obtained and purified by using the QIAquick PCR Purification Kit (Qiagen, USA). The purified 16S rDNA fragment was sequenced by First Base Company (Singapore). The obtained sequence was blasted on NCBI to identify the species.

2.3 EVALUATION OF THE MAIZE GROWTH PROMOTION OF BACTERIA UNDER GREENHOUSE CONDITION

The experiment was carried out in a greenhouse belonging to the Central Institute for Natural Resources and Environmental Studies, Vietnam National University Hanoi, Vietnam.

The soil of the tillage layer in the field at Na Ri, Bac Kan, Vietnam was collected. Soil samples have removed the gravel and weed dead branches, and mixed well through a 5 mm pore size sieve. The soil properties were organic matter 13.01 g kg^{-1}, total nitrogen 0.126 g kg^{-1}, alkaline nitrogen 81.03 mg kg^{-1}, available phosphorus 16.1 mg kg^{-1}, and available potassium 101.2 mg kg^{-1}. The soil was used to fill the pots.

The maize seeds (VN595 hybrid variety) were surface sterilized with 0.1 % $HgCl_2$ for 10 min and rinsed 5 times with sterile distilled water. The sterilized seeds were coated with bacteria by soaking in the bacterial solution (1×10^8 CFU ml^{-1}) for 1 hour. The bacterized seeds were sown 5 seeds per pot, and the soil moisture content was adjusted to 60 % of the maximum water-holding capacity in the field. Two treatments have been set up, each treatment was repeated 5 times. Every two weeks, 10 ml of bacterial solution (1×10^8 CFU ml^{-1}) was applied at the base of corn seedlings. For control treatment (CK), sterile water was used instead of the bacterial solution. Soil fertilization was performed as the recommendation for maize crops according to QCVN 01-56:2011/BNNPTNT.

The plant growth parameters (the root length, surface area, root tip number, plant height, SPAD value, and plant fresh mass) were measured after 49 days.

Steel tape measure and TYS- were selected for maize plant height and SPAD value, respectively-Type A chlorophyll analyzer determination; Aboveground fresh mass of the plant was measured on a one-percent scale (Lu et al., 2019). Maize root length, diameter, and surface area

with a root scanner (LA1600 + scanner, Canada) obtained images of the roots of individual plants for assays (Liu et al., 2017).

2.4 EVALUATION OF THE CELLULOSE DEGRADATION PROMOTION OF BACTERIA UNDER *IN VITRO* CONDITION

Weighted and crushed 5 g of sieved wheat straw powder in a 250 ml Erlenmeyer flask. Then added 30 ml of water, 2 g of sodium nitrate, and 2 ml of bacterial solution (1×10^8 CFU ml^{-1}) into the flash. After that, the inoculated mixtures were incubated on a constant temperature shaker at 28 °C, 120 rpm. After 15 days of incubation, the culture was centrifugated (5 000 rpm for 10 min) to remove the supernatant and washed the pellets with distilled water three times. The pellet was dried to constant mass at 80 °C. For the control experiment, replaced the bacterial solution with sterile water, other steps are consistent. Each treatment was done in triplicates. The decomposition rate of straw was determined by mass loss (%), which was calculated by the formula:

$$\text{Mass loss (\%)} = [(M - M_1)/M] \times 100$$

Where M and M1 are the initial and final mass, respectively.

2.5 EVALUATION OF SELECTED CELLULOSE-DEGRADING BACTERIA IN COMPOSTING AGRICULTURAL BYPRODUCTS UNDER GREENHOUSE CONDITIONS

By-product waste materials include 200 kg of straw, waste after mushroom cultivation; 120 kg of water hyacinth; and 80 kg of corn stalks, beans, and peanuts.

The selected bacteria were cultured in a mixture of rice bran and cornstarch (3 : 1 ratio) supplemented with 50 ml of sterile distilled water for 1 kg. The mixtures were incubated at laboratory temperature (28 ± 2 °C) and after 7 days counted the number of microbial cells. The results were: 5.21×10^8 CFU g^{-1}, in accordance with the standard of microbial production (> 10^8 CFU g^{-1}).

The composting experiment consisted of 2 formulas (Table 1) and was carried out on a cement base. A mixture of 200 kg of annealing material + 0.4 kg of lime and 0.5 kg of phosphate compounds was prepared and mixed well. The mixture was incubated for 7 days, then mixed well with the bacterial seed, stacked in 70 cm high piles, and covered with plastic. The incubation period was 30 days.

After 30 days of incubation assessed the total protein content (N %) according to TCVN6498:1999, the total P content (P_2O_5 %) according to TCVN 8940:2011; total potassium (K_2O %) according to TCVN 8660:2011 and cellulose content of the composting formula with the bacterial inoculation compared to controls (no bacterial inoculation) to assess the effectiveness of the microbial mixture.

2.6 EVALUATION OF THE PEANUT GROWTH PROMOTION OF NR1 STRAIN-PRODUCED COMPOST UNDER FIELD CONDITION

The experiments were carried out in Na Ri, Bac Kan, Vietnam. Different combinations were designed to evaluate the impact of compost on the growth and productivity of the L14 peanut variety, as in Table 2. The L14 peanut variety was cultivated on the field in spring 2021 (12/1–20/5/2021) with a planting density was 33 seedlings m^{-2}. The experiments were designed in completely randomized blocks (10 m^2) with 3 repetitions.

At harvest, the growth, development, and productivity of peanuts were collected according to the national technical regulation on testing for the value of cultivation and use of groundnut varieties (QCVN 01-57:2011/BNNPTNT).

Soil samples taken on the 0–20 cm depth before and after the experiment were dried in the air and analyzed the following indicators: pH_{KCl} by pH meter method, organic carbon content (OC) according to TCVN 8941:2011; total nitrogen by TCVN 6498:1999; total phosphorus (P_2O_5) according to TCVN 8940:2011; total potassium (K_2O) according to TCVN 8660:2011.

2.7 DATA ANALYSIS

All experiments were repeated three times the re-

Table 1: The formula for experimenting with composting

Formula	Amount of byproduct mixture (kg)	Bacterial addition	Seed inoculation rate (%)
I (Control)	200	No	0
II	200	Yes	5

Table 2: The formula for experimenting with composting

Experimental formulas	Amount of fertilizer for 1 hectare
CT1 (control) = Background	30 kg N + 60 kg P_2O_5 + 60 kg K_2O + 400 kg lime
CT2	7 tons of composting byproduct + Background
CT3	8 tons of composting byproducts + Background
CT4	9 tons of composting byproducts + Background

sults were presented as mean values ± SD. Data were statistically analyzed using Excel 2010 and SPSS 13.0 software, and the least significant difference (LSD) test was used for multiple comparisons ($p < 0.05$).

3 RESULTS AND DISCUSSION

3.1 ISOLATION AND IDENTIFICATION OF CELLULOSE-DEGRADING BACTERIA

From 12 soil samples, 17 strains of bacteria capable of degrading cellulose compounds were isolated with different shapes, sizes, colors, and cellulose degradation (Table 3).

As can be seen, bacterial strains have a diversity of colors: pale yellow, milky white, light yellow, light pink, and different cellulose-degrading abilities (Table 3). Among those, strains NR1 and NR10 presented strong cellulose degradation ability with a hydrolysis index (HI) larger than 20 mm; while 3 strains (NR7, NR9, and NR12) had a weak ability (HI < 10 mm) and other 12 isolates showed a medium ability with HI ranged from 10 to 20 cm.

3.2 SCREENING THE BACTERIAL ISOLATES FOR CELLULASE ENZYME AND IAA PRODUCTION CAPABILITIES

Seventeen strains of cellulose-degrading bacteria were screened for cellulase activity and IAA production (Figure 1). Strain NR1 produces the highest activity of CMC enzymes, up to 20.60 U ml^{-1}, which was significantly higher than that of other strains. This was followed by NR4, with a CMC enzyme activity of 17.98 U ml^{-1}, and

Table 3: Characterization of cellulose-degrading bacteria strains

Bacterial strain	Colony characteristics	Hydrolysis index (mm)
NR1	Milky white, round shape, serrated edge	24.8 ± 0.00 ^{a}
NR2	Pale yellow, viscous, irregular edge, flat	17.3 ± 0.33 ^{bc}
NR3	Pale yellow, viscous, flat, irregular edge	16.1 ± 037 ^{bc}
NR4	Milky white, flat, irregular edge	14.2 ± 0.32 ^{c}
NR5	Milky white, irregular edge, wrinkled	10.8 ± 0.23 ^{d}
NR6	Milky white, wrinkled, irregular edge	12.3 ± 0.22 ^{cd}
NR7	Milky white, irregular round, viscous	8.3 ± 0.26 ^{e}
NR8	Milky white, irregular round, viscous	18.7 ± 0.45 ^{bc}
NR9	Filamentous, uniform round, light pink	9.6 ± 0.01 ^{de}
NR10	Milky white, irregular round, viscous	20.2 ± 0.13 ^{b}
NR11	Milky white, rough, wrinkled	10.7 ± 0.23 ^{d}
NR12	Milky white, rough, wrinkled	7.6 ± 0.25 ^{e}
NR13	Milky white, rough, wrinkled	10.2 ± 0.12 ^{d}
NR14	Pale yellow, flat, wrinkled, with concentric rings	10.9 ± 0.04 ^{d}
NR15	Light yellow, slightly viscous, round edges	10.1 ± 0.33 ^{d}
NR16	Transparent, viscous, round, concentric ring	13.4 ± 0.43 ^{cd}
NR17	Milky white, uniformly round, concentric ring, viscous	12.2 ± 0.26 ^{cd}

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

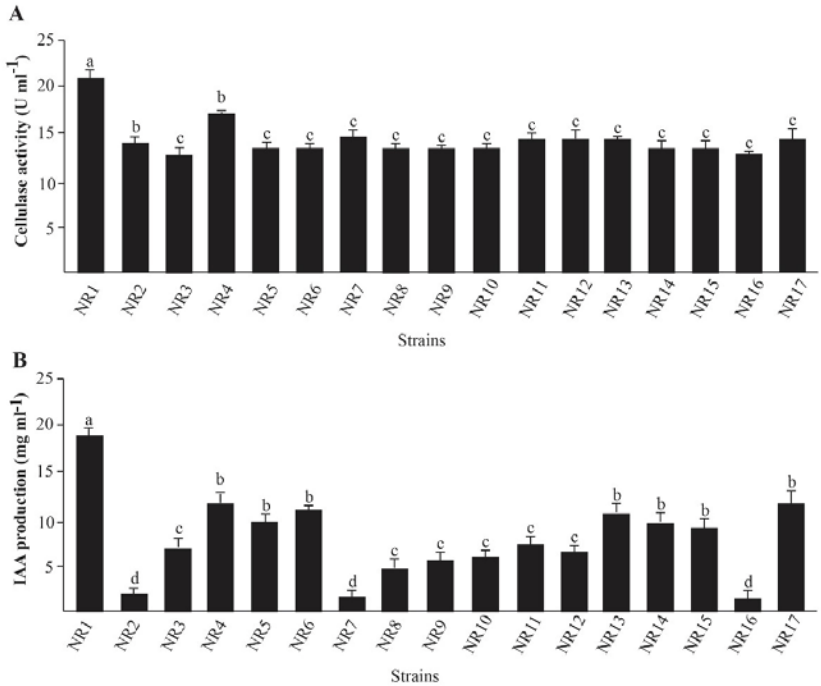

Figure 1: The ability of different strains to produce CMC enzymes (A) and IAA (B). Plotted data are means ± SD (n = 3). The same letter(s) are not significantly different as determined by the least significant difference (LSD) test ($p < 0.05$)

there was no significant difference in CMC enzyme activity among other strains (Figure 1A). The IAA-producing capacity of the NR1 strain is also the strongest, with concentrations of up to 19.83 mg l^{-1}, significantly higher than other strains ($p < 0.05$), and none of the NR2, NR7, and NR16 strains produced IAA (Figure 1B). Therefore, we selected strain NR1 as a multifunctional strain capable of degrading cellulose and synthesizing IAA and carried out straw degradation experiments and pot experiments to verify its decay-promoting and growth-promoting effects.

The 16S rDNA sequence of the NR1 strain was compared to the NCBI database for homology using the blast function. The results showed that the NR1 strain has the highest homology (identity percentage of 99.47 %) and is most closely related to *Bacillus amyloliquefaciens* Priest et al., 1987. The 16S rDNA sequence was deposited on Genbank with the accession number MZ484519.

3.3 EVALUATION OF PLANT GROWTH PROMOTION OF NR1 STRAIN

The greenhouse experiment showed that maize plant and root traits were significantly improved after inoculating with the NR1 strain. The root length, the mean diameter of the root, and the SPAD value increased significantly by 18.3 %, 22.0 %, and 5.24 % respectively ($p < 0.05$, Table 4), compared with the control.

It was reported that IAA participates in many physiological and biochemical regulations in plants, such as cell elongation, cell division, etc., and can promote plant growth (Yue et al., 2005). In this study, the IAA production of the strain NR1 reached 20.15 mg l^{-1}, which is significantly higher than the average amount of the screened strains in the previous reports (Sun et al., 2020). As the plant growth showed a low-promotion and high-inhibition effect with the increase in IAA concentration

Table 4: Effect of strain NR1 on straw degradation promotion and maize growth

Formula	Strawdegradation rate (%)	SPAD value	Average diameter (mm)	Root length (cm)	Root surface area (cm^2)	Plant height (cm)	Aboveground fresh plant mass (g)
Control	9.76 ± 0.90 [a]	40.2 ± 1.62 [a]	0.41 ± 0.04 [a]	23.5 ± 2.29 [a]	111.5 ± 23.0 [a]	51.8 ± 1.25 [a]	3.91 ± 0.42 [a]
NR1	15.1 ± 0.12 [b]	42.3 ± 0.82 [b]	0.50 ± 0.05 [b]	27.8 ± 5.02 [b]	114.3 ± 43.9 [a]	54.4 ± 1.71 [b]	4.12 ± 0.25 [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 least significant difference (LSD) test ($p < 0.05$)

(Jiang et al., 2000). Therefore, the growth-promoting effect of IAA-producing strains must be comprehensively analyzed with pot experiments. The experiments showed that the SPAD values of maize plants inoculated with strain NR1 were significantly increased compared with the control. This may be because the SPAD value represents the chlorophyll content of the plant, and the higher the value, the stronger the plant's photosynthetic ability. Strain NR1 could belong to nitrogen-fixing bacteria, which can promote the absorption and accumulation of nitrate nitrogen in maize after inoculation (Wu et al., 2011). Nitrogen is an important component of chlorophyll. Therefore, the SPAD value of maize plants significantly increased (Wu et al., 2011). The root length and average diameter of the corn inoculated strain NR1 also increased, indicating that the inoculated maize formed a more developed root system. This confirmed the previous research that IAA produced by microorganisms can promote cell division and differentiation changing the root morphology of plants (Xi et al., 2005); The root surface area is not significantly increased compared with the control, which may be related to external pressure and soil type (Zhang et al., 2018). The condition of the root system directly affects plant growth and nutrient supply, and a well-developed root system can fully interact with nutrients in the soil, thereby improving nutrient utilization and promoting growth (Liu et al., 2017; Nguyen & Nguyen, 2018). However, in this study, the plant height and aboveground fresh mass of inoculated corn increased by 5.0 % and 5.4 % respectively compared with the control, which did not reach a significant correlation level, which may be attributed to the influence of plant species and cultivation conditions (Yu et al., 2015).

3.4 EVALUATION OF STRAW DEGRADATION-PROMOTING ABILITY OF STRAIN NR1 UNDER *IN VITRO* CONDITION

The liquid shake flask test showed that the degradation rate of wheat straw inoculated with strain NR1 reached 15.1 %, which was 54.71 % higher than that of the control ($p < 0.01$, Table 4). These results suggest strain NR1 could produce external cellulase that degrades cellulose into monosaccharides.

The result of this study showed that the CMC enzyme-producing ability of strain NR1 was as high as 20.60 U ml^{-1}. A variety of cellulose-degrading strains have been found. For example, the decomposing bacteria ZJA-6 isolated by Wei et al. (2015) exhibited a CMC enzyme activity of 13.20 U ml^{-1}; Li et al. (2019) reported the enzyme activity of Actinomycetes C31 reached 4.8 U ml^{-1}; the enzyme activity of *Burkholderia* ME27-1 reported by Liang et al. (2014) was only 2.08 U ml^{-1} under optimized conditions. The CMC enzyme activity of strain NR1 was 1.56 times, 4.29 times, and 9.90 times that of strains ZJA-6, C31, and ME27-1, respectively, indicating that strain NR1 had relatively high CMC enzyme activity. It should be noted that the application of the strain NR1 needs to be comprehensively judged in combination with CMC enzyme activity and straw degradation test (Wang et al., 2016) because straw is composed of cellulose, hemicellulose, and lignin through covalent bonds, hydrogen bonds and it is a water-insoluble polymer compound composed of a variety of molecular forces such as wax bonds. The outside of cellulose is tightly wrapped by lignin and hemicellulose, which is difficult to be decomposed by cellulase (Yu & Guo, 2019). The straw degradation test showed that the straw degradation rate of strain NR1 reached 15.1 % in 15 days, which was 54.71 % higher than that of natural degradation straw. These results indicated that the addition of strain NR1 can significantly improve the straw degradation yield. Therefore, the strain NR1 isolated in this study not only exhibits high enzyme activity but also can accelerate the process of straw degradation, which is expected to improve the comprehensive utilization rate of straw in practical applications.

3.5 EVALUATION OF THE ABILITY TO DEGRADE AGRICULTURAL BYPRODUCTS OF NR1 STRAIN

The strain NR1 was investigated for its potential ability to decompose cellulose-rich agricultural byproducts under natural conditions. The results are presented in Table 5.

The data in Table 5 showed that the cellulose content was dramatically reduced at the formula inoculated with NR1 strain (66.7 %) while a slight decrease of it was observed for the control. These results suggest strain NR1 still kept its strong ability to degrade indigestible organic compounds very well by secreting the external cellulase under natural conditions.

Protein, phosphorus, and potassium are the necessary nutritional elements that determine crop yield. Determination of the total protein, phosphate, and potassium amount in compost plays an important role in considering the possibility of supplying N, P, and K from the manure. The results in Table 5 showed that the total amount of protein, phosphorus, and potassium content in the formula for supplementing strain NR1 all increased and were higher than the formula without supplements of strain NR1. Compared to research reports on the quality of microbial compost composted from agricultural byproducts, the total protein, phosphate, and potassium

Table 5: Effect of strain NR1 on cellulose degradation and compost quality

	Cellulose content (%)		Compost quality (%)		
Formula	Before incubation	After incubation	Total N	Total P_2O_5	Total K_2O
I (Control)	14,5 ± 0.3 [a]	11,2 ± 0.2[a]	0,83 ± 0.2 [a]	0,36 ± 0.3 [a]	0,63 ± 0.4 [a]
II	14,4 ± 0.4 [a]	4,8 ± 0.3 [b]	1,22 ± 0.1 [b]	0,45 ± 0.2 [a]	0,76 ± 0.2 [a]

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

content in the compost of this study is equivalent to or higher than some other studies (Tran et al., 2011).

3.6 EVALUATION OF THE ABILITY TO STIMULATE PEANUT GROWTH UNDER FIELD CONDITIONS OF NR1-PRODUCED COMPOST

The results showed that the height of the peanut plant in the formulas added to the compost (CT2, CT3, and CT4) is higher than the one in the control (CT1, Table 6). Especially, the increase of compost (7, 8, and 9 tons ha^{-1}) enhanced the peanut plant height (33.34, 36.43, and 37.45 cm, respectively).

In addition, the addition of compost produced by the NR1 strain also enhanced the yield of the peanut plant (Table 6). As can be seen, all treatments added compost fertilizer did not affect the total number of fruits on the plant and the total number of fertilized fruits on the plant, with no statistically significant discrepancies. However, the mass of 100 fruits and the practical yield differed between the experimental formulas and the control and among the experimental formulas.

The mass of 100 fruits is the indicator that determines the productivity of the experimental formulas. The results showed that the highest mass of 100 fruits was observed in CT4 (160.34 g), followed by the one in CT3 (154.12 g), and the lowest values were in CT1 (142.85 g) and CT2 (147.31 g). Moreover, practical yield is an important indicator for assessing the effectiveness of the compost in the growth, development, and productivity of peanut plants. The practical yield of peanut plants in CT2, CT3, and CT4 was 3.01, 3.35, and 3.48 t ha^{-1}, respectively, and was significantly different from the one of the control (2.83 t ha^{-1}). These results indicated that NR1 strain in the compost produced IAA compounds and the composition of nutrients in composted organic fertilizers has added timely nutrition for peanut growth and hence improved productivity.

The analysis results of some chemical properties of the soil before and after the experiment are presented in Table 7.

The data presented in Table 6 aligns with previous research, demonstrating a positive correlation between rice straw compost application and practical yield. Iqbal (2008) observed similar trends, reporting that a combination of rice straw compost and 75 % recommended nitrogen fertilizer yielded the highest protein content in grains. This finding can be attributed to the compost's contribution of readily available phosphorus (P) and potassium (K) nutrients, essential for protein and carbohydrate synthesis within the plant (Iqbal, 2008). Furthermore, Madejon et al. (2001) documented improvements in plant nutritional status, growth response, and overall productivity following compost amendments.

Our study reinforces the potential of composted agricultural by-products as organic fertilizers for soil enhancement. This aligns with the established role of organic matter in improving soil properties, as demonstrated by Giller et al. (1998) and Nguyen & Nguyen (2018). These prior investigations highlight the potential for or-

Table 6: Effect of NR1strain-produced compost on the plant development and yield of peanuts

Formula	Plant height (cm)	Total number of fruit/plant (fruit)	Number of fertilized fruits/plant (fruit)	Mass of 100 fruits (g)	Theoretical yield (tons ha^{-1})	Practical Yield (tons ha^{-1})
CT1	31.21 [a]	21.24 [a]	12.91 [a]	142.85 [a]	4.89 [a]	2.83 [a]
CT2	33.34 [ab]	22.58 [ab]	13.41 [ab]	147.31 [ab]	5.13 [ab]	3.01 [b]
CT3	36.43 [b]	23.17 [b]	14.53 [b]	154.12 [b]	5.49 [b]	3.35 [ab]
CT4	37.45 [b]	23.75 [b]	15.15 [c]	160.34 [c]	5.91 [c]	3.48 [c]
LSD0.05	3.56	5.34	3.64	9.11	8.94	4.17

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

Table 7: Effect of compost on some chemical properties of groundnut soil

Formula	pH_{KCl}	OC (%)	Total N (%)	Total K_2O (%)	Total P_2O_5 (%)
Before experiment	4,97	1,37	0,09	0,30	0,05
CT1	4,99	1,55	0,13	0,35	0,05
CT2	5,80	1,64	0,13	0,28	0,05
CT3	5,81	1,73	0,11	0,30	0,06
CT4	5,50	1,69	0,11	0,36	0,07

The data showed that fertilization not only increases the yield of peanuts but also improves soil fertility. After the experiment, the pH_{KCl} and organic content (OC) increased fluctuated in the experimental formulas from 4.99–5.81 and 1.55-1.73, respectively. Other factors (total N, K_2O, and P_2O_5) showed no difference between the treatment and the control. These results suggest the NR1-produced compost could be applied to increase the soil's OC and balance pH

ganic fertilizers to replace chemical fertilizers partially or fully in agricultural practices.

4 CONCLUSIONS

In this study, *Bacillus amyloliquefaciens* NR1 screened from the root rhizosphere has both straw degradation and crop growth-promoting abilities. The above conclusions have a certain positive significance for guiding the creation and application of multifunctional straw-degrading bacteria in composting agricultural waste that could improve plant growth, plant yield, and soil fertility.

5 ACKNOWLEDGEMENTS

This research has been supported by the VNU-Central Institute for Natural Resources and Environmental Studies (VNU-CRES). The VNU-CRES provided funds for collecting samples and buying chemicals for experiments and supported experimental facilities to carry out experiments.

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doi:10.14720/aas.2024.120.2.13490 Original research article / izvirni znanstveni članek

Exogenous nano-selenium alleviates heat-induced oxidative damage in date palm seedlings by modulating the plant hormones and antioxidant defense

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Received April 29, 2023; accepted March 21, 2024.
Delo je prispelo 29. aprila 2023, sprejeto 21. marca 2024.

Exogenous nano-selenium alleviates heat-induced oxidative damage in date palm seedlings by modulating the plant hormones and antioxidant defense

Abstract: Crops are destroyed by extreme heat, which also limits their growth and yield. The present study sought to determine whether selenium (0, 15, or 30 mg l^{-1}) impacted 'Barhee' date palm seedling's development under heat stress (in the field and canopy temperature). The growth parameters, chlorophyll and relative water content, ascorbic acid, catalase activity, and phytohormones in seedlings were reduced under heat stress. At the same time, ascorbate peroxidase activity, proline, phenols, malondialdehyde, hydrogen peroxide, and abscisic acid in seedlings increased. Enhancing growth features, chlorophyll content, relative water content, ascorbic acid, catalase activity, plant hormones, proline, phenols, and ascorbate peroxidase activity with exogenous nano-Selenium (15 mg l^{-1}) reduced the negative impacts of heat stress. Date palm seedlings can be protected from high temperatures by using nano-selenium. Selenium reverses heat-induced oxidative damage by enhancing the antioxidative mechanism, improving reactive oxygen species scavenging, lowering lipid peroxidation, and modulating plant hormone levels.

Key words: nano-selenium, antioxidant enzymes, ascorbic acid, abscisic acid, malondialdehyde, phytohormones

Dodatek nano selena zmanjšuje od vročine povzročene oksidativne poškodbe v sejankah dateljeve palme s spremembami v rastlinskih hormonih in antioksidativni obrambi

Izvleček: Gojene rastline uničuje ekstremna vročina, ki zmanjšuje njihovo rast in pridelek. V raziskavi se je poskušalo določiti učinek dodatka selena (0, 15, or 30 mg l^{-1}) na razvoj sejank dateljeve palme 'Barhee' v razmerah vročinskega stresa (na prostem in temperaturi krošnje). Rastni parametri kot so vsebnost klorofila, relativna vsebnost vode, vsebnost askorbinske kisline in fitohormonov ter aktivnost katalaze so se v sejankah zmanjšali v razmerah vročinskega stresa. Istočasno so se v sejankah povečali parametri kot so vsebnosti prolina, fenolov, malondialdehida, vodikovega peroksida in abscizinske kisline ter aktivnost askorbat peroksidaze. Povečanje rastnih parametrov, vsebnosti klorofila, relativne vsebnosti vode, vsebnosti askorbinske kisline, prolina, fenolov in povečanje aktivnosti katalaze in askorbat peroksidaze je povzročil dodatek nano selena (15 mg l^{-1}), ki je tako zmanjšal negativne učinke vročinskega stresa. Sejanke dateljeve palme bi tako lahko zaščitili pred visokimi temperaturami z uporabo nano selena. Selen odpravlja od vročine povzročene oksidativne poškodbe s povečanjem antioksidativnih mehanizmov, izboljšanjem nevtralizacije reaktivnih zvrsti kisika, zmanševanjem peroksidacije maščob in uravnavanjem ravni rastlinskih hormonov.

Ključne besede: nano selen, antioksidacijski encimi, askorbinska kislina, abscizinska kislina, malondialdehd, fitohormoni

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1 INTRODUCTION

One negative effect of global warming is that plants are predicted to suffer from heat stress (Raza et al., 2019). Although some plants adapt to rising temperatures, temperatures that exceed adaptation cause heat stress, significantly impacting metabolism and production (Hatfield & Prueger, 2015). Plants contain many temperature-sensitive biochemical reactions that depend on temperature and duration of exposure (Missaoui et al., 2017)and to validate the hypothesis that genes underlying stem determinacy might be involved in the mechanism of summer dormancy. Our results suggest that vernalization is an important requirement in the onset of summer dormancy in tall fescue. Non-vernalized tall fescue plants do not exhibit summer dormancy as vernalized plants do and behave more like summer-active types. This is manifested by continuation of shoot growth and high root activity in water uptake during summer months. Therefore, summer dormancy in tall fescue should be tested only in plants that underwent vernalization and are not subjected to water deficit during summer months. Total phenolic concentration in tiller bases (antioxidants. Because reactive oxygen species (ROS) are produced uncontrollably under conditions of high temperature, oxidative stress results (Manafi et al., 2021). Plants have an adequate antioxidant defense system that involves enzymes and non-enzymatic antioxidants that play an essential part in ROS signaling to adjust for the damage caused by ROS (Mohi-ud-din et al., 2021). Non-enzymatic antioxidants include proline, phenolic compounds, ascorbate, and others (Khan et al., 2019). In contrast, enzymatic antioxidants include many enzymes, such as catalase and ascorbate peroxidase, which work according to antioxidant mechanisms to eliminate ROS toxins and defend plant cells against oxidative stress (Awan et al., 2020).

Heat stress causes an imbalance in hormonal levels and decreases growth-promoting plant hormones such as auxins (IAA), cytokinins (CK), and gibberellins (GA) (Al-Zahrani et al., 2022). At the same time, heat stress increases abscisic acid (ABA), which is known as a component of transduction signaling (Ryu & Cho, 2015)as a sessile organism, rely on the endogenous regulators for the modulation of growth and development under severe stress conditions for their survival. Plant hormones have long been considered as essential endogenous molecules involved in regulating plant development and tolerance or susceptibility of diverse stresses including salinity stress. Plants are frequently exposed to numerous adverse environmental factors such as drought, cold, heat and high salinity. Under high salinity, plants rapidly reduce the growth and developmental programs in response to the stress due to either the effects of specific ions on metabolism, or adverse water relations. Recent investigations on the functional roles of plant hormones in response to unfavorable environmental conditions have eventually unravel their potentials in coffering tolerance to such conditions including salinity stress. In this review, we will present recent progress of our understanding to the important role of plant hormones including abscisic acid (ABA. ABA transduction signaling leads to the induction of genes for proteins necessary for the protection of plants under stress conditions (Zhu, 2016).

Young date palms, such as seedlings or tissue culture plantlets, are exposed to leaf dryness and reduced growth in dry regions, especially in the summer, due to high temperatures and rapid moisture loss from the soil and plant (Shareef & Al-Khayri, 2021). Although plant reactions to heat stress are widespread, the full explanation of the mechanisms of heat tolerance is still limited. Botanists are responsible for researching ways to reduce environmental stress. Abiotic stresses can be reduced by using fertilizer or foliar spraying.

It has been reported that selenium (Se) has a beneficial role in plants. Some plant species that have been treated with selenium have also demonstrated increased tolerance to specific abiotic stresses such as salinity and drought (Alharby et al., 2021), temperature extreme (Safari et al., 2018), mineral toxicity (Zhang et al., 2020), and ultraviolet radiation (Banerjee & Roychoudhury, 2019). There is evidence that selenium plays a role in encouraging antioxidant system mechanisms in plants that lead to removing ROS toxins from the plant (Abbas, 2018). Three possible mechanisms are proposed in ROS scanning in response to the Se application. These mechanisms involve superoxide (O^{2-}) breakdown in hydrogen peroxide (H_2O_2), direct cooling of O^{2-} and hydroxide ion (OH^-), and antioxidant activity regulation by various enzymes. (Balal et al., 2016). Data on the interaction of selenium in plant hormone levels under high temperatures are unavailable. Recently Li et al. (2021) reported on the interaction of nano selenium in limiting cadmium damage on pepper plants (*Capsicum annuum* L.) improves plant hormone content, including jasmonic acid (JA), ABA, salicylic Acid (SA), and brassinolide (BL).

Despite numerous studies on Se's role in mitigating the toxicity associated with heavy metals or salinity, there are only a few reports about the mitigating effect of Se in case of heat stress. As a result, treating plants with selenium foliar may adjust oxidative stress and antioxidant metabolism in young date palm plants, enabling them to withstand high temperatures. This research aims to assess selenium's capacity to prevent oxidative damage caused by high temperatures and to encourage the development of date palm seedlings under heat stress.

2 MATERIALS AND METHODS

The experiment was carried out in Date Palm Research Center, University of Basrah. Seeds of 'Barhee' date palm were approved by Date Palm Research Center, Basrah University to conduct the experiments in 2021 and 2022. For germination, seeds were grown in unadulterated sand soil in an incubator for two months at a temperature of 27 ± 2 °C. Seedlings were separately moved to plastic pots (5 kg) filled with sand with particle size 0.7-2.0 mm, and peat moss (White Sphagnum Peat, H 1-3 von post) in a 2:1 proportion. Seedlings were developed in the wooden canopy at 30 ± 2 °C, with a relative humidity of about 20 %, and photoperiod maintained at 12 h d^{-1}. Thirty seedlings were selected to experiment. The development of 15 seedlings in the wooden canopy continued under the previous temperature (27 ± 2 °C), for two consecutive years (2021-2022), while the other fifteen seedlings were transported to the field in 2022. The service process and irrigation are carried out evenly, regularly, and as needed. On 1st May 2021, the experiment used a randomized block design with two factors (heat stress) at two levels (field and canopy temperature) and three concentrations of nano-selenium* (0, 15, and 30 mg l^{-1}). Each of the six treatments had five replications. A year later, in 2022, the selenium treatments were applied again on 1st May 2022. Temperature, humidity, and illumination intensity are measured inside and outside the canopy for five months (Table 1).

Five seedlings per treatment were utilized for growth analysis on 1st October 2022. The number and length of leaves were recorded. The leaves of each seedling were isolated and weighed to establish the fresh weight. Dehydration in an oven at 70 °C for two days defines the dry matter.

2.1 CHLOROPHYLL CONTENT IN LEAVES

According to Lichtenthaler and Wellburn (1983), 100 mg of fresh leaves were squashed in 10 ml $(CH_3)_2$ CO acetone (80 %) and centrifuged at 2000 rpm for five minutes. Chlorophyll content was colorimetry estimated at 663 and 645 nm. According to the following equation: Total chlorophyll (mg l^{-1}) = 20.2 (O.D. 645) + 8.02 (O.D. 663).

2.2 PROLINE CONCENTRATION IN LEAVES

Proline content was assessed according to Bates et al. (1973). The leaf sample (0.5 g) was homogenized with 5 ml of 3 % sulfosalicylic acid. This mixture was separated, and 3 ml was mixed with ninhydrin reagent (3 ml) and glacial acetic acid (3 ml). This mix was warmed in a bubbling water bath for an hour until it reached 90 °C and quickly cooled to 25 °C. A chromophore was shaped by adding 4 ml toluene to the cold solution. The absorbance was measured at 520 nm using the UV-VIS spectrophotometer. Proline solution (0-10 μg ml^{-1}) was used as a standard.

2.3 RELATIVE WATER CONTENT (RWC) IN LEAVES

Plant leaves were weighed (fresh biomass) instantly

Table 1: Light intensity and temperature change during the growing season of 2022

		Months				
Weather elements	location	May	June	July	August	September
Maximum temperature (°C)	Field	43.31	46.86	48.34	47.32	42.23
	Canopy	37.42	42.57	43.61	41.54	36.87
Minimum temperature (°C)	Field	25.73	26.55	32.34	29.94	26.21
	Canopy	19.45	20.45	26.43	23.56	21.34
Relative humidity (%)	Field	21.18	26.34	32.45	34.74	28.54
	canopy	28.54	32.56	38.43	38.43	34.56
the light intensity μmol m^{-2} s^{-1}	Field	341.14	370.832	382.463	389.528	352.547
	canopy	325.832	362.834	365.743	370.264	328.496
Rainfall (mm)	Field	0	0	0	0	0
	canopy	0	0	0	0	0

Selenium nanoparticles, purity: 99.9 %, APS: less than 80 nm, stock no: NS6130-01-171. Nanoshel LLC company, 3422 Old Capitol Suit 1305, Willmington DE – 19808, United States

upon harvesting, drenched in distilled water at 25 °C for 24 h to calculate turgid mass, and afterward dried in the oven at 80 °C for 48 h to calculate dry biomass. The following equation was used to calculate *RWC: RWC = (fresh mass-dry mass) / (turgid mass-dry mass)* x 100.

2.4 ASCORBIC ACID CONTENT IN LEAVES

The method of Luwe et al. (1993) was used to measure ascorbic acid (AsA). 10 ml of 6 % trichloroacetic acid was used to homogenize leaves tests (0.5 g). The concentrate was combined with 2 ml of 2 % dinitrophenylhydrazine (pH 5) and one drop of 10 % thiourea (in 70 % ethanol). After bubbling for 15 minutes in a water bath, the mixture cooled at room temperature before mixing with 5 ml of 80 % (v/v) H_2SO_4 at 0 °C. The absorbance was measured at 265 nm. A standard bend plotted with its known focus was used to calculate the ascorbic acid content.

2.5 TOTAL PHENOLIC ASSAY OF LEAVES

The Folin-Ciocalteu technique was used to evaluate the phenol extract (Waterman & Mole, 1994). A 25 µl concentrate (500 µg ml^{-1}) was used, along with 25 µl of (1:1) Folin-Ciocalteu reagent and 100 µl of 7.5 % sodium bicarbonate solution, and hatched at room temperature for 2 hours in dim conditions. The absorbance was measured at 765 nm using a UV-VIS spectrophotometer. Gallic acid from 0 to 100 µg ml^{-1} was used as a standard to calculate the phenol content of the sample.

2.6 MALONDIALDEHYDE (MDA) CONTENT IN LEAVES

Davey et al. (2005) report that leaves (0.2 g) were homogenized in ten quantities of 80 % ethanol on frozen ground and separated by centrifugation at 14000 x.g. for 15 min. The permeate was incubated at 97 °C for 20 minutes with an equivalent amount of 0.70 % (w/v) thiobarbituric acid (TBA) solution that contains 200 percent (w/v) trichloroacetic acid and 0.01 % oxytoluenes. 5 µl of the supernatant was used for HPLC analysis using an ODS column (4.6 mm) acclimatized to 35 % methanol through 60 mM buffer with potassium phosphate (pH = 6.8) after cooling and centrifugation. MDA was measured at 540 nm after being eluted at 1.5 ml min^{-1}. MDA prepared chemically by acid-hydrolysis of tetra ethoxy propane was used for calibration.

2.7 HYDROGEN PEROXIDE (H_2O_2) IN LEAVES

The hydrogen peroxide was extracted to cold acetone using the method described by Tabatabai (1998). The extract was quantitatively mixed with titanium tetrachloride and ammonia to create a peroxide-Ti complex. Centrifugation was used to collect the complex, which was then dissolved in 2 M sulfuric acid. The solution's absorbance was measured at 420 nm, and the H_2O_2 content was calculated using the standard curve.

2.8 ANTIOXIDANT ENZYMES IN LEAVES

Activities of ascorbate peroxidase (APX) and catalases (CAT) were determined using the protocol of Radić et al. (2009) using a spectrophotometer.

2.9 HORMONES ANALYSIS IN LEAVES

To ensure data reliability, indoleacetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA3), and zeatin (ZT) were determined using the same tissue extracts. Date fruit samples were washed and dried with a paper towel. They were immediately placed in liquid nitrogen, and stored at -20 °C for 48 hr. One gram of fresh mass (FM) samples were ground in liquid nitrogen and medium-term extracted with 30 ml of 80 % cold methanol at 4 °C. The concentrate was centrifuged for 15 minutes at 2000 x.g. and 4 °C and the supernatant was collected. At that point, new cold methanol was used to fill the remainder, extracted four times using the methods described above. The all-out methanolic separate was dried in a rotary evaporator and divided into 10 ml aliquots of methanol. According to Tang et al. (2011) IAA, ABA, GA3, and ZT were determined by infusing the concentrate into a turnaround stage HPLC on a switch stage C18 section (250 4.60 mm, 5 microns) in an isocratic elution mode utilizing a portable stage comprised of acetonitrile: water (26:74) with 30 mM phosphoric acid.

2.10 STATISTICAL ANALYSIS

Analysis of variance (ANOVA) was performed on the data using SPSS variant 21.0 (SPSS, Chicago, IL), and the means were separated using the Duncan test at the 5 % significance level.

3 RESULTS

3.1 NANO-SELENIUM PROMOTES THE GROWTH OF DATE PALM SEEDLINGS

Field temperature of heat stress reduced the growth of seedlings by reducing plant height and leaf numbers (Fig. 1). Treatment of selenium 15 mg l^{-1} in canopy and field temperature significantly ($p \leq 0.05$) increased the plant height and leaf numbers. While the control treatment recorded the lowest plant height and number of leaves under the field temperature. No significant differences between the canopy and field temperature under selenium 30 mg l^{-1} in leaf numbers.

3.2 NANO-SELENIUM ENHANCES THE CHLOROPHYLL, RWC, PROLINE, AND ASCORBIC ACID CONTENTS IN DATE PALM SEEDLINGS

The contents of chlorophyll, RWC, and AsA in seedlings were reduced significantly ($p \leq 0.05$) by field temperature. At the same time, proline levels increased (Fig. 2). Selenium spraying improved total Chl, RWC, Pro, and AsA levels in seedlings. Under canopy temperature, selenium increased total Chl and proline. In the field, however, selenium increased total chlorophyll and Pro, RWC, and AsA compared to the control. Under field and canopy temperature, a selenium concentration of 15 mg l^{-1} increases total Chl. There were no significant differences between Se treatments at 15 and 30 mg l^{-1} selenium in RWC in AsA under canopy and temperature.

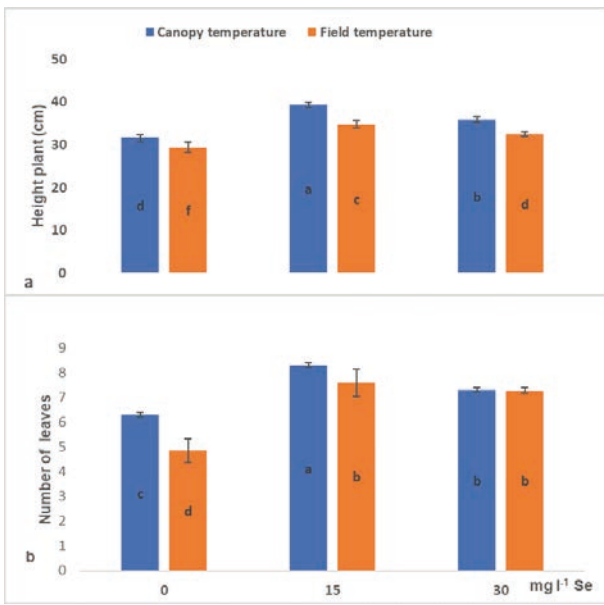

Figure 1: The response of date palm seedlings to nano-selenium in (a) plant height and (b) leaves number under field and canopy temperature (n = 5; means ± SE). Means denoted by different letters differ significantly at $p \leq 0.05$

3.3 NANO-SELENIUM ENHANCES TOTAL PHENOL AND REDUCES OXIDATIVE STRESS IN DATE PALM SEEDLINGS

Phenols, MDA, and H_2O_2 increased significantly ($p \leq 0.05$) under field temperature (Figure 3). Selenium increased total phenols under the canopy and field temperature. Se at 15 mg l^{-1} increased total phenol under field temperature, whereas MDA and H_2O_2 decreased. No significant differences were shown between Se at 15 and 30 mg l^{-1} in the content of MDA and H_2O_2 in field temperature.

3.4 NANO-SELENIUM ENHANCES THE ACTIVITIES OF ENZYMES IN DATE PALM SEEDLINGS

In the field, APX activity increased while CAT activity decreased. Date palm seedlings' responses to selenium spray increased significantly ($p \leq 0.05$) the activity of APX and CAT enzymes (Figure 4). Selenium increased enzyme activity under canopy and field field temperature. Under canopy and field temperature, Se at 30 mg l^{-1} increased enzyme activities compared to 0 mg l^{-1}. In either the canopy or the field temperature, selenium at both concentrations had no significant effect on increasing the activities of the APX enzyme.

3.5 NANO-SELENIUM ENHANCES THE CONTENT OF GROWTH REGULATORS IN DATE PALM SEEDLINGS

High field temperature decreased IAA, GA, and CK while increasing ABA. Selenium spraying increased significantly ($p \leq 0.05$) IAA, GA, CK, and ABA levels in date palm seedlings (Fig. 5). Selenium increased GA, CK, and ABA while decreasing IAA under canopy temperature. In the field, Se at 15 mg l^{-1} increased IAA and GA while decreasing ABA compared to the control. Under field temperature, Se at 30 mg l^{-1} significantly ($p \leq 0.05$) increased CK and ABA levels.

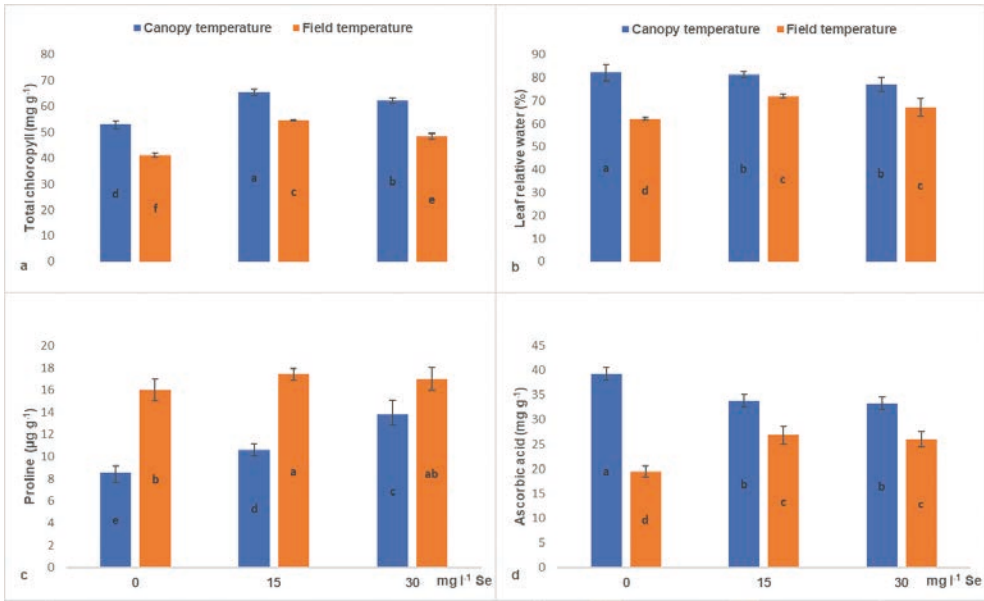

Figure 2: Date palm seedlings respond to nano-selenium in (a) Total chlorophyll, (b) RWC, (c) Proline, and (d) AsA under field and canopy temperature (n = 5; means ± SE). Means denoted by different letters differ significantly at $p \leq 0.05$

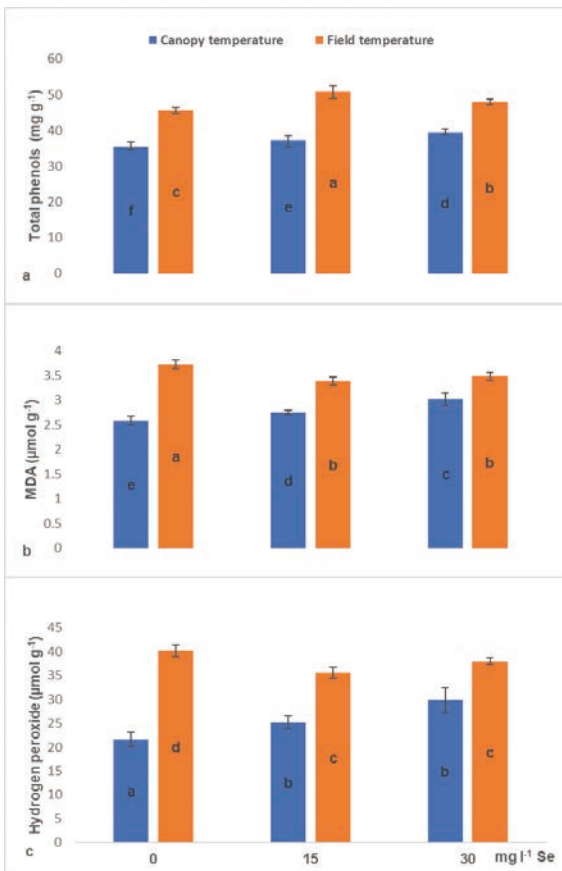

Figure 3: Response of date palm seedlings to nano-selenium in (a) Total phenols, (b) MDA, and (c) H_2O_2 under field and canopy temperature (n = 5; means ± SE). Means denoted by different letters differ significantly at $p \leq 0.05$

4 DISCUSSION

Heat stress is one of the primary causes of plant growth decline because the energy of the photon needed for photosynthesis raises the temperature of the tissues exposed to light (Missaoui et al., 2017)and to validate the hypothesis that genes underlying stem determinacy might be involved in the mechanism of summer dormancy. Our results suggest that vernalization is an important requirement in the onset of summer dormancy in tall fescue. Non-vernalized tall fescue plants do not exhibit summer dormancy as vernalized plants do and behave more like summer-active types. This is manifested by continuation of shoot growth and high root activity in water uptake during summer months. Therefore, summer dormancy in tall fescue should be tested only in plants that underwent vernalization and are not subjected to water deficit during summer months. Total phenolic concentration in tiller bases (antioxidants. The plant is subjected to oxidative stress when subjected to heat stress. Any factor that reduces oxidative stress damage, such as selenium, improves plant growth and tolerance to surrounding conditions (Alharby et al., 2021).

Although the mechanisms associated with the action of Se reduce the effect of specific environmental stresses such as light, high temperatures, drought, heavy metal, and salinity remain incomplete. Several studies have indicated that exogenous nano-Se improves plant growth under stressful or non-stressful conditions (Banerjee & Roychoudhury, 2019).

Several studies have shown an improvement in

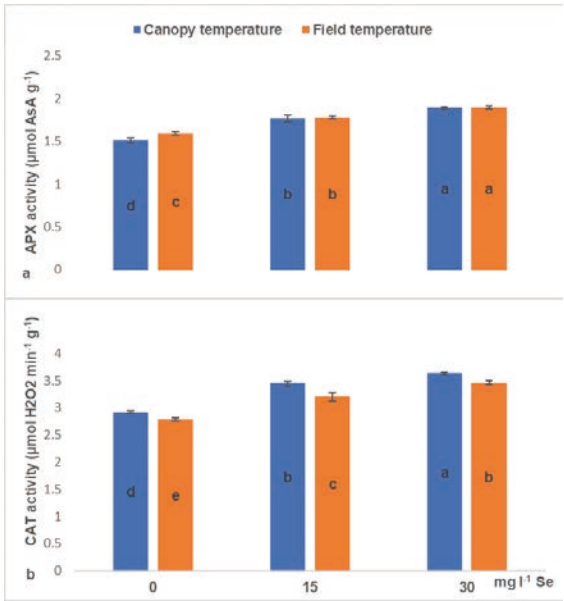

Figure 4: Date palm seedlings respond to nano-selenium in activities of enzymes (a) CAT and (b) APX under field and canopy temperature (n = 5; means ± SE). Means denoted by different letters differ significantly at $p \leq 0.05$

growth in selenium-treated plants such as lettuce (Hawrylak-Nowak et al., 2018), maize (Fernandez et al., 2018) Maize (Zea mays L., potatoes (Somalraju et al., 2022) solutions to manage late blight in organic systems are scarce. This study was undertaken to evaluate the effect of selenium (Se, and soybeans (Alharby et al., 2021) by regulating the water state of the plant or delaying aging. The results showed that nano-Se improved plant growth in both canopy and field temperature (Fig. 1). Our study showed that heat stress decreased the chlorophyll content, whereas Se increased the chlorophyll content in the field and canopy temperature (Figure 2a). Under field temperature, chlorophyll content decreases due to low chlorophyll biosynthesis and disrupted photosystem biochemical reactions (Mathur et al., 2014)ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco. Appropriate selenium levels can reduce chlorophyll damage and increase chlorophyll content in various plants (Missaoui et al., 2017)and to validate the hypothesis that genes underlying stem determinacy might be involved in the mechanism of summer dormancy. Our results suggest that vernalization is an important requirement in the onset

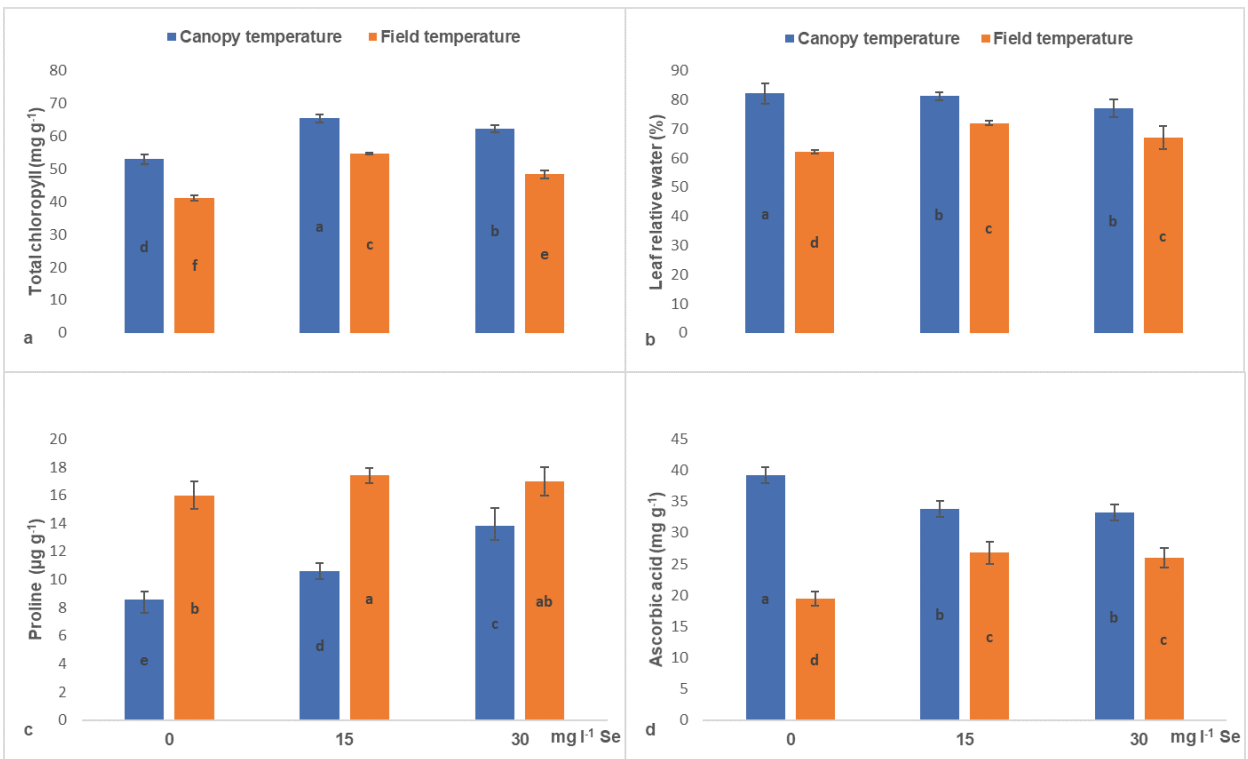

Figure 5: Date palm seedlings respond to nano-selenium in (a) IAA, (b) GA3, (c) CK, and (d) ABA under field and canopy temperature (n = 5; means ± SE). Means denoted by different letters differ significantly at $p \leq 0.05$

of summer dormancy in tall fescue. Non-vernalized tall fescue plants do not exhibit summer dormancy as vernalized plants do and behave more like summer-active types. This is manifested by continuation of shoot growth and high root activity in water uptake during summer months. Therefore, summer dormancy in tall fescue should be tested only in plants that underwent vernalization and are not subjected to water deficit during summer months. Total phenolic concentration in tiller bases (antioxidants. Selenium's beneficial effects on chloroplast enzymes increase the photosynthesis of photosynthetic pigments and, as a result, the chlorophyll content (Jiang et al., 2017)1, 5 and 25 µM Na 2 SeO 3.

Plants absorb less water when exposed to severe environmental conditions, including drought and extreme heat (Hussain et al., 2019). Therefore relative water content is considered an efficient parameter for evaluating plants to withstand those stresses (Pour-Aboughadareh et al., 2019). In our results, field temperature led to a significant reduction in RWC (Figure 2b) which is supported by other studies (Hasanuzzaman et al., 2014; Manafi et al., 2021). Spraying selenium on heat-stressed seedlings conserves cell water and improves water absorption (Malerba & Cerana, 2018). The use of selenium nanoparticles in the treatment of seedlings increased the reinforcement and protective capacity by providing an effective antioxidant system against heat stress. This is because nanoparticles can penetrate the leaves, improving the plant's ability to absorb and use water, which led to the creation of an enzymatic system and enhanced seedling growth (Wang et al., 2021).

Abiotic stresses cause plants to accumulate large amounts of proline, which helps in proteins, reduces hydroxyl radicals, regulates cellular pH, and maintains turgor pressure (Hao et al., 2021). The increase in proline accumulation under heat stress conditions could be attributed to a lack of the enzyme proline oxidase (POX) (Servet et al., 2012)as an initial set of indicators may need further development and refining. This chapter mentions several frameworks that have been proposed to measure the progress of societies. The point is simply that there are potentially useful frameworks that seek to capture the definition and scope of national wellbeing. They provide potential starting points. However, the framework that is most suitable for a given locality, country or group of countries should be determined through a process of deciding and meeting user requirements, which is explored in the chapter. For the wellbeing conceptual framework that the Organisation for Economic Co-operation and Development (OECD. Increased protein decomposition due to high temperatures caused by increased activity of the protease enzyme under stress conditions to release free amino acids, including proline, for storage, transport, or use in the plasmolysis modification (Alahmad et al., 2022). From our results, high field temperature significantly increased the proline content (Figure 2-c). Similar proline levels have been observed in sugarcane (*Saccharum officinarum* L.) under heat stress (Elsheery et al., 2020).

In contrast, selenium spraying increased proline content in heat-stressed plants, related to higher water content and reduced oxidative stress (Balal et al., 2016). The stressful environments lead to increased proline in the plant, which contributes to stress tolerance by preventing cell degradation and maintaining osmotic balance, and stability of membranes, thus preventing electrolyte leakage. Proline accumulation causes ROS concentrations to be placed within normal ranges, thus preventing the explosion of oxidation in plants (Yaish, 2015).

Increased production of phenolics in plants exposed to stress is an adaptation of the plant to the surrounding conditions (Šamec et al., 2021). From our results, field temperature significantly increased the phenol content in the leaves and nano-Se at 15 mg l^{-1} increased total phenol under field temperature (Figure 3a). Saffaryazdi et al. (2012) found that exogenous selenium on spinach (*Spinacia oleracea* L.) increased total phenols in the leaves. Selenium significantly increased phenolic contents by increasing phenylalanine ammonia-lyase activity (PAL) (Walaa et al., 2010).

Ascorbic acid acts as the main store of oxidation and reduction and as a cofactor for enzymes that regulate plant hormones, photosynthesis, antioxidant regeneration, cell division, and growth (Abdellatif & Ibrahim, 2018). By removing ROS, AsA protects cells and organelles from oxidative damage (Al-Zahrani et al., 2022). Heat stress reduced AsA. In turn, selenium increased the contents of ascorbic acid under heat stress (Figure 2, d). AsA is a precursor to the construction of chlorophyll and thus increases photosynthesis leading to the accumulation of various parts of soluble sugars in plant tissues under stress conditions, in addition to possibly mitigating the negative impacts of overheating through the removal of oxidative agents and the prevention of protein oxidation (Paciolla et al., 2019).

H_2O_2 can cause oxidative stress when its level rises in cells and can increase due to heat stress and other abiotic stresses (Hossain et al., 2015). Extreme heat raised MDA and H_2O_2 (Fig. 3, b-c). An ineffective antioxidant defense system under field temperature caused these findings. Due to high levels of antioxidants and the activities of antioxidant enzymes, plants treated with selenium showed lower MDA and H_2O_2 when exposed to heat stress (Gupta & Gupta, 2017). On the other hand, a

high concentration of selenium can likely harm plants by stimulating ROS generation (Lehotai et al., 2012).

APX hinders the accumulation of H_2O_2 by lowering it to H_2O in the AsA-GSH cycle as an enzyme's first line of defense (Hasanuzzaman et al., 2014). APX activity increased significantly under heat stress (Fig. 4, a). However, a high dose of selenium (30 mg l^{-1}) increased CAT and APX activity in stressful and non-stressful conditions (Fig. 4). Se increases antioxidant capacity, reduces free radical production, and promotes biomass accumulation under high temperature (Balal et al., 2016). Se-treated seedlings showed higher activity of CAT enzymes in canopy conditions than in field temperature. Catalase plays a vital role in converting H_2O_2 to H_2O. CAT activity reduces oxidative stress (Manafi et al., 2021). Furthermore, selenium nano-treatments significantly increased APX and CAT levels. Under heat stress, selenium is an active ingredient that can stimulate antioxidant enzyme gene expression and protein biosynthesis (Safari et al., 2018). Se increased the transcription of antioxidant defense genes, improving overall enzymatic activity in maize (*Zea mays* L.) (Jiang et al., 2017)1, 5 and 25 µM Na 2 SeO 3.

Thermal stress decreased the content of growth-promoting hormones IAA, GA, and CK and increased ABA (Fig. 5). Se reduced heat stress by increasing nutrient absorption, including nitrogen absorption (Shalaby et al., 2021). Nitrogen works to stimulate and produce auxin. Nitrogen is necessary for constructing the amino acid tryptophan, which forms the basis for the structure of indole acetic acid. IAA encourages the process of cell division and elongation of cells (Labeeuw et al., 2016). Plant hormones, on the other hand, such as IAA, stimulate the activation of the ATPases plasma membrane leading to hyperpolarization of the cell membrane (Zhang et al., 2017). The rise in the content of IAA, GA, and Ck plant hormones after selenium treatment under heat stress conditions could be related to selenium's beneficial role in reducing water loss by transpiration. Increased cell volume leads to plant hormone regulation and growth (Wang & Irving, 2011). Li et al. (2021) reported that the interaction of nano selenium in reducing cadmium damage on pepper plants improves the content of ABA in the roots and leaves. Malheiros et al. (2019) indicated that selenium regulates the development of primary and lateral roots in rice seedlings, resulting in novel patterns of root architecture through changes in auxin and ethylene levels. The high concentration of nano-selenium 30 mg l^{-1} showed a better effect than 15 mg l^{-1} in increased enzyme activities compared to controls.

Although nano-selenium reactive molecules may harm cell membranes and molecules, they may be essential signals in the reaction stages. These reactive molecules can activate cellular defense mechanisms, thereby mitigating the adverse effects of stress. When exposed to abiotic stress, the plants showed a specific response (Ramegowda & Senthil-Kumar, 2015). ABA appears as a chemical indicator sent by roots to the leaves to activate the mechanism of controlling water loss and closing stomata (Saddhe et al., 2017). Hormone results showed that nano-selenium stimulates the biosynthesis of natural hormones by increasing the levels of those substances in the leaves. The presence of abundant plant hormones may be crucial in regulating plant growth.

5 CONCLUSION

The findings showed that low concentrations of nano-selenium significantly improved the enzymatic and non-enzymatic antioxidant defense components in date palm seedlings exposed to heat stress. The enhanced antioxidant defense system shields seedlings from lipid peroxide and H_2O_2 overproduction. Nano-Se affects the metabolism of stressed and non-stressed plants. Nano-selenium improves the antioxidant defense system under heat stress by regulating growth regulators (IAA, GA, CK, and ABA), enzymatic activities, and osmolyte soluble such as proline, ascorbate, and phenols. Nano-selenium can be used to protect seedlings of date palms against high temperatures.

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doi:10.14720/aas.2024.120.2.15297 Original research article / izvirni znanstveni članek

Comparative assessment for nutritional and antinutritional qualities revealed better performance of traditional white-fleshed sweet potatoes than orange-fleshed sweet potatoes

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Received July 31, 2023; accepted June 03, 2024.
Delo je prispelo 31. julija 2023, sprejeto 3. junija 2024.

Comparative assessment for nutritional and antinutritional qualities revealed better performance of traditional white-fleshed sweet potatoes than orange-fleshed sweet potatoes

Abstract: Recent introduction of beta-carotene rich orange-fleshed sweet potatoes (OFSP) has resulted to consumers' low demands for traditional white-fleshed sweet potatoes (TWFSP), without due consideration of their nutritional qualities. This study appraised the nutritional compositions of OFSP and TWFSP. They were analyzed for mineral content, antinutrients, and phytochemicals at National Root Crops Research Institute, Umudike. The field experiment was conducted using randomized complete block design with three replicates. TWFSP showed higher concentrations of minerals, anti-nutrients and phytochemicals than OFSP. In TWFSP, potassium ranged from 1879.20 ± 0.01 mg kg^{-1} ('B_3V_3') to 1960.30 ± 0.01 mg kg^{-1} ('B_2V_2') while in OFSP it varied from 1162.60 ± 0.02 mg kg^{-1} ('$B_{26}T_{26}$') to 1800.20 ± 0.01 mg kg^{-1} ('$B_{10}T_{10}$'). The antinutrients and phytochemicals results showed that flavonoids in TWFSP ranged from 0.30 ± 0.01 mg TAE kg^{-1} ('B_1V_1') to 970.50 ± 0.02 mg TAE kg^{-1} (B_3V_3) while it varied from 0.20 ± 0.01 mg TAE kg^{-1} ('B_4T_4') to 670.30 ± 0.01 mg TAE kg^{-1} ('B_8T_8') in OFSP. Heritability estimates were high for all antinutrients and minerals while genetic advance was high only for potassium (42.206) and phosphorus (10.288) traits. Variation between phenotypic coefficient of variation and genotypic coefficient of variation was negligible, with the former higher for most minerals and antinutrients. TWFSP were found richer than OFSP, and suggests improvement by selection.

Key words: antinutrient, cultivars, phytochemicals, minerals, nutrition, sweet potatoes

Primerjalna ocena hranilne in nehranilne kakovosti je odkrila, da ima sladki krompir z belim založnim parenhimom boljše lastnosti kot tisti z oranžnim

Izvleček: Nedavna uvedba sladkega krompirja z oranžnim mesom (OFSP) bogatega z beta-karotenom, je povzročila slabše povpraševanje potrošnikov po tradicionalnem sladkem krompirju z belim mesom (TWFSP), ne da bi ustrezno upoštevali njegove prehranske lastnosti. Ta študija preučuje prehransko sestavo sladkega krompirja z belim in oranžnim mesom (založnim parenhimom). Vzorci so bili analizirani na vsebnost elementov, bioaktivnih sestavin in antinutrientov na National Root Crops Research Institute, Umudike. Nigerija. Terenski poskus je bil izveden z uporabo naključne blokovne zasnove s tremi ponovitvami. Krompir z belim mesom je pokazal večje vsebnosti mineralov, antihranil in bioaktivnih sestavin v primerjavi z oranžnim sladkim krompirjem. V krompirju z belim mesom se je kalij gibal od 1879,20 ± 0,01 mg kg^{-1} ('B_3V_3') do 1960,30 ± 0,01 mg kg^{-1} ('B_2V_2'), medtem, ko se je v krompirju z oranžnim mesom gibal od 1162,60 ± 0,02 mg kg^{-1} ('$B_{26}T_{26}$') do 1800,20 ± 0,01 mg kg^{-1} ('$B_{10}T_{10}$'). Rezultati antinutrientov in bioaktivnih sestavin so pokazali, da je bila vsebnost flavonoidov v krompirju z belim mesom od 0,30 ± 0,01 mg TAE kg^{-1} ('B_1V_1') do 970,50 ± 0,02 mg TAE kg^{-1} (B_3V_3), v krompirju z oranžnim mesom pa od 0,20 ± 0,01 mg TAE kg^{-1} ('B_4T_4') do 670,30 ± 0,01 mg TAE kg^{-1} ('B_8T_8'). Določitve dednih znakov so bile velike za vse antinutriente in vsebnosti elementov, genetska prednost je bila večja samo za kalij (42,206) in fosfor (10,288). Razlike med fenotipičnim in genotipičnim koeficientom spremenljivosti so bile zanemarljive, pri čemer so bile razlike v genotipičnem koeficientu večje za vsebnosti večine elementov in antinutrientov. Sorte z belim založnim parenhimom so se izkazale po večji vsebnosti koristnih snovi, kar nakazuje, da jih je potrebno uporabiti v žlahtniteljskih programih.

Ključne besede: antihranilo, sorte, fitokemikalije, minerali, prehrana, sladki krompir

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1 INTRODUCTION

Most traditional white-fleshed sweet potato (TWFSP) especially cultivars from Abakaliki, Nigeria, seem to have gone into extinction as a result of introduction of the orange-fleshed sweet potatoes (OFSP) (Mazuze, 2004). Beta-carotene, a known vitamin A precursor and carotenoids are plentiful in OFSP and their regular consumption can prevent blindness especially night blindness (Ndirigue, 2004; Park et al., 2016). TWFSP cultivars have been grown among local farmers for several years due to their high yielding capability, and consumer's acceptability despite its low beta-carotene contents. However, the high adoption rate of the recently introduced OFSP cultivars seemed to have caused a total replacement of TWFSP cultivars among farmers and consumers (Mazuze, 2004).

Although OFSP cultivars have been recognized for better beta-carotene contents than TWFSP cultivars, there is no elaborate record that compared other nutritional and antinutritional properties between them. Generally, sweet potatoes have been a vital food supply for the poorest farmers and food-insecure people around the world (Sugri et al., 2017). Studies reported that after maize, rice, and wheat, sweet potato is the fifth most important food crop in the developing world, with over 110 million metric tonnes produced per annum (Kanu et al., 2018). Sweet potatoes are abundant in protein, dietary fiber, polyphenols, vitamins and minerals but low in fat, which perhaps made it an ideal food for a greater percentage of the world's populace (Kanu et al., 2018; Tunio et al., 2019).

In many countries, including countries of sub-Saharan Africa, sweet potatoes especially white flesh sweet potatoes are the most utilized traditional root crops when compared to other root crops. In most Nigerian localities, TWFSP cultivars appear to be the most prevalent choice among local farmers probably among other benefits, because of their enormous volatile organic compounds (flavor) content (Mazuze, 2004; Kanu et al., 2018).

Previous studies have examined the nutritional properties of sweet potato cultivars grown in different countries of the world and have found considerable variation in nutrients among different sweet potato cultivars (Sanoussi et al., 2016; Kanu et al., 2018). The differences observed could be attributed to soil, climate, growing conditions, varying genetic make-up of cultivars and other factors (Mwanri et al., 2011; Sanoussi et al., 2016). However, none of these studies investigated the nutritional potentials of a given white fleshed sweet potato cultivar(s) in comparison with the OFSP cultivars, which recently have seemingly dominated greater parts of sub-Saharan African countries since development and introduction.

We hypothesize that the nutritional potentials of different OFSP and TWFSP cultivars will vary but to what extent and which would be better we cannot ascertain. Hence, the present study was undertaken to make a comparative assessment or appraisal of the nutritional, antinutritional and phytochemical properties of the available traditional white-fleshed sweet potatoes and the orange-fleshed sweet potato cultivars in the region and check for variability and trait association for better future sweet potato breeding.

2 MATERIALS AND METHODS

2.1 MATERIALS, EXPERIMENTAL SITE, DESIGN AND AGRONOMIC PRACTICE

Twenty-two cultivars of orange-fleshed sweet potato genotypes were collected from the National Root Crops Research Institute (NRCRI) Umudike, Abia State, Nigeria and three traditional white fleshed cultivars of sweet potato were collected from farmers in Abakaliki, Ebonyi State, Nigeria. The summary and description of each sweet potato cultivar are presented in Table 1.

The stems cuttings were collected and planted at the research and teaching farm of the Department of Crop Production and Landscape Management, Ebonyi State University, Abakaliki, Nigeria during raining season precisely June, 2019 which lasted till September the same year. The experimental design utilized was a randomized complete block design with three replicates. Standard agronomic management practices included; weeding, fertilizer (NPK 20:10:10), fungicide (mancozeb/chlorothalonil) and insecticide (malathion1/cypermethrin) applications at the rates provided on the labels, respectively. Tuber weighing 10 kg was harvested from each cultivar for nutritional analysis. All tubers were harvested, washed, and sliced before freeze-drying.

The dried potato slices were then pulverized, sieved through a 100-mesh sieve, and stored at –20 °C for all analysis. The laboratory analysis was carried out at the NRCRI central molecular biology laboratory, Umudike.

2.2 MINERAL ANALYSIS

The method of the Association of Official Agricultural Chemists (AOAC) in 2010 was used for the determination of mineral content; 1 g of the pulverized samples was placed in a crucible and ignited in a muffle furnace at 550 °C for 6 hours. The resulting ash was dissolved in

Table 1: Description of 25 sweet potato cultivars

S/N	Genotypes	Sources	Sowing/Harvesting date	Skin and flesh color
1	'B_6T_6'	NRCRI	June. 28, 2019/September. 28, 2019	purple/yellow
2	'B_1T_1'	NRCRI	June. 28, 2019/September. 28, 2019	purple/yellow
3	'B_3V_3'	ABAKALIKI	June. 28, 2019/September. 28, 2019	White/white
4	'$B_{17}T_{17}$'	NRCRI	June. 28, 2019/September. 28, 2019	purple/yellow
5	'B_8T_8'	NRCRI	June. 28, 2019/September. 28, 2019	purple/yellow
6	'$B_{28}T_{28}$'	NRCRI	June. 28, 2019/September. 28, 2019	purple/yellow
7	'$B_{13}T_{13}$'	NRCRI	June. 28, 2019/September. 28, 2019	purple/yellow
8	'$B_{26}T_{26}$'	NRCRI	June. 28, 2019/September. 28, 2019	purple/yellow
9	'$B_{10}T_{10}$'	NRCRI	June. 28, 2019/September. 28, 2019	purple/yellow
10	'$B_{16}T_{16}$'	NRCRI	June. 28, 2019/September. 28, 2019	purple/yellow
11	'$B_{29}T_{29}$'	NRCRI	June. 28, 2019/September. 28, 2019	Brown/Yellow
12	'$B_{19}T_{19}$'	NRCRI	June. 28, 2019/September. 28, 2019	Brown/yellow
13	'B_2V_2'	ABAKALIKI	June. 28, 2019/September. 28, 2019	Light purple/white
14	'$B_{11}T_{11}$'	NRCRI	June. 28, 2019/September. 28, 2019	Brown/yellow
15	'B_5T_5'	NRCRI	June. 28, 2019/September. 28, 2019	Brown/yellow
16	'$B_{18}T_{18}$'	NRCRI	June. 28, 2019/September. 28, 2019	Brown/yellow
17	'B_7T_7'	NRCRI	June. 28, 2019/September. 28, 2019	Brown/yellow
18	'$B_{15}T_{15}$'	NRCRI	June. 28, 2019/September. 28, 2019	Brown/yellow
19	'$B_{14}T_{14}$'	NRCRI	June. 28, 2019/September. 28, 2019	Brown/yellow
20	'$B_{20}T_{20}$'	NRCRI	June. 28, 2019/September. 28, 2019	Brown/yellow
21	'B_3T_3'	NRCRI	June. 28, 2019/September. 28, 2019	Red/yellow
22	'B_1V_1'	ABAKALIKI	June. 28, 2019/September. 28, 2019	Red/White
23	'B_2T_2'	NRCRI	June. 28, 2019/September. 28, 2019	Red/yellow
24	'B_4T_4'	NRCRI	June. 28, 2019/September. 28, 2019	Red/yellow
25	'B_9T_9'	NRCRI	June. 28, 2019/September. 28, 2019	Red/yellow

NRCRI: National Root Crops Research Institute, Umudike, Abia State, Nigeria

10 ml of 10 % HNO_3 and heated slowly for 20 minutes. After heating, it was filtered and the filtrate was used for the determination of mineral content. Atomic Absorption Spectrophotometer (AAS) was used in all analyses.

2.3 EXTRACTION OF PHENOLICS AND FLAVONOIDS

Total phenolics and flavonoids in freeze-dried OFSP and TWFSP roots were determined through colorimetric assay using the method of Abidemi (2013). Briefly, 1 g of the freeze-dried root powdered sample was weighed into clean propylene tubes before the addition of 10 ml of 80 % methanol, vortexed, shaken on a mechanical shaker, and incubated at a temperature of 25 °C for 12 hours. The mixture was then centrifuged at 3226 × g for 10 min, and the supernatant aliquot was collected to determine the total phenolics and total flavonoid contents.

2.4 DETERMINATION OF THE TOTAL POLYPHENOL CONTENT

The method of Baba and Malik (2015) was used to quantify the total phenolic content using the Folin-Ciocalteu technique. Exactly 20 ml of the sample blank solution (80 % methanol), gallic acid standards (0.001–0.1 kg ml^{-1}), and 5 ml of samples were pipetted into their corresponding test tubes, followed by the addition of 100 ml of 10 % Folin–Ciocalteu reagent and the mixtures are then shaken thoroughly. After 5 minutes, 80 ml of 7 % sodium carbonate was added and gently mixed before the plate was covered with aluminum foil and the reaction was allowed to incubate for 90 minutes at room temperature.

The absorbance value was then taken at 725 nm in a spectrophotometer. The concentration of total phenolic compounds in mg kg^{-1} of the dry sample as gallic acid equivalent was determined using an external standard calibration procedure (mg GAE).

2.5 ANALYSIS OF FLAVONOIDS

Exactly 250 ml titration flask, 0.005 kg of each plant sample was weighed, and 100 ml of 80 percent aqueous methanol was added at room temperature and agitated in an electric shaker for 4 hours. This process was repeated with the entire solution filtered through Whatman filter paper no. 42. The filtrate was then placed in a crucible and evaporated to dryness over a water bath before being weighed (Abidemi, 2013).

2.6 ANALYSIS OF TANNIN

Tannin was analyzed using the method of Ejikeme et al. (2014). Exactly 0.001 kg of the samples was weighed into a plastic bottle followed by the addition of 1000 ml of water and shaken for 1 hour in a shaker. It was then filtered, and 10 ml of the extract was measured into a test tube, along with 3 ml of 0.1 N HCl and three drops of ferrocyanide. It was let to stand for 10 minutes before being measured in a UV-Spectrophotometer at a wavelength of 605 nm.

$$\text{Tannic acid (mg kg}^{-1}) = C \times \text{extract volume} \times 0.1$$

$$\text{Aliquot volume} \times \text{mass of the sample}$$

Where, C is the concentration of tannic acid read.

2.7 EXTRACTION AND DETERMINATION OF OXALATE

Extraction of total oxalate was done as reported by Liu et al. (2009) and Nguyễn and Savage (2013). 1 g of each powdered sample was added to 0.5 mol l^{-1} of HCl before being diluted in 1 ml distilled water. The homogenate was put in 10 ml graduated tubes and cooked for 20 minutes in a boiling water bath. After the homogenate had cooled, distilled water was added to each tube to bring the total volume to 10 ml. About 1 ml of the homogenate was clarified the next day at 4 8C by centrifugation (12,000 g, 10 min). After that, 0.016 ml NaOH (2 mol l^{-1}) was carefully added to 0.5 ml supernatant.

Initially, a 2 ml test tube was added 20 mg of oxalate oxidase and then filled with other ingredients, including 0.06 ml of distilled water, 0.08 ml of colorant (10 mg of 4-aminoantipyrine), 25 ml of N, N-dimethylaniline, 0.04 ml of horseradish peroxide and 0.05 ml oxalate extract. The reaction mixture's absorbance at 555 nm was measured in a spectrophotometer after 90 minutes of incubation at room temperature. The oxalate content was calculated using a standard curve made by mixing 0, 2, 4, 6, 8, and 10 mg oxalic acid into a 1 ml reaction system, respectively. The results are given as mean mg oxalate kg^{-1} (Liu et al., 2009).

2.8 SAPONINS ANALYSIS

Saponin was analyzed according to the methods of Akpe et al. (2021). In a beaker, 0.005 kg of sample was added in 50 ml of 20 % ethanol. The suspension was heated for four hours in a hot water bath with constant stirring at a temperature of 60 °C. The mixture was filtered after 4 hours, and the residue was extracted again with another 25 ml of 20 % ethanol. The combined extract was concentrated and reduced to 40 ml in a water bath at 90 °C. The sample was placed in a separator-funnel and 20 ml diethyl ether was added and thoroughly shaken. The extracts' aqueous layer was recovered, while the other layers were discarded. Exactly 60 ml of n-butanol was then added and the extract was washed twice with 10 ml of 5 % aqueous sodium chloride. The remaining extracts were evaporated in a water bath and dried in an oven to a constant mass and weighed.

2.9 ALKALOIDS ANALYSIS

5 g of sample was weighed into a beaker; 100 ml of 100 % acetic acid in ethanol (1:1) was measured into the sample container and covered for 4 hours. After four hours, the extracted sample was filtered. It was then concentrated to a fraction of its original volume using a water bath. Drop-by-drop, ammonia solution was added to the concentrated extract, allowing the precipitate to settle before being filtered and washed with dilute ammonium hydroxide. The crude alkaloid was extracted from the residue and dried in an oven before being weighed.

2.10 ANTHOCYANINS ANALYSIS

The total anthocyanin content was calculated using Giusti and Wrolstad's method (2001) and Wegdan et al. (2020). In summary, two dilutions of the sample extract were made as follows 1 ml of the extracted sample so-

lution was added to a 10 ml volumetric flask each. One dilution volume was adjusted using potassium chloride buffer (pH 1.0), while the other was adjusted with sodium acetate buffer (pH 4.5). For equilibration, the dilution was allowed to sit for 15 minutes. Each dilution's absorbance was measured against water at 510 and 700 nm. The diluted sample's absorbance (A) was determined as follows:

$$A = (A_{510} - A_{700})\ \text{pH 1.0} - (A_{510} - A_{700})\ \text{pH 4.5}$$

The concentration of monomeric anthocyanin pigment was determined using the following formula: Monomeric anthocyanin pigment

$$(\text{mg } 100\ \text{g}^{-1}) = (A \times MW \times \text{DF} \times 1000) / (\varepsilon \times 1)$$

Where *MW* is the molecular weight, DF is the dilution factor, and ε is the molar absorptivity, calculate pigment content as cyanidin-3-glucoside.

Where *MW* = 449.2 and ε = 26,900

2.11 STATISTICAL ANALYSIS

All analyses such as ANOVA, genetic variability including phenotypic coefficient of variation percentage (% PCV) and genotypic coefficient of variation percentage (% GCV), Pearson correlation, Clusters and PCA were performed using the R statistical package (R-4.2.1) version (R Core Team, 2022).

3 RESULTS AND DISCUSSION

The results of the mineral contents of orange-fleshed sweet potatoes and indigenous or traditional white-fleshed sweet potatoes are presented in Table 2, Table 3, and Figure 1 while those of the antinutrients and phytochemicals are presented in Table 4, Table 5, and Figure 2.

3.1 COMPARATIVE ANALYSIS OF VARIATION AMONG OFSP AND TWFSP

3.1.1 Minerals

The results showed significant variation for the variables, and that both OFSP and TWFSP cultivars contained all the eight minerals including calcium, iron, potassium, phosphorus, sodium, magnesium, manganese, and zinc (Table 2). The studies of Mwanri et al. (2011) and Sanoussi et al. (2016) reported the presence of these minerals in orange-flesh sweet potatoes. Their concentrations as observed in the present study showed evidence that sweet potatoes possess high nutritional value. Minerals are needed in the body to keep the heart beating, blood clotting, nerve responses and reactions, and most importantly keep the body fluid balance in check (Mwanri et al., 2011; Sanoussi et al., 2016). The proper consumption of minerals is essential for human health. For instance, potassium is highly needed for proper neuronal transmission, and protein synthesis (Sebeo et al., 2009). Of the eight minerals studied, the concentrations of six minerals including zinc, calcium, iron, potassium, phosphorus, and sodium were found to be higher in TWFSP compared to the OFSP. The higher concentrations of these minerals in traditional white-fleshed cultivars suggested the presence of variation in their genetic make-up, and showed that this group may possess more nutrient and health benefits than orange-fleshed sweet potatoes (Table 3). Zinc and iron contents were higher in TWFSP cultivar 'B_3V_3' with values of 8.30 ± 0.01 mg kg^{-1} and 12.60 ± 0.01 mg kg^{-1}, respectively. Comparatively, OFSP cultivars, B_8T_8 and '$B_{26}T_{26}$' expressed the values 7.60 ± 0.02 mg kg^{-1} and 12.40 ± 0.02 mg kg^{-1} for zinc and iron, respectively. Although there is no literature on the TWFSP cultivars used in this study, the values we had for OFSP cultivars were close to those of Sanoussi et al. (2016). Zinc has been reported as a catalyst in a variety of activities in our bodies including involvement in macromolecules metabolism and required for cell division, tissue repair and normal reproductive development (Sebeo et al., 2009). Furthermore, iron has been implicated in the formation of hemoglobin in red blood cells, hence TWFSP cultivars, especially 'B_3V_3' will be of health importance for people with a metabolism health related problems and those suffering from iron deficiency compared to OFSP cultivars with lower mean concentrations. Phosphorus and sodium were also higher in TWFSP cultivar 'B_1V_1' with mean concentrations of 486.40 ± 0.03 mg kg^{-1} and 374.20 ± 0.02 mg kg^{-1}, respectively compared to the values of phosphorus (295.80 ± 0.01 mg kg^{-1}) and sodium (216.60 ± 0.01 mg kg^{-1}) in OFSP cultivars '$B_{17}T_{17}$' and 'B_6T_6', respectively. Magnesium and manganese with values 301.20 ± 0.03 mg kg^{-1} and 3.30 ± 0.03 mg kg^{-1} were the only minerals that had higher mean concentrations found in OFSP cultivars '$B_{18}T_{18}$' and '$B_{26}T_{26}$', respectively. These values were against the lowest mean concentrations of 260.50 ± 0.02 mg kg^{-1} and 1.70 ± 0.01 mg kg^{-1} for magnesium and manganese observed in TWFSP cultivars 'B_1V_1' and 'B_3V_3', respectively. These mean concentrations varied compared to 235.00 mg kg^{-1} reported for magnesium in an OFSP cultivar (Sanoussi et al., 2016). The reason for this variation may be attributed to differences in edaphic factors of the locations they were

Table 2: Mineral content (mg kg^{-1}) of orange-fleshed sweet potatoes and Abakaliki indigenous white-fleshed sweet potatoes

Genotypes	Ca	Na	Mg	P	K	Fe	Zn	Mn
'B_6T_6'	271.20 ± 0.02^{i}	218.80 ± 0.01^{a}	243.40 ± 0.02^{j}	301.30 ± 0.01^{c}	1274.30 ± 0.02^{b}	6.80 ± 0.01^{c}	5.20 ± 0.02^{e}	1.20 ± 0.00^{de}
'B_1T_1'	248.60 ± 0.01^{d}	226.20 ± 0.02^{c}	228.60 ± 0.01^{f}	393.10 ± 0.01^{h}	1451.70 ± 0.01^{h}	10.50 ± 0.01^{hi}	6.80 ± 0.01^{i}	1.20 ± 0.02^{cde}
'B_3V_3'	363.20 ± 0.01^{v}	372.50 ± 0.01^{r}	260.50 ± 0.02^{o}	442.30 ± 0.02^{t}	1879.20 ± 0.01^{w}	12.60 ± 0.01^{l}	8.30 ± 0.01^{l}	2.20 ± 0.01^{h}
'$B_{17}T_{17}$'	220.50 ± 0.01^{c}	301.70 ± 0.01^{i}	221.60 ± 0.02^{d}	295.80 ± 0.01^{a}	1332.60 ± 0.02^{d}	5.40 ± 0.02^{b}	6.10 ± 0.01^{g}	1.80 ± 0.01^{g}
'B_8T_8'	293.70 ± 0.02^{l}	358.70 ± 0.02^{o}	270.40 ± 0.01^{s}	437.10 ± 0.01^{s}	1492.70 ± 0.02^{k}	11.50 ± 0.02^{k}	7.60 ± 0.02^{k}	1.50 ± 0.01^{def}g
'$B_{28}T_{28}$'	300.40 ± 0.01^{n}	339.10 ± 0.01^{l}	256.80 ± 0.01^{n}	403.30 ± 0.01^{k}	1631.60 ± 0.02^{q}	12.40 ± 0.02^{l}	7.20 ± 0.01^{j}	2.30 ± 0.01^{h}
'$B_{13}T_{13}$'	294.40 ± 0.04^{m}	346.30 ± 0.03^{n}	213.60 ± 0.02^{b}	401.40 ± 0.03^{j}	1425.40 ± 0.01^{g}	5.60 ± 0.02^{b}	6.40 ± 0.02^{gh}	1.50 ± 0.02^{defg}
'$B_{26}T_{26}$'	216.60 ± 0.02^{b}	251.50 ± 0.01^{d}	200.50 ± 0.02^{a}	385.50 ± 0.01^{f}	1162.60 ± 0.02^{a}	2.50 ± 0.02^{a}	7.10 ± 0.01^{j}	3.30 ± 0.03^{i}
'$B_{10}T_{10}$'	314.30 ± 0.02^{r}	382.50 ± 0.02^{s}	256.10 ± 0.01^{m}	433.70 ± 0.02 ^{r}	1800.20 ± 0.01^{v}	11.50 ± 0.01^{k}	6.20 ± 0.02^{g}	2.20 ± 0.01^{h}
'$B_{16}T_{16}$'	286.80 ± 0.01^{j}	316.30 ± 0.01^{k}	261.90 ± 0.01^{p}	405.30 ± 0.01^{m}	1508.30 ± 0.02^{l}	10.50 ± 0.02^{hi}	4.80 ± 0.01^{d}	0.80 ± 0.01^{bc}
'$B_{29}T_{29}$'	253.20 ± 0.02^{e}	301.40 ± 0.01^{i}	225.80 ± 0.01^{e}	300.20 ± 0.01^{b}	1401.30 ± 0.02^{e}	12.40 ± 0.02^{l}	4.40 ± 0.03^{c}	1.50 ± 0.02^{defg}
'$B_{19}T_{19}$'	270.50 ± 0.01^{h}	223.70 ± 0.03^{b}	251.40 ± 0.02^{k}	331.60 ± 0.02^{d}	1285.30 ± 0.01^{c}	7.40 ± 0.02^{d}	4.60 ± 0.02^{cd}	1.40 ± 0.03^{defg}
'B_2V_2'	386.30 ± 0.03^{x}	391.40 ± 0.01^{t}	281.50 ± 0.02^{u}	473.40 ± 0.01^{x}	1960.30 ± 0.02^{y}	11.60 ± 0.02^{k}	7.50 ± 0.02^{k}	1.70 ± 0.03^{fg}
'$B_{11}T_{11}$'	303.60 ± 0.01^{p}	345.30 ± 0.02^{m}	263.50 ± 0.03^{q}	447.40 ± 0.02^{u}	1612.80 ± 0.01^{o}	11.70 ± 0.02^{k}	5.20 ± 0.01^{e}	1.70 ± 0.08^{g}
'B_5T_5'	321.90 ± 0.01^{s}	360.50 ± 0.03^{p}	301.20 ± 0.01^{v}	401.60 ± 0.02 ^{j}	1402.50 ± 0.01^{f}	8.40 ± 0.02^{e}	3.80 ± 0.01^{b}	1.70 ± 0.01^{g}
'$B_{18}T_{18}$'	325.30 ± 0.02^{t}	296.50 ± 0.03^{h}	264.50 ± 0.03^{r}	391.30 ± 0.03^{g}	1452.60 ± 0.02^{i}	10.40 ± 0.02^{h}	5.50 ± 0.03^{f}	1.10 ± 0.01^{cd}
'B_7T_7'	301.20 ± 0.02^{o}	263.90 ± 0.01^{f}	270.50 ± 0.03^{s}	452.50 ± 0.02^{w}	1771.30 ± 0.01^{u}	11.10 ± 0.01^{j}	4.70 ± 0.02^{d}	1.80 ± 0.00^{g}
'$B_{15}T_{15}$'	254.20 ± 0.01^{f}	312.50 ± 0.03^{j}	233.60 ± 0.02^{g}	428.30 ± 0.02^{q}	1620.40 ± 0.02^{p}	9.30 ± 0.02^{g}	6.30 ± 0.01^{gh}	2.30 ± 0.01^{h}
'$B_{14}T_{14}$'	206.40 ± 0.03^{a}	301.60 ± 0.03^{i}	254.40 ± 0.02^{l}	398.40 ± 0.02^{i}	1487.20 ± 0.02^{j}	8.80 ± 0.01^{f}	7.60 ± 0.01^{k}	1.60 ± 0.02^{efg}
'$B_{20}T_{20}$'	267.70 ± 0.02^{g}	254.50 ± 0.02^{e}	228.80 ± 0.01^{f}	404.20 ± 0.01^{l}	1692.10 ± 0.01^{s}	7.40 ± 0.02^{d}	6.50 ± 0.01^{h}	1.80 ± 0.01^{g}
'B_3T_3'	314.50 ± 0.03^{r}	365.60 ± 0.03^{q}	241.50 ± 0.03^{i}	375.50 ± 0.04^{e}	1550.30 ± 0.02^{m}	6.80 ± 0.02^{c}	6.40 ± 0.01^{gh}	0.30 ± 0.00^{a}
'B_1V_1'	374.20 ± 0.02^{w}	410.40 ± 0.02^{u}	274.70 ± 0.03^{t}	486.40 ± 0.03^{y}	1901.50 ± 0.02^{x}	10.80 ± 0.01^{i}	6.50 ± 0.03^{h}	1.70 ± 0.01^{fg}
'B_2T_2'	340.70 ± 0.03^{u}	338.80 ± 0.01^{l}	264.20 ± 0.03^{r}	422.50 ± 0.03^{p}	1596.60 ± 0.04^{n}	8.70 ± 0.02^{ef}	3.50 ± 0.02^{a}	1.40 ± 0.02^{def}g
'B_4T_4'	305.40 ± 0.03^{q}	312.50 ± 0.04^{j}	238.90 ± 0.01^{h}	413.10 ± 0.01^{o}	1632.70 ± 0.03^{r}	7.50 ± 0.04^{d}	4.70 ± 0.02^{d}	1.30 ± 0.02^{def}
'B_9T_9'	288.70 ± 0.02^{k}	294.30 ± 0.02^{g}	216.50 ± 0.02^{c}	407.50 ± 0.01^{n}	1701.80 ± 0.02^{t}	7.50 ± 0.03^{d}	4.50 ± 0.03^{cd}	0.60 ± 0.02^{ab}

The results are the standard deviations of three duplicate samples; values in the same column with similar letters are not significantly different at 0.05, Keys: Ca = calcium, Na = sodium, P = phosphorus, K = potassium, Zn = zinc, Fe = iron, Mg = magnesium, Mn = manganese

planted and fertilizer application as has been suggested in potato (*Solanum tuberosum* L.) (Liang et al., 2019). Manganese is very important component of glucose tolerance factor (GTF), which regulates blood glucose levels while magnesium is an essential component in many enzyme reactions and has an important role in the immune system regulation (Siddiqui et al., 2014; Konieczynski et al., 2022).

3.1.2 ANTINUTRIENTS AND PHYTOCHEMICALS

The antinutrients (oxalate, tannins, saponins and alkaloids) and phytochemicals (total polyphenols, anthocyanins and flavonoids) quantified in this study were all present in all the 25 cultivars at varying concentrations (Table 4). TWFSP cultivars had higher concentrations of all the antinutrients when also compared with OFSP (Table 5). The alkaloids and anthocyanin were the two compounds higher in OFSP than TWFSP. The highest mean value of alkaloids concentrations in OFSP was 1.30 ± 0.01 mg kg^{-1} for cultivar 'B_6T_6' contrary to 1.20 ± 0.01 mg kg^{-1} for TWFSP cultivar 'B_1V_1'. The values we obtained for alkaloids in this study deferred from the 6.20 ± 0.01 mg kg^{-1} reported in different cultivars of OFSP (Ogah et al., 2014; Akpe et al., 2021). This may be due to differences in plant maturity date, post-harvest storage and processing, location, growth season, soil type and nutrients (Li et al., 2012). Alkaloids have been implicated in a wide range of pharmacological effects, including anti-malarial, anti-cancer (Kittakoop et al., 2014), anti-bacterial (Cushnie et al., 2014) and anti-hyperglycemic (Qiu et al., 2014). Other compounds quantify in this study including oxalate, flavonoids, tannin, polyphenols and saponin were higher in TWFSP than OFSP. The concentrations of total polyphenols, flavonoids, tannins and oxalate in TWFSP were 123.80 ± 0.01 mg kg^{-1}, 970.50 ± 0.02 mg kg^{-1}, 4.70 ± 0.01 mg kg^{-1}, and 382.20 ± 0.02 mg kg^{-1}, respectively for cultivars 'B_3V_3' compared to the maximum mean concentration values of 89.20 ± 0.02 mg kg^{-1}, 673.80 ± 0.0102 mg kg^{-1}, 3.90 ± 0.01 mg kg^{-1} and 330.30 ± 0.0 mg kg^{-1} respectively in OFSP cultivars 'B_1T_1', 'B_8T_8', 'B_4T_4' and '$B_{13}T_{13}$' respectively. The concentrations obtained for tannin, flavonoids, and total polyphenols varied compared to the studies by Akpe et al. (2021) that reported 2.80 ± 0.01 mg kg^{-1}, 9.70 ± 0.01 mg kg^{-1}, and 7.20 ± 0.01 mg kg^{-1} for tannins, flavonoids, and total polyphenols, respectively. Flavonoids have antioxidant effects and have been shown to inhibit the initiation, promotion, and progression of tumors and can equally reduce coronary heart disease (Ezeonu & Ejikeme, 2016). Tannins have also been implicated in antiviral, antibacterial, and antitumor activity (Ezeonu & Ejikeme, 2016) while saponins are helpful in treating yeast and fungal infections. TWFSP also has higher content of oxalate with mean concentrations of 7.20 ± 0.01 mg kg^{-1} compare to 6.20 mg kg^{-1} in OFSP. Oxalate can prevent calcium absorption and utilization in the body. This eventually could lead to disorders like osteomalacia and rickets in the human body (Reddy & Pierson, 1994).

Table 3: The basic statistics description of mineral content of TWFSP and OFSP

TWFSP (Traditional white-fleshed sweet potatoes)								
Parameter	Ca	Na	Mg	P	K	Fe	Zn	Mn
Mean	374.60	391.40	272.20	467.30	1913.70	11.70	7.40	1.90
Median	374.20	391.40	274.70	473.40	1901.50	11.60	7.50	1.90
Minimum	363.10	372.40	260.30	442.10	1879.10	10.70	6.20	1.40
Maximum	386.60	410.60	281.60	486.60	1960.40	12.70	8.40	2.30
Range	235.00	38.20	21.30	44.50	81.30	2.00	2.20	0.90
OFSP (Orange-fleshed sweet potatoes)								
Parameter	Ca	Na	Mg	P	K	Fe	Zn	Mn
Mean	281.80	305.10	248.10	392.30	1513.00	8.80	5.70	1.50
Median	291.20	307.10	251.40	402.50	1500.50	8.80	5.90	1.60
Minimum	206.20	218.70	200.30	295.70	1162.40	2.50	3.30	0.30
Maximum	340.90	382.60	333.40	452.70	1800.20	12.50	7.80	3.50
Range	134.70	163.90	133.10	157.00	637.80	10.00	4.50	3.20

Ca = calcium, Na = sodium, P = phosphorus, K = potassium, Zn = zinc, Fe = iron, Mg = magnesium, Mn = manganese

Table 4: Anti-nutrient and phytochemical compositions (mg kg^{-1}) of orange-fleshed sweet potatoes and Abakaliki indigenous white-fleshed sweet potatoes

Genotypes	Oxalate	Tannin	Saponin	Alkaloids	Polyphenol	Flavonoids	Anthocyanin
'B$_6$T$_6$'	4.50 ± 0.01^{fa}	3080 ± 0.01^{de}	0.70 ± 0.01^{ghi}	1.30 ± 0.00^{a}	81.80 ± 0.01^{b}	592.10 ± 0.01^{bc}	478.10 ± 0.01^{b}
'B$_1$T$_1$'	2.80 ± 0.01^{m}	2.20 ± 0.01^{o}	1.10 ± 0.01^{de}	0.70 ± 0.00^{bc}	89.20 ± 0.02^{b}	601.50 ± 0.01^{bc}	403.20 ± 0.02^{bc}
'B$_3$V$_3$'	7.20 ± 0.01^{a}	4.70 ± 0.01^{a}	1.50 ± 0.01^{b}	0.40 ± 0.00^{a}	123.80 ± 0.01^{a}	970.50 ± 0.02^{a}	0.40 ± 0.04^{a}
'B$_{17}$T$_{17}$'	4.10 ± 0.01^{hi}	3.20 ± 0.01^{ghi}	0.80 ± 0.01^{fg}	0.10 ± 0.00^{bcd}	76.20 ± 0.02^{b}	534.10 ± 0.01^{bc}	313.70 ± 0.01^{c}
'B$_8$T$_8$'	3.80 ± 0.02^{j}	3.10 ± 0.01^{hij}	0.50 ± 0.01^{jk}	0.50 ± 0.00^{dcf}	92.60 ± 0.01^{b}	673.80 ± 0.02^{b}	445.70 ± 0.02^{c}
'B$_{28}$T$_{28}$'	4.30 ± 0.01^{gh}	3.70 ± 0.01^{de}	0.70 ± 0.00^{fgh}	0.80 ± 0.01^{b}	80.40 ± 0.01^{b}	645.20 ± 0.02^{b}	319.30 ± 0.02^{c}
'B$_{13}$T$_{13}$'	6.20 ± 0.01^{b}	4.10 ± 0.01^{bc}	1.40 ± 0.01^{bc}	0.70 ± 0.01^{bc}	82.10 ± 0.01^{bcd}	572.20 ± 0.01^{bc}	382.80 ± 0.02^{bc}
'B$_{26}$T$_{26}$'	4.10 ± 0.01^{l}	3.20 ± 0.01^{ghi}	1.10 ± 0.01^{e}	0.30 ± 0.01^{gh}	49.50 ± 0.03^{c}	403.60 ± 0.01^{c}	283.00 ± 00.03^{c}
'B$_{10}$T$_{10}$'	5.20 ± 0.02^{d}	3.30 ± 0.03^{fgh}	1.20 ± 0.01^{de}	0.70 ± 0.01^{bcd}	0.70 ± 0.00^{d}	0.80 ± 0.00^{d}	70.50 ± 0.01^{d}
'B$_{16}$T$_{16}$'	2.40 ± 0.01^{n}	3.20 ± 0.02^{ghi}	0.40 ± 0.01^{k}	0.60 ± 0.00^{bcd}	1.40 ± 0.01^{d}	0.70 ± 0.01^{d}	58.10 ± 0.01^{d}
'B$_{29}$T$_{29}$'	1.50 ± 0.01^{o}	2.80 ± 0.01^{jk}	0.60 ± 0.01^{hij}	0.80 ± 0.01^{b}	1.20 ± 0.03^{d}	0.50 ± 0.04^{d}	49.90 ± 1.17^{d}
'B$_{19}$T$_{19}$'	3.40 ± 0.01^{kl}	2.40 ± 0.04^{mn}	1.10 ± 0.01^{de}	0.40 ± 0.01^{fgh}	1.10 ± 0.00^{d}	0.30 ± 0.00^{d}	41.70 ± 0.00^{d}
'B$_2$V$_2$'	5.10 ± 0.01^{d}	3.40 ± 0.01^{fg}	1.20 ± 0.01^{de}	0.20 ± 0.01^{h}	1.20 ± 0.01^{d}	0.70 ± 0.01^{d}	0.20 ± 0.01^{d}
'B$_{11}$T$_{11}$'	5.60 ± 0.02^{c}	3.90 ± 0.01^{cd}	0.90 ± 0.01^{f}	0.70 ± 0.01^{bcd}	0.80 ± 0.06^{d}	0.70 ± 0.01^{d}	38.70 ± 0.21^{d}
'B$_5$T$_5$'	4.90 ± 0.01^{e}	3.20 ± 0.01^{ghi}	1.20 ± 0.01^{de}	0.30 ± 0.01^{gh}	0.40 ± 0.01^{d}	0.60 ± 0.00^{d}	40.10 ± 0.01^{d}
'B$_{18}$T$_{18}$'	4.20 ± 0.02^{hi}	3.00 ± 0.00^{ij}	0.70 ± 0.01^{ghi}	0.20 ± 0.00^{h}	0.60 ± 0.01^{d}	0.80 ± 0.01^{d}	39.30 ± 0.01^{d}
'B$_7$T$_7$'	4.60 ± 0.01^{f}	2.70 ± 0.01^{ghi}	0.50 ± 0.01^{fg}	0.40 ± 0.01^{bcd}	0.80 ± 0.03^{b}	0.60 ± 0.03^{bc}	45.20 ± 0.83^{c}
'B$_{15}$T$_{15}$'	3.60 ± 0.01^{jk}	3.00 ± 0.01^{ij}	0.60 ± 0.01^{hij}	0.60 ± 0.01^{bcd}	1.20 ± 0.01^{d}	0.40 ± 0.01^{d}	51.40 ± 0.01^{d}
'B$_{14}$T$_{14}$'	3.20 ± 0.01^{l}	3.50 ± 0.01^{ef}	0.70 ± 0.01^{ghi}	0.80 ± 0.01^{b}	1.20 ± 0.01^{d}	0.20 ± 0.01^{d}	62.30 ± 0.01^{d}
'B$_{20}$T$_{20}$'	3.80 ± 0.01^{j}	3.20 ± 0.01^{ghi}	1.10 ± 0.01^{de}	1.20 ± 0.02^{a}	1.10 ± 0.02^{d}	0.40 ± 0.03^{d}	61.30 ± 0.14^{d}
'B$_3$T$_3$'	4.20 ± 0.01^{hi}	4.10 ± 0.01^{bc}	0.80 ± 0.01^{fg}	0.70 ± 0.01^{bc}	0.90 ± 0.01^{d}	0.70 ± 0.00^{d}	60.20 ± 0.01^{d}
'B$_1$V$_1$'	6.10 ± 0.01^{b}	4.30 ± 0.01^{b}	1.70 ± 0.01^{a}	1.20 ± 0.02^{a}	1.20 ± 0.01^{d}	0.30 ± 0.01^{d}	0.50 ± 0.01^{d}
'B$_2$T$_2$'	4.10 ± 0.01^{l}	2.60 ± 0.01^{klm}	1.10 ± 0.01^{e}	0.40 ± 0.00^{efg}	0.10 ± 0.00^{d}	0.30 ± 0.01^{d}	48.90 ± 0.04^{d}
'B$_4$T$_4$'	4.30 ± 01^{gh}	2.50 ± 0.00l^{mn}	1.30 ± 0.00^{cd}	0.60 ± 0.00^{bcd}	0.70 ± 0.01^{d}	0.20 ± 0.00^{d}	46.30 ± 0.01^{d}
'B$_9$T$_9$'	5.30 ± 0.01^{d}	2.30 ± 0.02^{no}	0.80 ± 0.01^{fg}	0.60 ± 0.01^{cde}	0.50 ± 0.01^{d}	0.40 ± 0.01^{d}	51.10 ± 0.01^{d}

The results are the standard deviations of three duplicate samples; values in the same column with similar letters are not significantly different at 0.05

Table 5: The basic statistics description of antinutrient and phytochemical contents of TWFSP and OFSP

TWFSP (Traditional white-fleshed sweet potatoes)							
Parameters	OXA	TAN	SAP	ALK	POL	FLA	ANT
Mean	6.10	4.10	1.40	0.60	42.00	323.80	259.70
Maximum	7.20	4.70	1.80	1.30	123.90	970.60	690.60
Range	2.20	1.40	0.70	1.20	122.80	970.40	653.60
Minimum	5.00	3.30	1.10	0.10	1.10	0.20	37.00
Median	6.10	4.30	1.50	0.40	1.20	0.70	51.70
OFSP (Orange-fleshed sweet potatoes)							
Parameters	OXA	TAN	SAP	ALK	POL	FLA	ANT
Mean	4.10	3.10	0.80	0.60	25.70	183.20	154.10
Median	4.10	3.10	0.80	0.60	1.10	0.70	59.10
Minimum	1.40	2.10	0.30	0.20	0.30	0.10	37.20
Maximum	6.20	4.20	1.50	1.30	98.30	806.50	495.30
Range	4.80	2.10	1.20	1.10	98.00	806.40	458.10

OXA = Oxalate, TAN = Tannins, SAP = Saponins, ALK = Alkaloids, POL = Polyphenol, FLA = Flavonoids, ANT = Anthocyanins

3.2 TRAIT ASSOCIATION, GENETIC VARIABILITY AND PRINCIPAL COMPONENT ANALYSIS

The comprehensive correlations between the levels of all the minerals, antinutrients and phytochemicals in OFSP and TWFSP were investigated using Pearson's correlation analysis.

Among the minerals, positive significant correlations across the minerals were observed except for zinc and manganese, which had either negative or positive insignificant correlation with other minerals. Potassium showed substantial positive correlation with phosphorus (r = 0.776) and calcium (r = 0.707) (Figure 1). The result of this study is in congruence with that of Sanoussi et al. (2016) who reported high significant correlation between calcium and magnesium in sweet potatoes. Among the phytochemicals, total polyphenols showed the highest significant positive correlation with anthocyanins and flavonoids with correlation values of 0.980 and 0.980 respectively. Oxalate, which is an antinutrient, showed significant association with tannins and saponins with values of 0.477 and 0.593, respectively (Figure 2). The strong positive correlation observed among traits suggested high relatedness among them and that any trait can influence the other in the same direction.

Considering minerals, antinutrients and phytochemicals, the percentage value of PCV were higher than the percentage values of GCV showing how little the environment affected each trait. PCV values for minerals ranged from 39.981 to 10.581, while GCV values ranged from 37.875 % to 8.9714 %. PCV values for antinutrients and phytochemicals ranged from 159.890 % to 20.302 % while GCV values ranged from 149.450 % to 19.824 % (Table 6). The difference between GCV and PCV was very small and ranged from 2.1058 to 0.000 for minerals, while the difference between PCV and GCV for antinutrients and phytochemicals ranged from 10.4906 % to 0.0942 %. The PCV and GCV reported in our study are higher than the values of 14.41 % and 15.98 % reported for Iron by Amoros et al. (2020) and lower than the 254.75 % and 253.96 % respectively, reported for anthocyanin by Dutta et al. (2022). The higher percentage values of PCV than GCV showed that environment influences the expression of minerals and antinutrients in sweet potatoes, while the extremely small (less than 10 %) difference between PCV and GCV confirmed that environment indeed interacts in the expression of all traits studied (Uyeda et al., 2015). This equally implies that selection and hybridization may not be suitable for improving the content of sweet potatoes (Uyeda et al., 2015).

Heritability (H2b) in the broader sense was generally high for antinutrients, phytochemicals and minerals, ranging from 0.7189 to 1.0 for minerals and from 0.8731 to 0.9934 for antinutrients and phytochemicals (Table 6). This result varied slightly higher than the 0.81 reported for iron and zinc by Uyeda et al. (2015) and the same with the studies of Dutta et al. (2022) who reported 0.99 for anthocyanin. The genetic advance (GA) was relatively low for all traits except potassium (42.206) and phosphorus (10.288) among the other traits for minerals. The genetic advance for phytochemicals was higher for flavonoids and anthocyanin with values of 35.699 and 57.526, respectively. The low genetic advance with high heritability observed in all minerals, antinutrients and

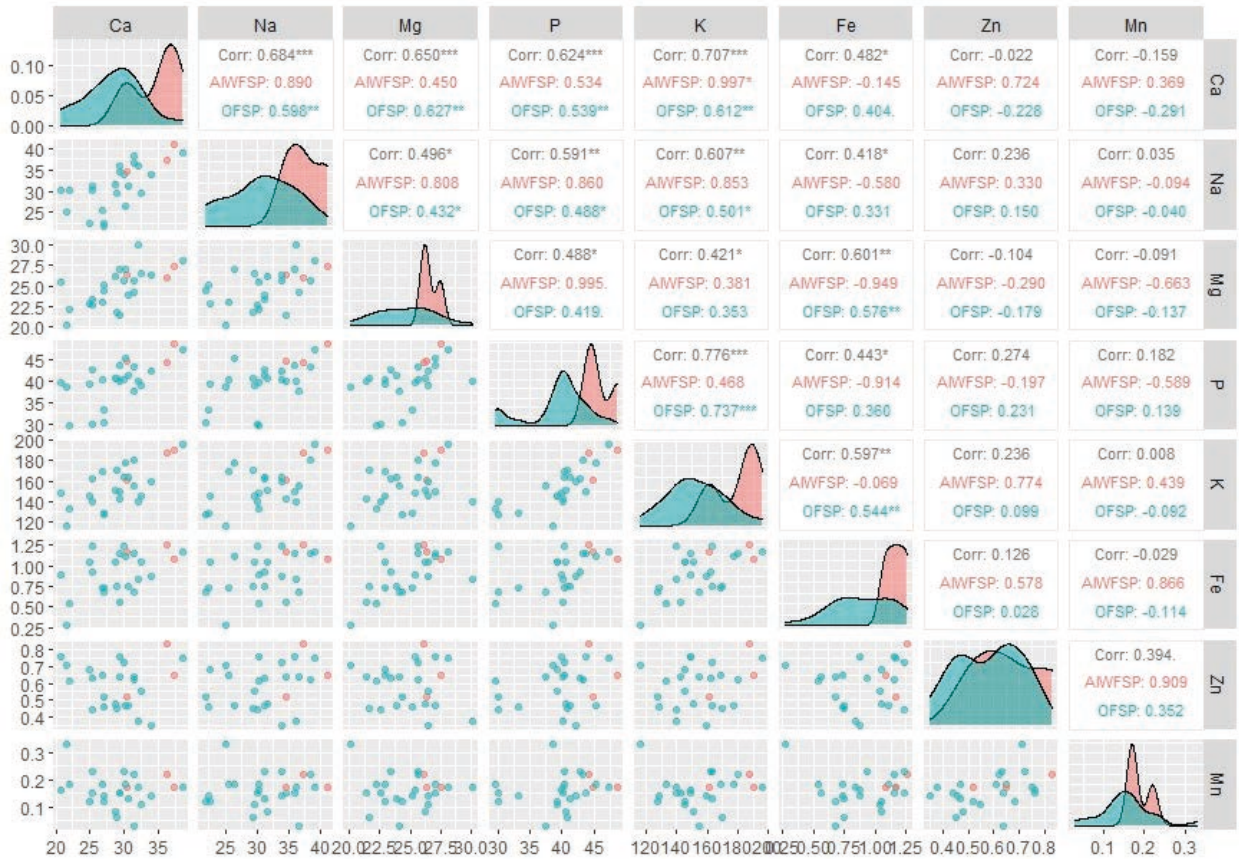

Figure 1: Pearson correlation plot for minerals. Ca = calcium, Na= sodium, P= phosphorus, K= potassium, Zn = zinc, Fe = iron, Mg = magnesium, Mn= manganese, Red color = Orange-fleshed sweet potatoes, Green = Traditional white-fleshed sweet potatoes

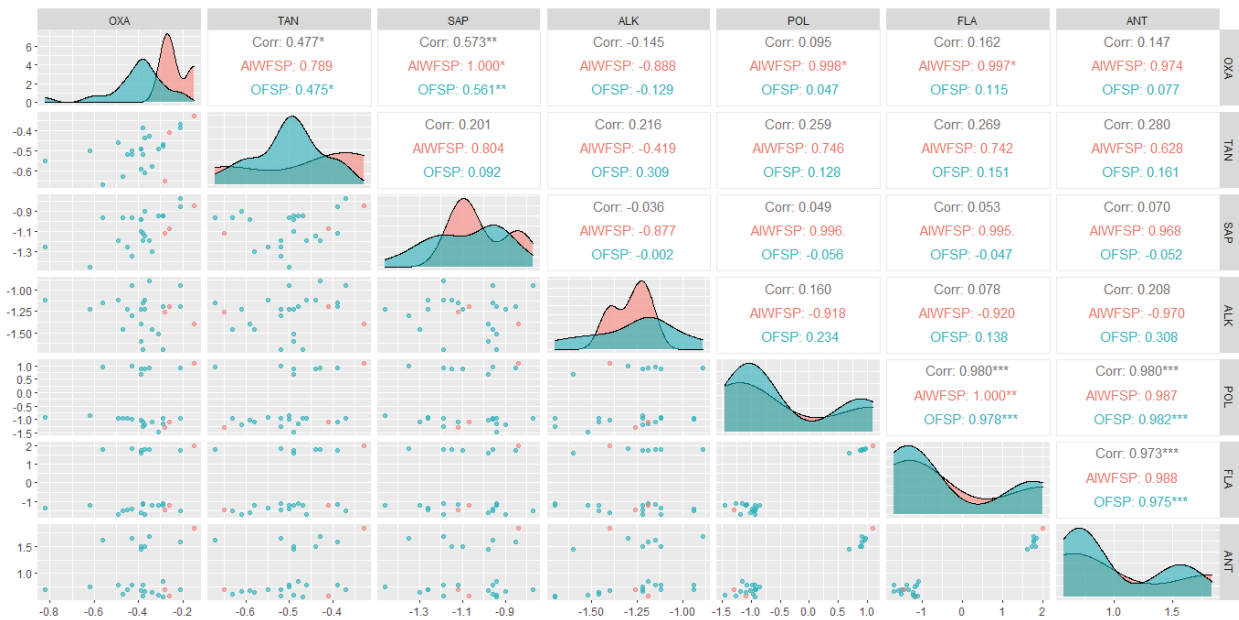

Figure 2: Pearson correlation plot for antinutrients. OXA = Oxalate, TAN = Tannins, SAP = Saponins, ALK = Alkaloids, POL =Total polyphenols, FLA = Flavonoids, ANT = Anthocyanins, Red color = Orange-fleshed sweet potatoes, Green = Traditional white-fleshed sweet potatoes

Table 6: Estimates of genetic parameters of antinutrients and phytochemicals from different cultivars of orange-fleshed and sweet potatoes

Traits	Gra. M	Min	Max	VE	VG	PV	ECV %	GCV %	PCV %	PCV-GCV	H_2b	GA	GAM
Ca	29.29	20.62	38.66	0.001	21.249	21.25	0.09	15.737	15.737	0.0003	1	9.4959	32.418
Na	31.544	21.87	41.06	0.0008	28.958	28.959	0.0871	17.059	17.06	0.0002	1	11.085	35.142
Mg	25.096	20.03	33.34	1.9817	5.0691	7.0508	7.0508	8.9714	10.581	1.6093	0.7189	3.9326	15.67
P	40.13	29.57	48.66	0.0006	24.942	24.942	0.061	12.445	12.445	0.0002	1	10.288	25.636
K	156.11	116.24	196.04	0.0005	419.78	419.78	0.0139	13.125	13.125	0	1	42.206	27.037
Fe	0.915	0.25	1.27	0.0006	0.0663	0.0669	2.6294	28.141	28.268	0.127	0.991	0.528	57.705
Zn	0.5886	0.33	0.84	0.0004	0.0168	0.0172	3.5196	22.021	22.282	0.2606	0.9767	0.2639	44.835
Mn	0.1562	0.03	0.35	0.0004	0.0035	0.0039	12.265	37.875	39.981	2.1058	0.8974	0.1155	73.944
Traits	Gra. M	Min	Max	VE	VG	PV	ECV %	GCV %	PCV %	PCV-GCV	H_2b	GA	GAM
OXA	0.4312	0.14	0.72	0.0001	0.0151	0.0152	2.7326	28.498	28.592	0.0942	0.9934	0.2523	58.511
TAN	0.323	0.21	0.47	0.0002	0.0041	0.0043	4.3784	19.824	20.302	0.4778	0.9535	0.1288	39.876
SAP	0.0914	0.03	0.18	0.0001	0.0011	0.0012	10.551	36.287	37.901	1.6136	0.9167	0.0654	71.554
ALK	0.0604	0.01	0.13	0.0001	0.0008	0.0009	14.992	46.828	49.669	2.8406	0.8889	0.0549	90.894
POL	2.7634	0.03	12.39	1.9024	16.088	17.99	49.912	145.15	153.49	8.3421	0.8943	7.8135	282.75
FLA	20.004	0.01	97.06	129.84	893.19	1023	56.962	149.4	159.89	10.4906	0.8731	57.526	287.57
ANT	16.677	3.7	69.06	36.09	332.87	368.96	36.024	109.4	115.18	5.7783	0.9022	35.699	214.06

Ca = calcium, Na = sodium, P = phosphorus, K = potassium, Zn = zinc, Fe = iron, Mg = magnesium, Mn = manganese, OXA = Oxalate, TAN = Tannins, SAP = Saponins, ALK = Alkaloids, POL = Polyphenols, FLA = Flavonoids, ANT = Anthocyanins, VE = Environmental variance, VG = Genotypic variance, PV = Phenotypic variance, ECV = Environmental coefficient of variation, GCV = Genotypic coefficient of variation, PCV = Phenotypiccoefficient of variation, H2b = Broad sense heritability, GA = Genetic advance, GAM = Genetic advance as a percentage of mean, Min = Minimum, Max = Maximum, Gra. M = Grand mean

phytochemicals studied indicates that these traits are significantly influenced by environmental factors and phenotypic selection may not be possible for enhancement (Uyeda et al., 2015).

The PCA biplot loading for the minerals revealed an overall variance of 68.3 % for dimensions 1 and 2. Dimension (PC1) explained the highest variation at 48.9 % (Figure 3). The plot of different dimensions for the minerals showed that dimension 2 had the highest concentrations or magnitude of zinc and manganese while iron and sodium were higher in dimensions 4 and 5, respectively (Figure 4). Magnesium had higher concentration

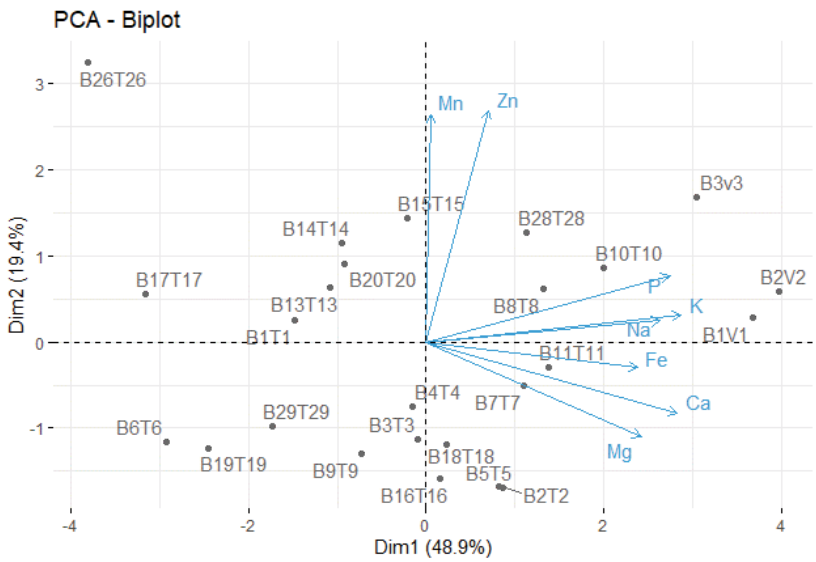

Figure 3: Principal component analysis for minerials. Ca = calcium, Na= sodium, P = phosphorus, K = potassium, Zn = zinc, Fe = iron, Mg = magnesium, Mn = manganese

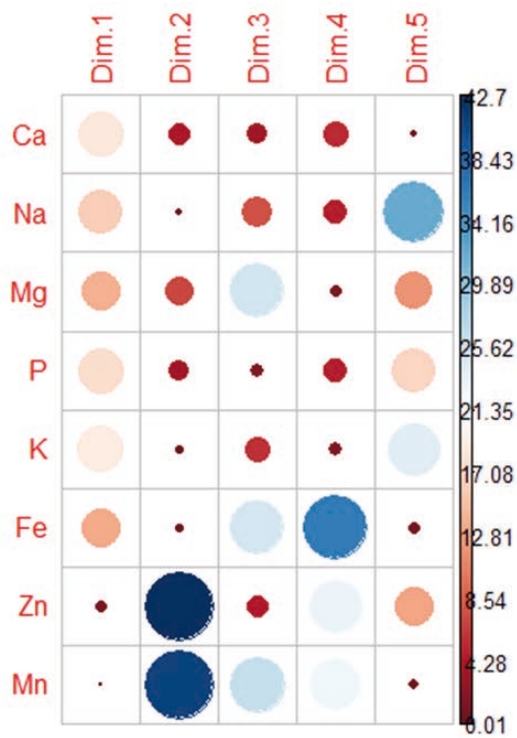

Figure 4: Dimension plots analysis for minerals. Ca = calcium, Na = sodium, P = phosphorus, K = potassium, Zn = zinc, Fe = iron, Mg = magnesium, Mn = manganese

in dimension 3 compared to other dimensions whereas potassium was higher in dimension 5, comparatively. A PCA biplot demonstrates how each trait affects a principal component and how they are related to one another. Based on the factor loading, manganese, phosphorus, potassium, zinc, and sodium contributed most to the variation observed in PC1 suggesting a positive and high correlation with some of the cultivars such as '$B_{28}T_{28}$', '$B_{10}T_{10}$', 'B_2V_2', 'B_8T_8', 'B_3V_3', and 'B_1V_1' since the smaller angle (less than 90 degree) between the two vectors indicates positive and greater correlation (Olanrewaju et al., 2021). It is clear from the PCA biplot that accessions loading in PC1 had a larger content of minerals (Mn, P, K, Zn, and Na) than accessions loading in PC2. The present result is similar to the studies of Laurie et al. (2022) which found sweet potatoes major nutrient in PC1.

The biplot of the principal component analysis for antinutrients revealed an overall variance of 78.7 % for dimension 1 and 2. Dimension (PC1) explained the highest variation at 48.8 % (Figure 5). The plot of different dimensions for antinutrients showed that dimensions 3 and 5 had the highest concentrations of alkaloid and oxalate, respectively (Figure 6). Among the 5 dimensions considered, saponin was higher in dimension 4. Based on the factor loading, saponin, oxalate, tannin, and alkaloids contributed most to the variation observed in PC1 indicating a positive and high correlation with some of the cultivars such as 'B_1V_1', '$B_{13}T_{13}$', and 'B_3V_3'. The PCA biplot clearly showed that the accessions loading in PC1 had a higher content of antinutrients than the accessions loading in PC2. The analysis also revealed that out of the three cultivars that showed strong and positive association with these antinutrients (saponin, oxalate, alkaloid, and tannin), two ('B_1V_1' and 'B_3V_3') were of traditional white flesh sweet potato cultivars. Although, studies are limited based on the samples used in the present study, however, our findings are similar to those of Ellong et al. (2014) which also reported strong relationship between polyphenols or phenoics and sweet potatoes.

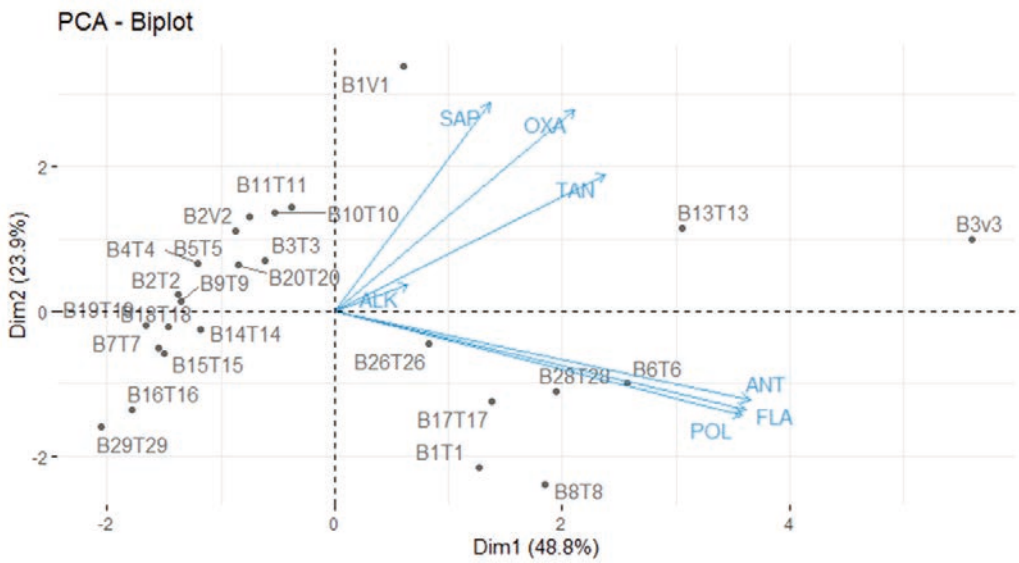

Figure 5: Principal component analysis for anti-nutrient. OXA = Oxalate, TAN = Tannins, SAP = Saponins, ALK = Alkaloids, POL = Total Polyphenols, FLA = Flavonoids, ANT = Anthocyanins

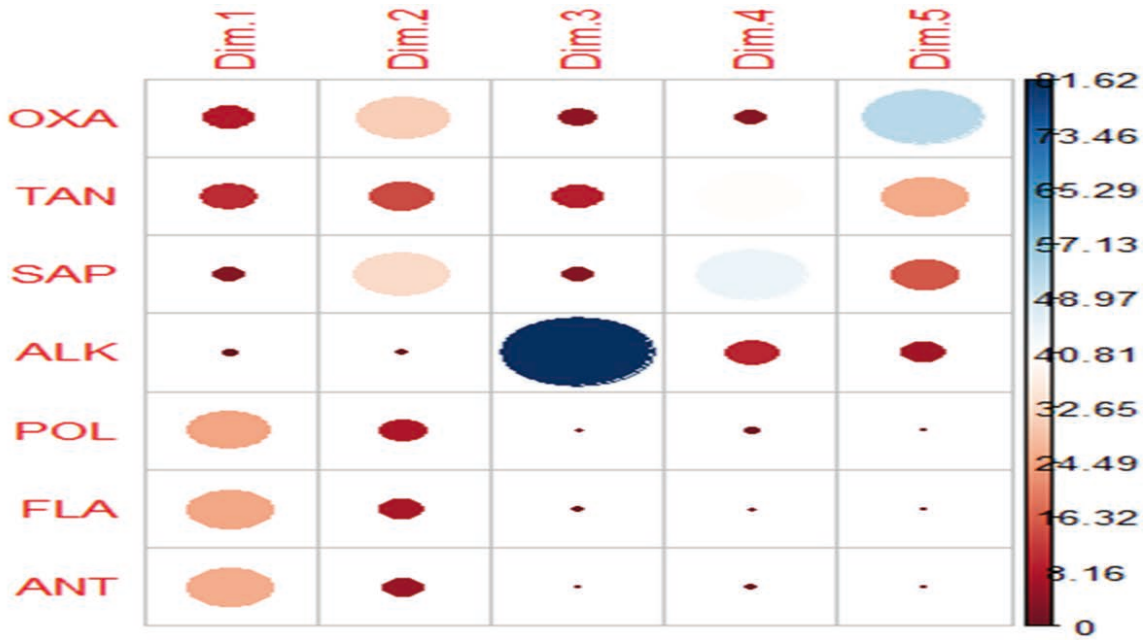

Figure 6: Dimension plot analysis for antinutrient. OXA = Oxalate, TAN = Tannins, SAP = Saponins, ALK = Alkaloids, POL = Total Polyphenols, FLA = Flavonoids, ANT = Anthocyanins

3.3 CLUSTER ANALYSIS

The hierarchical clustering analysis constructed using pvclust cluster method with AU/BP Pvalues in percentages and the bootstrapping of 10,000 are shown below in figures 7 and 8 for mineral and antinutrients,

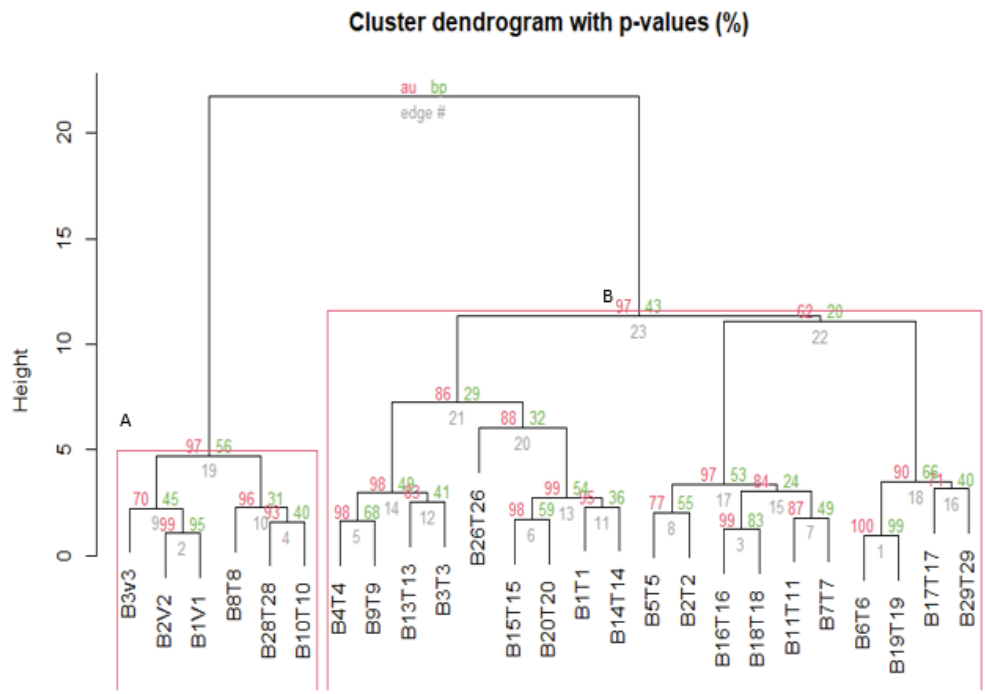

Figure 7: Cluster dendrogram with au/bp values (%) based mineral. The values at the edges of the cluster are P-values (%) calculated over a multiscale bootstrap with 1000 resamples. Values on the left in red = au (approximate unbiased) P-values, and values on the right in green = bp (bootstrap probability) values. Clusters with au above 95 % are highlighted in blocks suggest high relatedness.

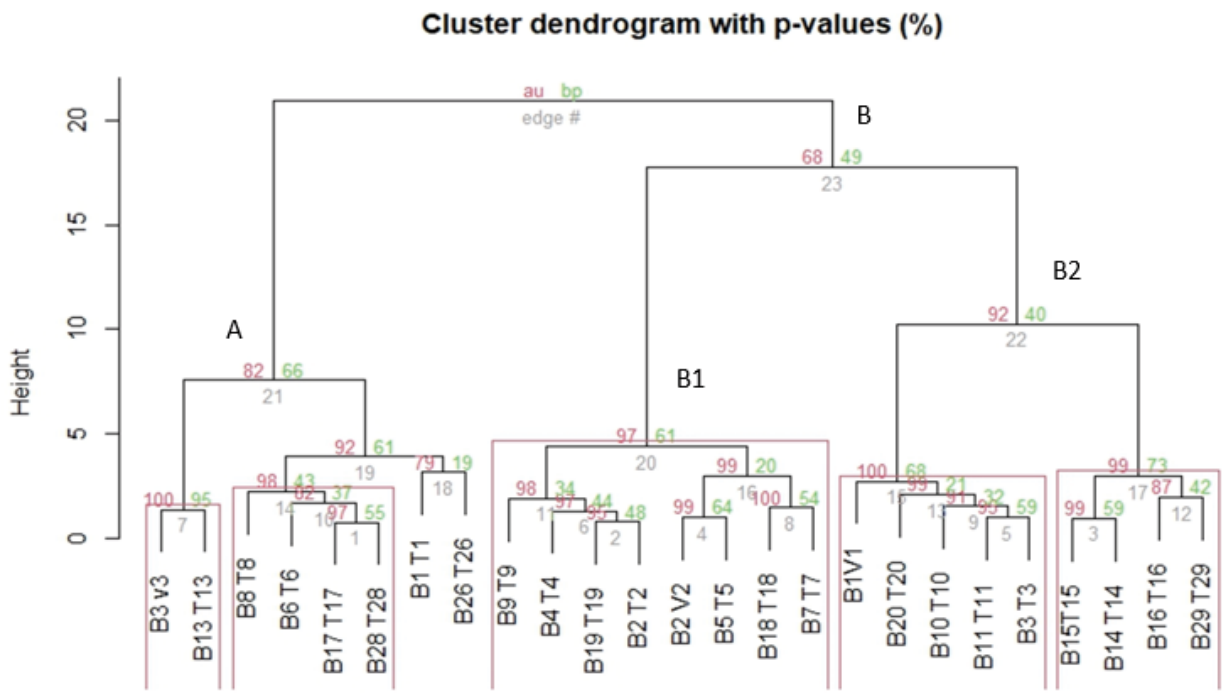

Figure 8: Cluster dendrogram with au/bp values (%) for antinutrient analysis. The values at the edges of the cluster are P-values (%) calculated over a multiscale bootstrap with 1000 resamples. Values on the left in red = au (approximate unbiased) P-values, and values on the right in green = bp (bootstrap probability) values. Clusters with au above 95 % are highlighted in block suggest high relatedness

respectively. This method offers two types of p-values: AU (Approximately Unbiased) p-value and BP (Bootstrap Probability) value. The AU p-value calculated by multiscale bootstrap resampling is a better approximation of the unbiased p-value than the BP value calculated by normal bootstrap resampling. Therefore, AU p-values above 95 % indicate significant clusters (Suzuki & Shimodaira, 2009; de Croos & Pálsson, 2012). The cluster dendrogram for the minerals grouped the genotypes into two groups (A and B) according to the relatedness of their mineral composition. Cluster A contains only six cultivars, including all traditional white-fleshed sweet potatoes ('B_1V_1', 'B_2V_2' and 'B_3V_3') and a few OFSP ('$B_{10}T_{10}$,' 'B_8T_8' and '$B_{28}T_{28}$'). Cluster B consists of 19 genotypes and included all OFSPs.

The cluster dendrogram for antinutritional analysis (Figure 8) was also divided into two clusters A and B. Cluster B was further divided into B1 and B2. Cluster A had eight genotypes, including '$B_{13}T_{13}$,' 'B_6T_6,' '$B_{17}T_{17}$,' '$B_{26}T_{26}$,' 'B_1T_1,' 'B_8T_8,' '$B_{28}T_{28}$' and 'B_3V_3.' 'B_3V_3' was the only TWFSP in cluster A. However, cluster B had two TWFSPs, B_2V_2 in cluster B1 and 'B_1V_1' in cluster B2. Cluster B had a total of 17 genotypes. The appearance of 'B_3V_3' of TWFSP among the cultivars of OFSP in cluster A showed that the grouping pattern of the genotypes did not completely follow their source or geographical distribution. This suggests that 'B_3V_3' which fell into cluster A despite its origin or geographical distribution showed a sign of broad genetic base of the genotype. Lee et al. (2019) reported that breeding has enhanced the diversity of cultivated potatoes, especially with its related wild relatives at both phenotypic and genotypic levels. This type of clustering was also reported by Lee et al. (2015) where OFSP cultivars fell into the same cluster compared to TWFSP.

4 CONCLUSIONS

The study revealed significant variation for the traits in both TWFSP and OFSP cultivars. Of the eight minerals studied, the concentrations of six minerals including zinc, calcium, iron, potassium, phosphorus, and sodium were found to be higher in TWFSP compared to the OFSP which suggest that the former may possess more nutrient and health benefits than the latter.

Except for alkaloids and anthocyanin, TWFSP cultivars had higher concentrations for all the antinutrients compared to OFSP cultivars.

The positive significant correlations across the minerals and phytochemicals suggested high relatedness among traits and this can encourage the selection of fewer traits in future trials, which would reduce cost in traits measurement and management without undermining experiment precision.

The high genetic advance with high heritability observed for potassium and phosphorus (minerals), flavonoids and anthocyanin (phytochemicals) indicates that these traits would respond to selection as the best improvement approach.

Despite the lack of carotene, the traditional white-fleshed sweet potatoes proved to possess higher concentrations of these minerals and phytochemicals than orange-fleshed sweet potatoes of which the three varieties of TWFSP involved in the study stood out the best, comparatively.

4.1 ACKNOWLEDGEMENTS

We are grateful to the National Root Crops Research Institute, Umudike, Nigeria, for providing us with the facility and genetic materials.

4.2 DATA AVAILABILITY STATEMENT

All data set generated during and/or analyzed to support the findings of this study are phenotypic data and can be made available from the corresponding author on request.

4.3 COMPETING INTERESTS

The authors declare that there are no conflicts of interest, financial or nonfinancial, directly or indirectly.

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doi:10.14720/aas.2024.120.2.15456 Original research article / izvirni znanstveni članek

Podnebne projekcije temperature zraka in padavin za porečja Ledave, Pesnice in Vipave do konca 21. stoletja

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Received August 08, 2023; accepted April 05, 2024.
Delo je prispelo 8. avgusta 2023, sprejeto 5. aprila 2024.

Climate projections of air temperature and precipitation for the Ledava, Pesnica and Vipava basins in the 21st century

Abstract: As part of the project ‚CeVoTak', Integrated Management of Small Water Retention and Soil Erosion Prevention Measures in Agricultural Catchments, we studied changing temperature and precipitation conditions up to the year 2100. The study was conducted on agricultural lands in the catchments of the Ledava and Pesnica rivers in the sub-Pannonian region and the Vipava river in the sub-Mediterranean region. A common climate database was used to create the climate projections - RCM (Regional Climate Model) simulations from the project EURO-CORDEX and scenarios RCP (Representative Concentration Pathway), RCP2.6, 4.5 and 8.5. The projections were prepared for three time periods 2011-2040; 2041-2070 and 2071-2100 for 6 different regional climate models for average, minimum and maximum air temperatures and precipitation. Analysis of the ensemble of model simulations for all scenarios shows similar results for the basin of all rivers, an increase in temperature (maximum in winter, minimum in spring), with high confidence for all scenarios and periods. Projections of precipitation are less reliable, but show an increase in annual precipitation due to the winter increase. The use of climate change projections with expert interpretation is essential for determining the vulnerability of individual areas and building resilience through the implementation of climate change adaptation.

Key words: project ‚CeVoTak', climate projections, Ledava, Pesnica, Vipava, air temperature, precipitation

Podnebne projekcije temperature zraka in padavin za porečja Ledave, Pesnice in Vipave do konca 21. stoletja

Izvleček: V okviru raziskovalnega projekta 'CeVoTak', celovito upravljanje malih ukrepov za zadrževanje vode in preprečevanje erozije tal v kmetijskih povodjih, smo naredili oceno spremenjenih temperaturnih in padavinskih razmer do leta 2100. Raziskava je potekala na kmetijskih površinah v povodjih rek Ledave in Pesnice v omiljenem celinskem in reke Vipave v omiljenem sredozemskem podnebju. Za pripravo podnebnih projekcij smo uporabili skupno klimatsko podatkovno bazo - simulacije RCM (Regional Climate Model) iz projekta EURO--CORDEX in scenarije RCP (Representative Concentration Pathway), RCP2.6, 4.5 in 8.5. Projekcije so bile pripravljene za tri obdobja: 1-bližnja prihodnost: 2011–2040; 2-sredina stoletja: 2041–2070 in 3-daljna prihodnost: 2071–2100 za 6 različnih regionalnih podnebnih modelov za povprečne, najnižje in najvišje temperature zraka ter količino padavin. Analiza ansambla modelskih simulacij za vse tri scenarije kaže podobne rezultate za porečje vseh treh rek, in sicer naraščanje temperature (največ pozimi, najmanj spomladi), zanesljivost spremembe je visoka za vse scenarije in obdobja. V primerjavi s temperaturami zraka so projekcije padavinskih razmer manj zanesljive, kažejo pa naraščanje letne količine padavin zaradi dviga pozimi. Uporaba projekcij podnebnih sprememb s strokovno razlago je nujna pri določanju ranljivosti posameznih območij in grajenju odpornosti z uvajanjem ukrepov prilagajanja na podnebne spremembe.

Ključne besede: projekt ‚CeVoTak', podnebne projekcije, Ledava, Pesnica, Vipava, temperatura zraka, padavine

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1 UVOD

Podnebne spremembe, ki so posledica vpliva človeka predvsem zaradi spreminjanja rabe tal ter izpustov toplogrednih plinov (TGP), in presegajo naravno podnebno spremenljivost, se kažejo v povišanju temperature ozračja, oceanov in tal ter posledično v pogostejših in intenzivnejših ekstremnih vremenskih dogodkih. Hitre in obsežne spremembe v ozračju, oceanih, kriosferi in biosferi so povzročile številne škodljive vplive za naravo in ljudi, nekateri od teh so nepopravljivi, saj so naravni in človeški sistemi potisnjeni preko svojih zmožnosti prilagajanja (IPCC, 2022). Medvladni panel za podnebne spremembe (IPCC) je konec marca 2023 objavil zbirno poročilo *Podnebne spremembe 2023, Povzetek za odločevalce*, ki spada v zadnji del šestega poročevalskega cikla IPCC. To sintezno poročilo IPCC zagotavlja najobsežnejšo in najboljšo razpoložljivo znanstveno oceno podnebja, ki temelji na osemletnem projektu, v katerem je sodelovalo več sto znanstvenikov (IPCC, 2023). Povprečna globalna temperatura ozračja je v letih 2011–2020 dosegla zvišanje za 1,1 °C glede na obdobje 1850–1900. Že leta 2018 je IPCC v posebnem poročilu (IPCC, 2018) opozoril, da se bližamo mejniku zvišanja temperature za 1,5 °C, ki bi še omogočal obvladljivo soočanje s podnebno krizo. Že zdaj se s povišanjem globalne temperature za 1,1 °C v vseh regijah sveta dogajajo spremembe podnebnega sistema, dodatno segrevanje pa bo še povečalo njihov obseg. Projekcije kažejo, da se bo ob dvigu temperature za 1,5 °C skoraj milijarda ljudi po vsem svetu soočala s pomanjkanjem vode, vročinskim stresom in dezertifikacijo, medtem ko se bo delež svetovnega prebivalstva, izpostavljenega poplavam, povečal za 24 % (IPCC, 2023). Vse več pozornosti v zadnjem času se namenja tudi t.i. sestavljenim ekstremnim dogodkom, kot so suše, poplave, požari, ki so lahko posledica sočasnega vpliva več različnih dejavnikov (Lawrence in sod., 2020; Hillier in sod., 2020). Sočasne visoke temperature zraka, suša in močan veter so lahko vzrok za obsežne požare, sočasne intenzivne padavine in taljenje snežne odeje pa za poplave. Predvideva se, da se bo pogostost in intenziteta sestavljenih ekstremov v prihodnosti povečala (Ribeiro in sod., 2020; Simpson in sod., 2021), zaradi biofizikalne soodvisnosti med temperaturo, vodo ter fiziološkimi procesi rastlin pa neto učinki takih prihodnjih dogodkov ostajajo negotovi (Lesk in sod., 2022). Pogostost in jakost dogodkov z izjemnimi padavinami sta se od 50. let prejšnjega stoletja povečali skoraj nad vsem kopnim, zaradi povečanega izhlapevanja pa so pogostejše tudi kmetijske suše površinskega sloja tal (ARSO, 2021). Zadnje poročilo IPCC (2023) navaja, da se bodo glede na projekcije tveganja suše v 21. stoletju povečala v številnih regijah, prav tako tudi povečanje intenzivnosti padavin, kar bo povečalo lokalne poplave.

Analiza podnebne spremenljivosti za Slovenijo je pokazala, da je bila temperatura ozračja v prvih dveh desetletjih tega tisočletja (2001–2020) za 1,8 °C (razpon 1,5–2,0 °C) višja glede na obdobje 1850–1900, v zadnjem desetletju (2011–2020) pa za 2,1 °C (razpon 1,9–2,4 °C) (Dolinar in sod., 2018; Berkley Earth, 2023). Od začetka šestdesetih let prejšnjega stoletja se je višina padavin na letni ravni zmanjševala, po letu 2000 ponovno večala, razlike pa niso statistično značilne (ARSO, 2021). Izhlapevanje se je v obdobju 1971–2012 povečalo za okoli 20 %, najbolj zaradi povečanja spomladi in poleti, višina novo-zapadlega snega pa se je zmanjšala za približno 40 %. Srednji pretoki rek v Sloveniji se od šestdesetih let prejšnjega stoletja zmanjšujejo, največji upad je značilen za pomlad in jesen, se je pa pogostost velikih pretokov ponekod v osrednjem in v vzhodnem delu države povečala (Vertačnik in sod., 2018).

Segrevanje je bilo v obdobju 1961–2011 večinoma močnejše v vzhodnem kakor zahodnem delu Slovenije, količina padavin pa se je v istem obdobju na letni ravni zmanjšala bolj v zahodnem delu, za okoli 15 %, v vzhodnem delu pa za 10 %. Gledano v celoti so bile najizrazitejše spremembe podnebja v tem obdobju v poletnem času v delu južne in jugozahodne Slovenije, kjer so poletja v zadnjih desetletjih toplejša, bolj sončna in bolj sušna (Bertalanič in sod., 2018).

Podobne spremembe temperatur zraka in padavin so bile opažene tudi v sosednjih državah. Povprečna letna temperatura zraka se je v Italiji v zadnjih 100 letih povečala za 1 °C, pri čemer se je segrevanje v zadnjih 50 letih pospešilo, hkrati se je povprečna letna količina padavin nekoliko zmanjšala (IEA, 2022; Straffelini in Tarolli, 2023). Tudi povprečna letna temperatura zraka v Avstriji narašča hitreje od svetovnega povprečja in se je od leta 1880 zvišala za 2 °C. Ekstremne padavine so postale pogostejše, ni pa jasnega trenda glede povprečne količine padavin (IEA, 2022; Olefs in sod., 2021). Povprečna letna temperatura zraka na Madžarskem se je med letoma 1907 in 2017 dvignila za 1,2 °C, še posebej izrazito je segrevanje poleti. Trend letne količine padavin v istem obdobju ni bil zaznan, opazne pa so spremembe v sezonskosti tveganja poplav in suš ter v regionalnih vzorcih padavin (IEA, 2022; Pinke in Lövei, 2017).

Po vsej Evropi se hidrološki cikel spreminja kot posledica antropogeno povzročenega globalnega segrevanja. Narejene so bile številne ocene vpliva teh sprememb na rečne režime v prihodnosti, za glavna porečja Severne in Južne Evrope, vključno s porečjem Save (Sperna Weiland in sod., 2021; Miro in sod., 2021), za porečja Mure, Drave in Donave (Probst in Mauser, 2023; Zlatanović, 2022), reke Pad (Boyko in sod., 2022), reke Vipave

(Cvejić in sod., 2020; Filmon, 2022) in druge. Študije so pokazale, da se bodo podnebni vplivi razlikovali za južno in severno Evropo, kar bo zelo verjetno vodilo do večjih sezonskih omejitev vode v južni Evropi in obilne razpoložljivosti vode v severni Evropi (ICPDR, 2019). Velik del Evrope je v prehodnem območju med bolj vlažnim severnim in bolj suhim južnim podnebjem v prihodnosti, kjer se podnebni modeli pogosto ne ujemajo glede znaka spremembe (ICPDR, 2019; Probst in Mauser, 2023; Sperna Weiland in sod., 2021).

V okviru raziskovalnega projekta ‚CeVoTak' (ARRS projekt L4-2625: celovito upravljanje malih ukrepov za zadrževanje vode in preprečevanje erozije tal v kmetijskih povodjih, https://cris.cobiss.net/ecris/si/sl/project/18388), smo preučevali vpliv podnebnih sprememb na ekonomsko trajnostno gospodarjenje z vodo v kmetijskih tleh. Raziskava je potekala na kmetijskih površinah v treh topološko in pedo-klimatsko različnih povodjih rek Ledave in Pesnice v omiljenem celinskem in reke Vipave v omiljenem sredozemskem podnebju. Vsa tri povodja so močno izpostavljena različnim vremenskim pojavom, tako sušam kot poplavam, glede na zadnje projekcije podnebnih sprememb za 21. stoletje (ARSO, 2021; Bertalanič in sod., 2018; IPCC, 2022) pa za vsa tri povodja pričakujemo veliko izpostavljenost podnebnim spremembam. V prispevku predstavljamo podnebne projekcije temperature zraka in padavin za porečja rek Ledave, Pesnice in Vipave do leta 2100, razpon pričakovanih sprememb in zanesljivost teh sprememb glede na upoštevane scenarije prihodnjih družbenih sprememb.

2 MATERIAL IN METODE DELA

Oceno spremenjenih temperaturnih in padavinskih razmer do leta 2100 smo naredili za porečja rek Ledave, Pesnice in Vipave. Ledava je reka v severovzhodni Sloveniji, nižinski vodotok, ki ima skoraj v celoti močno preoblikovano umetno strugo, večji del teče po severnem delu Murske ravnine. Na najvzhodnejši točki Slovenije, slovensko-hrvaško-madžarski tromeji, se izliva v Muro, površina porečja pa meri 1940 km^2. Tudi Pesnica je reka v severovzhodni Sloveniji, levi pritok Drave, izvira v Avstriji, površina porečja v Sloveniji pa znaša 539 km^2. Reka Vipava ima površino porečja 604 km^2, ki je na submediteranskem ozemlju v jugozahodni Sloveniji. Izvira iz več kraških izvirov v Vipavi, teče nato proti zahodu in se izliva v Sočo v Italiji (ARSO, 2023).

Za pripravo podnebnih projekcij smo uporabili skupno klimatsko podatkovno bazo podnebja - simulacije RCM (Regional Climate Model) iz projekta EURO-CORDEX (Jacob in sod., 2014) in scenarije RCP (Representative Concentration Pathway), RCP2.6, 4.5 in 8.5, ki so označeni glede na sevalni prispevek ob koncu 21. stoletja. Scenarij RCP2.6, t.i. 'optimističen scenarij', predvideva izrazito blaženje podnebnih sprememb in posledično zelo majhne izpuste TGP, sevalni prispevek doseže vrh v prvi polovici 21. stoletja pri 3,0 W m^{-2} in do leta 2100 upade na 2,6 W m^{-2}. Scenarij RCP4.5, t.i. 'zmerno optimističen' ali stabilizacijski, predpostavlja z začetkom druge polovice 21. stoletja postopno zmanjševanje izpustov, sevalni prispevek se ustali kmalu po letu 2100 in znaša ob koncu stoletja 4,5 W m^{-2}. Scenarij RCP8.5, t.i. 'pesimistični scenarij', ne predvideva blaženja podnebnih sprememb, ampak velike izpuste TGP in naraščanje njihove vsebnosti v ozračju tudi po letu 2100, ob koncu stoletja pa sevalni prispevek znaša 8,5 W m^{-2} (van Vuuren in sod., 2011). Osnovni podatki baze EURO-CORDEX so za Slovenijo na voljo v prostorski resoluciji 12,5 km, na katerih potem izvedemo povečanje ločljivosti na mrežo v resoluciji 1 km.

Popravek napak (bias correction) je bil izveden z uporabo podatkov reanalize ERA5-Land z neparametričnim empiričnim kvantilnim kartiranjem, referenčno obdobje je bilo 30-letno obdobje 1981–2010. Splošni postopek popravka napak temelji na primerjavi porazdelitev modelskih podatkov in meritev v primerjalnem obdobju ter oceni razlik po kvantilih te porazdelitve. Ocenjene razlike nato služijo kot popravki modelskih podatkov za podnebne projekcije za prihodnost pri izbranem kvantilu (Bertalanič in sod., 2018). Projekcije so bile pripravljene za tri obdobja: 1-bližnja prihodnost: 2011–2040; 2-sredina stoletja: 2041–2070 in 3-daljna prihodnost: 2071–2100 za 6 različnih regionalnih podnebnih modelov za 4 spremenljivke (povprečna, najnižja in najvišja temperatura zraka na 2 m v °C, količina padavin v mm). V preglednici 1 je seznam uporabljenih prilagojenih simulacij za 6 modelov za scenarija RCP4.5 in RCP8.5 ter 2 modela za scenarij RCP2.6.

Nabor rezultatov 6 različnih modelov omogoča vrednotenje modelske negotovosti in opredelitev možnih razponov prihodnjih sprememb. Odkloni meteoroloških spremenljivk so podani za leto in meteorološke letne čase: pomlad (marec, april, maj), poletje (junij, julij, avgust), jesen (september, oktober, november) in zimo (december, januar, februar). Običajni pristop pri obravnavi negotovosti modelskih projekcij vključuje izračun mediane modelov, ki predstavlja oceno reprezentativne vrednosti ansambla, izračun minimalne ter maksimalne vrednosti modelskih rezultatov.

Za analizo niza podnebnih simulacij smo izračunali spremembe za tri 30-letna projekcijska obdobja glede na referenčno obdobje 1981–2010 (izmerjeni podatki) za vsako simulacijo modela RCM. Nato smo sestavili ansambel, kjer so bili člani ansambla simulacije modela RCM (6 članov), in izračunali razpone ansambla (maksi-

Preglednica 1: Seznam prilagojenih simulacij ter oznake modelov (1 do 6) za posamezne RCP scenarije
Table 1: List of used simulations and models (1 to 6) for individual RCP scenarios

Globalni model	Regionalni model	RCP2.6	RCP4.5	RCP8.5
CNRM-CM5	CCLM4		1	1
MPI-ESM-LR	CCLM4		2	2
EC-EARTH	HIRHAM5	1	3	3
IPSL-CM5A-MR	INERIS		4	4
HadGEM2-ES	RACMO22E	2	5	5
MPI-ESM-LR	RCA4		6	6

malna– minimalna vrednost, reprezentativna vrednost je ocenjena z mediano). Spremembe so bile ocenjene na letni ravni in na ravni meteoroloških sezon. Skladnost podnebnega ansambla smo ocenili s kazalcem, imenovanim »zanesljivost spremembe«, ki nam pove, ali člani ansambla kažejo podobne spremembe. Kazalnik za zanesljivost podnebnih sprememb je izračunan na podlagi absolutne vrednosti vsote predznakov spremembe za vse modele ansambla, ki kažejo statistično značilno spremembo. Kazalnik je predstavljen v treh stopnjah – velika zanesljivost, nizka zanesljivost in brez sprememb, temelji pa na statistični zanesljivosti izračunanih sprememb. Za vsakega člana ansambla in vsako projekcijsko obdobje smo izračunali Mann-Whitney-Wilcoxov test in ga združili s predznakom (smerjo) izračunane spremembe. Ničelna hipoteza neparametričnega Mann-Whitney-Wilcoxonovega testa je, da je pri naključni izbiri vrednosti iz prvega vzorca enako verjetno, da je ta vrednost manjša ali večja od naključno izbrane vrednosti drugega vzorca (Bertalanič in sod., 2018).

Podroben opis celotne metodologije, to je izbora klimatskih spremenljivk, izbire EURO-CORDEX RCM simulacij, postopka popravkov napak, interpolacije modelskih simulacij, evaluacije ter analize simulacij sta opisala Honzak in Pogačar (2022).

3 REZULTATI Z DISKUSIJO

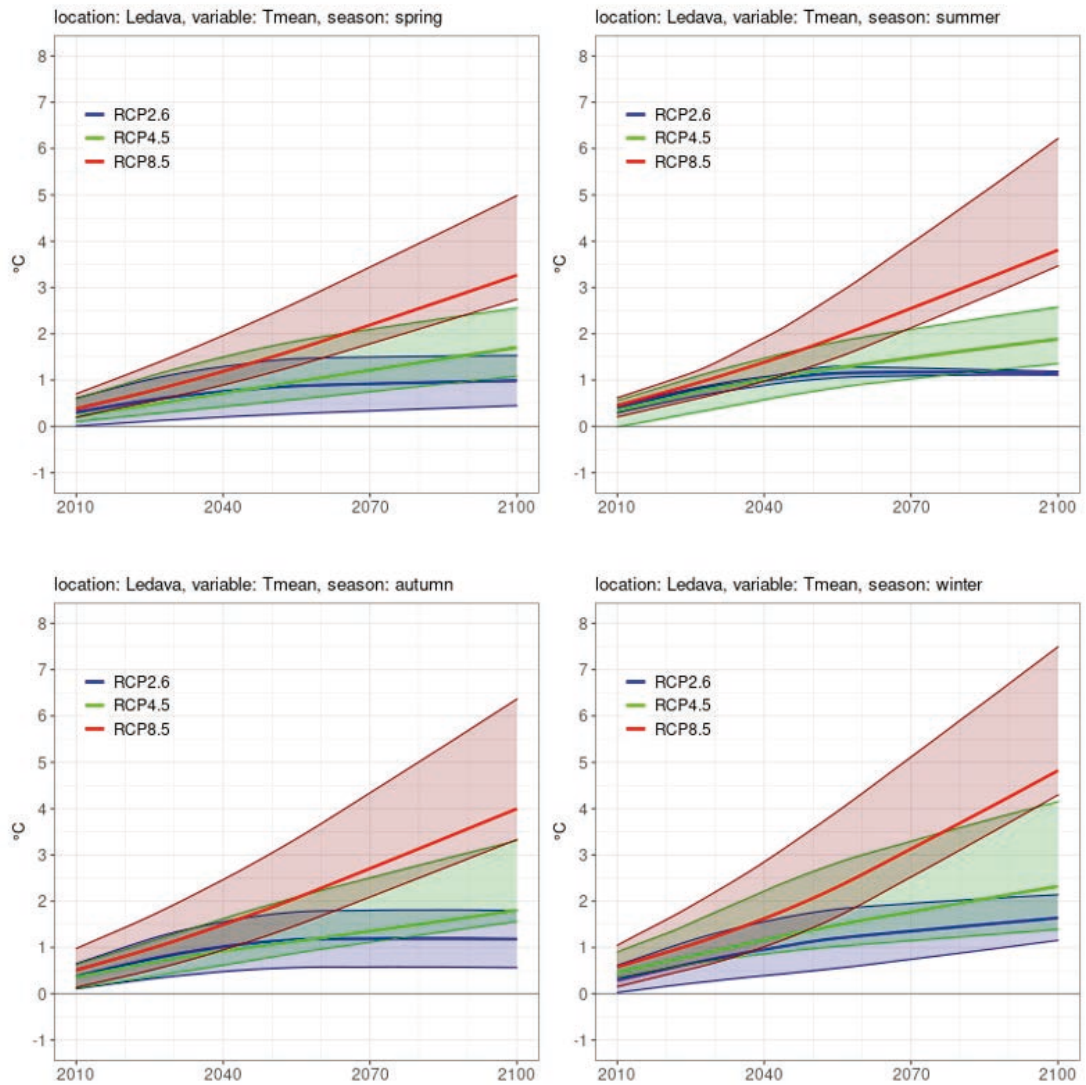
3.1 PODNEBNE PROJEKCIJE ZA POREČJE REKE LEDAVE

Vsi trije podnebni scenariji do leta 2100 za porečje Ledave (Slika 1) predvidevajo naraščanje povprečnih letnih temperatur zraka (Tpov), in sicer RCP2.6 za 1,3 °C (razpon 0,7–1,9 °C), RCP4.5 za 1,7 °C (razpon 1,4–2,7 °C) in RCP8.5 za 3,3 °C (razpon 3,0–5,3 °C). V prvih dveh scenarijih Tpov sprva narašča, a se do konca 21. stoletja ustali, pri RCP8.5 pa izrazito narašča tudi v zadnjem obdobju. Če so v prvem in drugem obdobju temperaturna odstopanja od primerjalnega obdobja (1981–2010) med posameznimi scenariji še primerljiva, pa se v zadnjem obdobju (2071–2100) časovni poteki ločijo. Medtem ko projekcije po RCP2.6 in RCP4.5 za to obdobje kažejo dvig Tpov za 1,3 °C in 1,7 °C, pa se izrazitejša sprememba pričakuje po scenariju RCP8.5, ki kaže dvig Tpov za 3,3 °C, s širokim razponom od 3,0 °C do 5,3 °C. Predvideno segrevanje se po letnih časih nekoliko razlikuje (Slika 1). Do konca 21. stoletja naj bi se najbolj segrele zime, projekcija po RCP2.6 kaže povečanje za 1,5 °C, po RCP4.5 za 2,1 °C in po RCP8.5 kar za 4,0 °C. Odstopanje temperature pozimi bo ob koncu stoletja izrazito večje od letnega segrevanja, poletja pa naj bi se v povprečju segrela enako kot leto. Za pomlad kažejo projekcije nekoliko manjše segrevanje od letnega povprečja, od 1,0 °C po RCP2.6 do 2,7 °C po RCP8.5, jesenske temperature pa naj bi se zvišale od 1,1 °C po RCP2.6 do 3,5 °C po RCP8.5. V prvem obdobju (2011–2040) znašajo projekcije dviga temperature po letnih časih v večini primerov od 0,8 °C do 1,0 °C, še največje segrevanje kaže jesen. V drugem obdobju se po RCP2.6 najbolj segrevata poletje in jesen (za 1,3 °C), po RCP4.5 poletje in zima (za 1,4 °C), po RCP8.5 pa je povišanje največje za zimo (2,1 °C) in jesen (2,0 °C).

Tako kot za Tpov modeli tudi za maksimalne temperature (Tmax) kažejo z veliko zanesljivostjo, da se bodo do konca stoletja le-te višale, tako v letnem povprečju kot tudi po sezonah. Letne Tmax se bodo v prvem obdobju povišale za 0,8 °C, enako za vse tri scenarije, za drugo obdobje je pričakovano povišanje Tmax po RCP2.6 za 1,1 °C in po RCP8.5 za 1,6 °C. Večja razpršenost velja za tretje obdobje, ko je povišanje Tmax po RCP2.6 za 1,2 °C, največje odstopanje po RCP8.5 pa znaša 3,2 °C. Vse navedeno so mediane modelskih rezultatov. Pomembne za kmetijstvo so projekcije sprememb ekstremnih temperatur po sezonah. Najmanjše povišanje Tmax kažejo modeli za pomlad, v prvem obdobju ne predvidevajo večjega odklona od 0,8 °C, do konca stoletja pa bi lahko bile Tmax od 1,5 °C (RCP4.5) do 2,6 °C (RCP8.5) višje. Tudi za poletje in jesen se bodo Tmax povišale, vzorec za obe sezoni je precej podoben, poleti je npr. po RCP4.5 sprememba 1,7 °C, najbolj pesimističen scenarij pa kaže za 3,4 °C višje poletne Tmax. Povečanje števila ekstremno

toplih dni se kaže tudi za zimo, do konca stoletja lahko pričakujemo tudi do 4,1 °C višje Tmax, če bi se uresničil scenarij RCP8.5, srednji scenarij RCP4.5 pa kaže 2,2 °C višje Tmax. Zelo podobni kot za Tmax so tudi modelski rezultati za minimalne temperature (Tmin), stopnja zanesljivost je visoka. V letnem povprečju se bodo v prvem obdobju Tmin povišale nekaj manj kot za 1 °C po vseh treh scenarijih, do konca stoletja pa po RCP4.5 za 1,7 °C in po RCP8.5 za 3,4 °C. Pregled po sezonah pokaže, da bo sprememba Tmin najmanjša spomladi, od 1,1 °C po RCP2.6 do 2,7 °C po RCP8.5, izrazitejše povišanje pa kažejo projekcije za zimo, od 1,5 °C po RCP2.6 do 3,9 °C po RCP8.5.

Oceno zanesljivosti spremembe meteoroloških spremenljivk smo podali v treh stopnjah. Visoka stopnja zanesljivosti pomeni, da lahko z veliko verjetnostjo pričakujemo modelirane spremembe. Nizka stopnja zanesljivosti pomeni, da se modelski rezultati med seboj zelo razlikujejo in je verjetnost sprememb v smer naraščanja ali upadanja spremenljivke velika, a ne vemo, v katero smer bo šla. Tretja stopnja zanesljivosti je označena kot 'ni spremembe', pomeni pa, da so spremembe majhne in

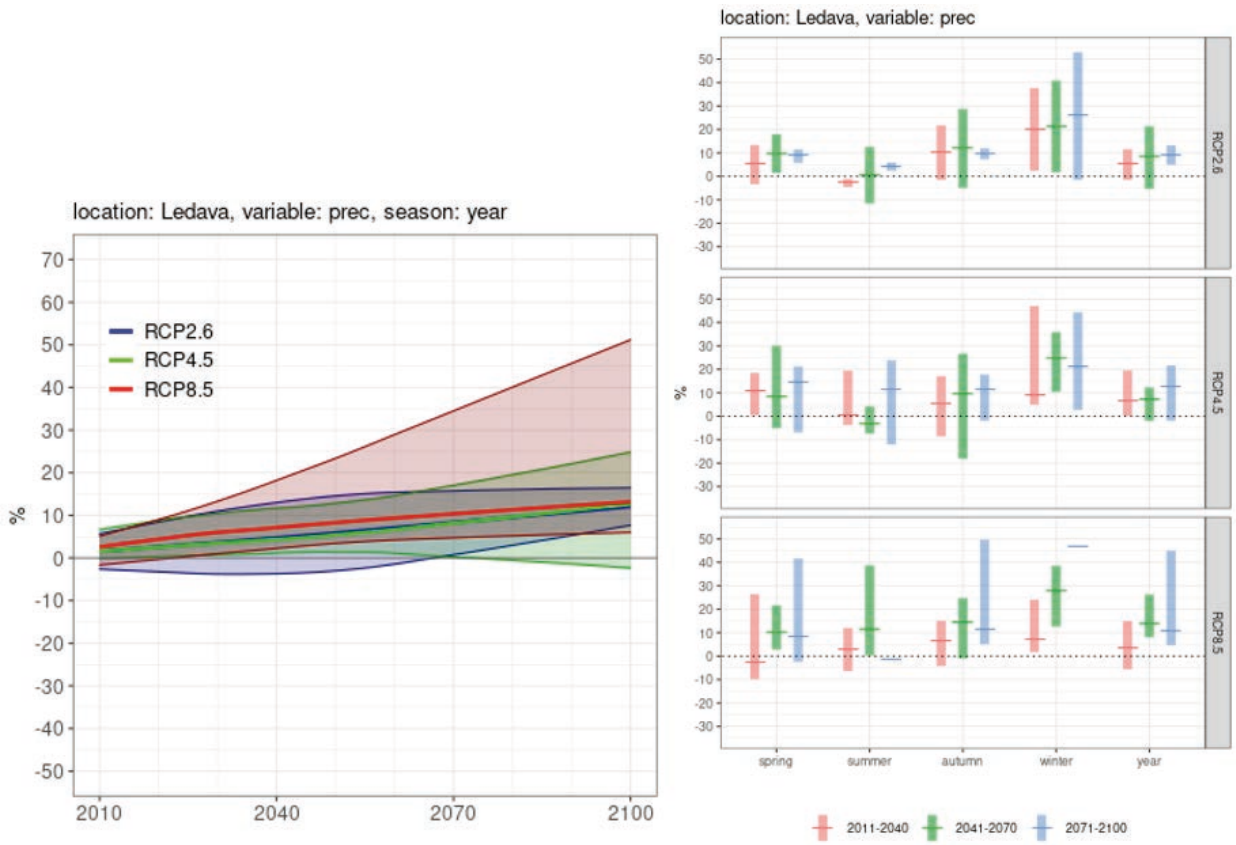


Slika 1: Časovni potek odklona Tpov po meteoroloških letnih časih z možnimi razponi do konca 21. stoletja za porečje Ledave glede na referenčno obdobje 1981–2010 za tri scenarije izpustov. Srednje črte za posamezen scenarij prikazujejo glajeno mediano modelskih projekcij, zgornji in spodnji rob pa največjo in najmanjšo vrednost
Figure 1: Mean air temperature change projections with possible ranges for the basin of Ledava until the end of the 21st century relative to 1981–2010 for three RCP scenarios by meteorological seasons. The middle lines for each scenario show the smoothed median of the model projections, and the upper and lower bounds show the maximum and minimum value

v okviru naravne spremenljivosti. Zanesljivost spremembe Tpov je visoka za vse scenarije in obdobja.

Na Sliki 1 je razvidna asimetričnost porazdelitve povprečne temperature, ki je najbolj izrazita pri scenariju RCP8.5. Številne raziskave so pokazale podobno asimetričnost tako za historične podatke, kot tudi v modelih bodočega podnebja. Povečanje dnevne oblačnosti tekom dneva v toplejšem podnebju je verjetno odgovorno za zmanjšanje dohodnega kratkovalovnega sevanja, kar vpliva na energijsko bilanco prizemne plasti zraka in s tem na večje razlike v spremembi minimalnih temperatur glede na maksimalne, asimetričnost sprememb pa pojasnjujejo tudi s spremenjenimi vzorci padavin ter vsebnostmi vode v tleh (Davy in sod., 2016; Doan in sod., 2022).

V primerjavi s temperaturami zraka so projekcije padavinskih razmer manj zanesljive. V prvem obdobju je za vse tri scenarije tako na letni skali kot tudi po sezonah stopnja zanesljivosti označena kot 'ni spremembe', kar pomeni, da so projekcije sprememb količine padavin majhne in statistično neznačilne, v okviru naravne spremenljivosti. V 2. in 3. obdobju se kaže porast letnih padavin (Slika 2) z visoko zanesljivostjo po RCP4.5 (6,9 % in 12,6 %) ter RCP8.5 (13,9 % in 10,4 %). Sezonske spremembe v 2. obdobju so opazne le po scenariju RCP8.5, za pomlad je projekcija povečanja količine padavin za okrog 10 %, za jesen za 15 % in za zimo za 28 %; za poletje pa ni statistično značilnih sprememb. Do konca stoletja projekcije kažejo le zanesljive spremembe v zimski količini padavin, ki naj bi se povečala za 21 % po RCP4.5 in za kar 46 % po scenariju RCP8.5.



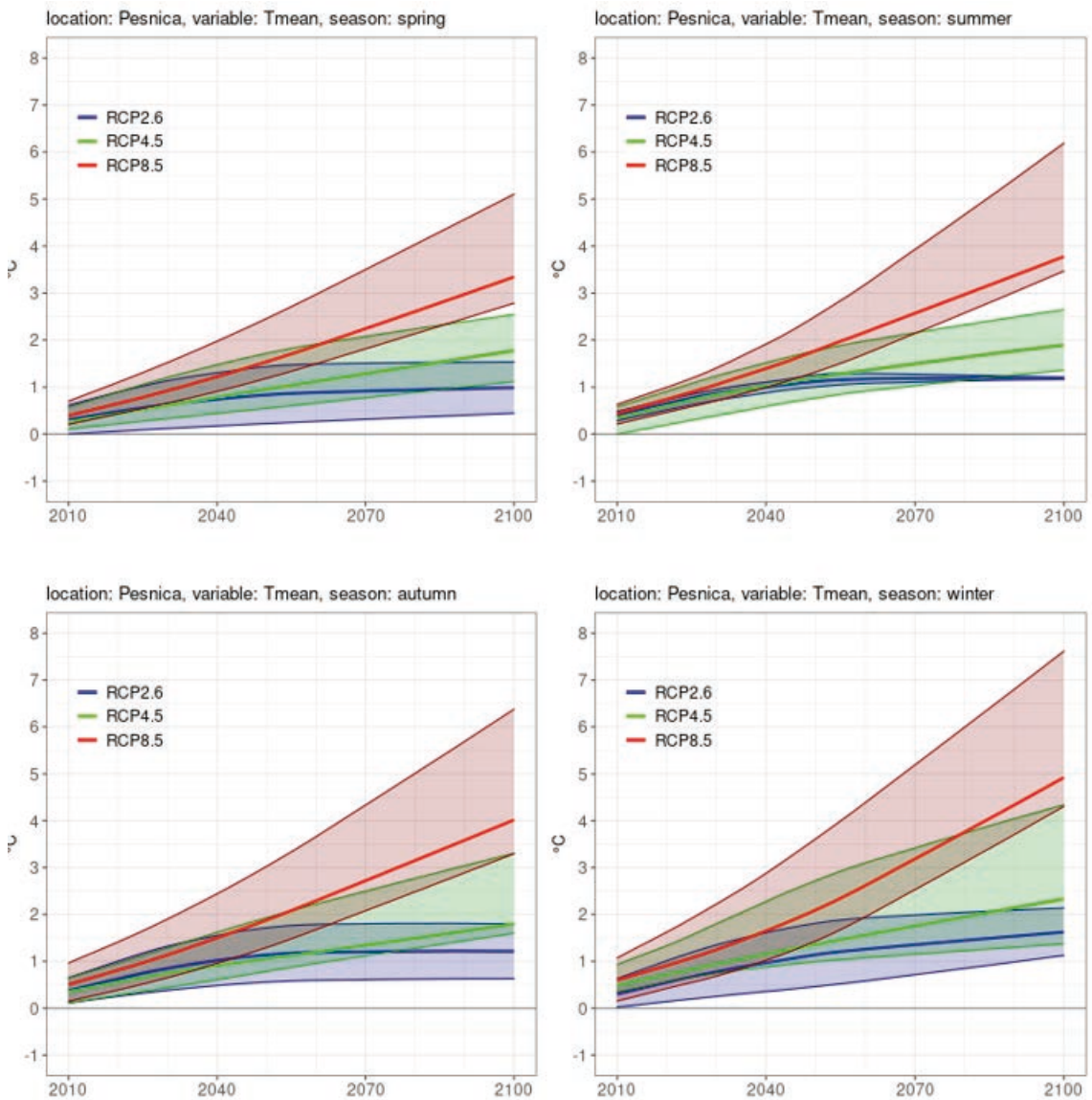
Slika 2: Levo: Letni časovni potek spremembe količine padavin (v %) z možnimi razponi do konca 21. stoletja za porečje Ledave glede na referenčno obdobje 1981–2010 za tri scenarije izpustov. Srednje črte za posamezen scenarij prikazujejo glajeno mediano modelskih projekcij, zgornji in spodnji rob pa največjo in najmanjšo vrednost; Desno: Povprečni razponi (minimalni, srednji, maksimalni) sprememb količine padavin po meteoroloških letnih časih in letno za tri scenarije. Vodoravna črta v stolpcu prikazuje mediano ansambla modelskih rezultatov, stolpec pa razpon vseh simulacij modelskega ansambla

Figure 2: Left: Annual precipitation change (%) projections with possible ranges for the basin of Ledava until the end of the 21st century relative to 1981–2010 for three RCP scenarios. The middle lines for each scenario show the smoothed median of the model projections, and the upper and lower bounds show the maximum and minimum value; Right: Average ranges (minimum, medium, maximum) of precipitation changes by meteorological seasons and annually for three scenarios. The horizontal line in the column shows the ensemble median, and the column shows the range of all simulations of the ensemble models

3.2 PODNEBNE PROJEKCIJE ZA POREČJE REKE PESNICE

Vsi trije podnebni scenariji do leta 2100 za porečje Pesnice predvidevajo naraščanje Tpov, in sicer RCP2.6 za 1,3 °C (razpon 0,7–1,9 °C), RCP4.5 za 1,7 °C (razpon 1,4–2,7 °C) in RCP8.5 za 3,3 °C (razpon 3,0–5,4 °C). V prvem scenariju Tpov sprva narašča, a se do konca 21. stoletja ustali, pri RCP 4.5 narašča tudi v tretjem obdobju, pri RCP8.5 pa izrazito narašča tudi v zadnjem obdobju. V prvem in drugem obdobju so temperaturna odstopanja od primerjalnega obdobja (1981–2010) med posameznimi scenariji še primerljiva, v zadnjem obdobju (2071-2100) pa se časovni poteki ločijo. Medtem ko projekcije po RCP2.6 in RCP4.5 za to obdobje kažejo dvig Tpov za 1,3 °C in 1,7 °C, se izrazitejša sprememba pričakuje po scenariju RCP8.5, ki kaže dvig Tpov za 3,3 °C, s širokim razponom od 3,0 °C do 5,4 °C.

Predvideno segrevanje se po letnih časih razlikuje (Slika 3). Do konca 21. stoletja naj bi se najbolj segrele

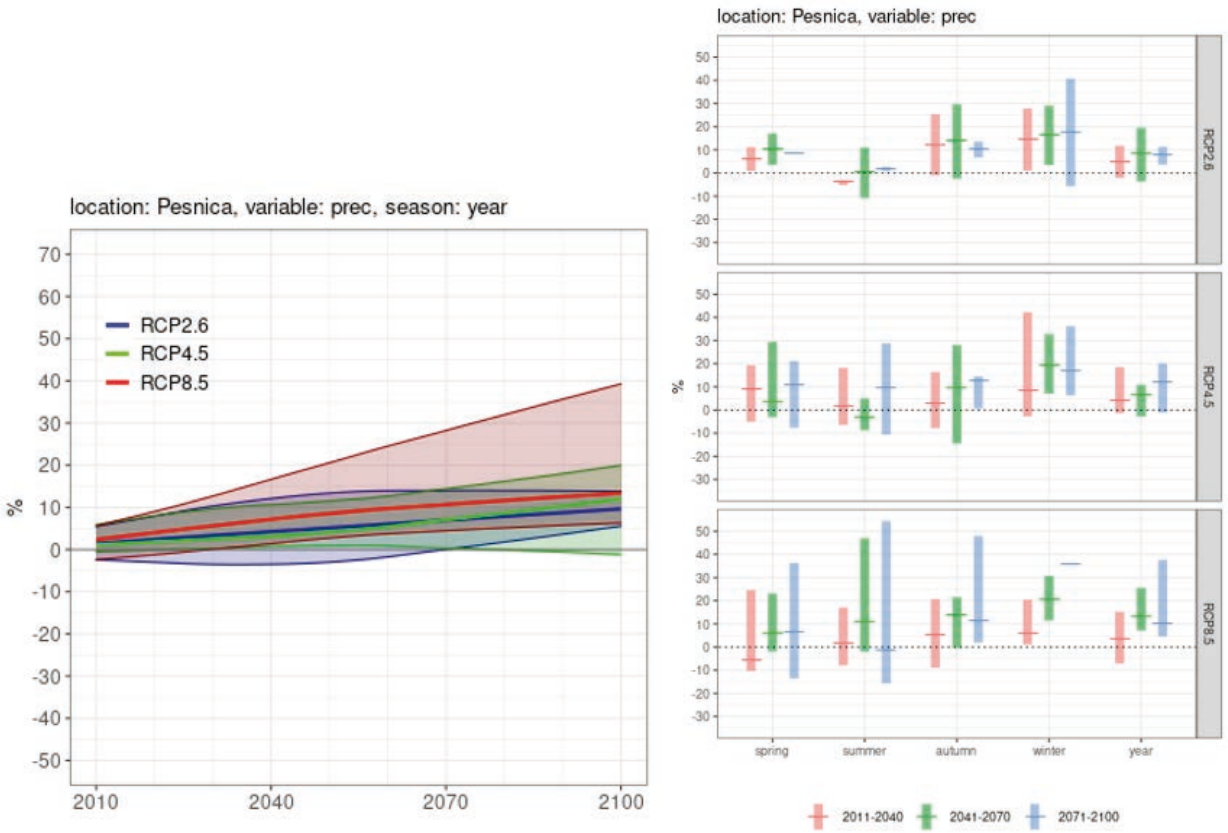


Slika 3: Časovni potek odklona Tpov po meteoroloških letnih časih z možnimi razponi do konca 21. stoletja za porečje Pesnice glede na referenčno obdobje 1981–2010 za tri scenarije izpustov. Srednje črte za posamezen scenarij prikazujejo glajeno mediano modelskih projekcij, zgornji in spodnji rob pa največjo in najmanjšo vrednost

Figure 3: Mean air temperature change projections with possible ranges for the basin of Pesnica until the end of the 21st century relative to 1981–2010 for three RCP scenarios by meteorological seasons. The middle lines for each scenario show the smoothed median of the model projections, and the upper and lower bounds show the maximum and minimum value

zime, projekcija po RCP2.6 kaže povečanje za 1,5 °C, po RCP 4.5 za 2,1 °C in po RCP8.5 kar za 4,1 °C. Odstopanje temperature pozimi bo ob koncu stoletja izrazito večje od letnega segrevanja, poletja pa naj bi se v povprečju segrela enako kot leto. Za pomlad kažejo projekcije nekoliko manjše segrevanje od letnega povprečja, od 1,0 °C po RCP2.6 do 2,8 °C po RCP8.5, jesenske temperature bi se naj zvišale od 1,1 °C po RCP2.6 do 3,5 °C po RCP 8.5. V prvem obdobju (2011–2040) znašajo projekcije dviga temperature po letnih časih v večini primerov od 0,8 °C do 1,0 °C, še največje segrevanje kaže jesen. V drugem obdobju se po RCP2.6 najbolj segrevata poletje in jesen (za 1,3 °C), po RCP4.5 poletje za 1,6 °C in zima za 1,4 °C, po RCP8.5 pa je povišanje največje za zimo in jesen (2,0 °C). Zanesljivost spremembe Tpov je visoka za vse scenarije in obdobja.

Projekcije tudi za Tmax kažejo z veliko zanesljivostjo, da se bodo do konca stoletja le-te višale, tako v letnem povprečju kot tudi po sezonah. Letne Tmax se bodo v prvem obdobju povišale za 0,8 °C, enako za vse tri scenarije, za drugo obdobje je pričakovano povišanje Tmax po RCP2.6 1,1 °C in po RCP8.5 1,6 °C. Večja razpršenost velja za tretje obdobje, ko je povišanje Tmax po RCP2.6 1,2 °C, največje odstopanje po RCP8.5 pa znaša 3,2 °C. Vse navedeno so mediane modelskih rezultatov. Najmanjše povišanje Tmax kažejo projekcije za pomlad, v prvem obdobju ne predvidevajo večjega odklona od 0,8 °C, do konca stoletja pa bi lahko bile Tmax od 1,5 °C (RCP4.5) do 2,7 °C (RCP8.5) višje. Tudi za poletje in jesen se bodo Tmax povišale, vzorec za obe sezoni je precej podoben, poleti je npr. po RCP2.6 sprememba 1,2 °C, po RCP4.5 1,7 °C, najbolj pesimističen scenarij pa kaže za

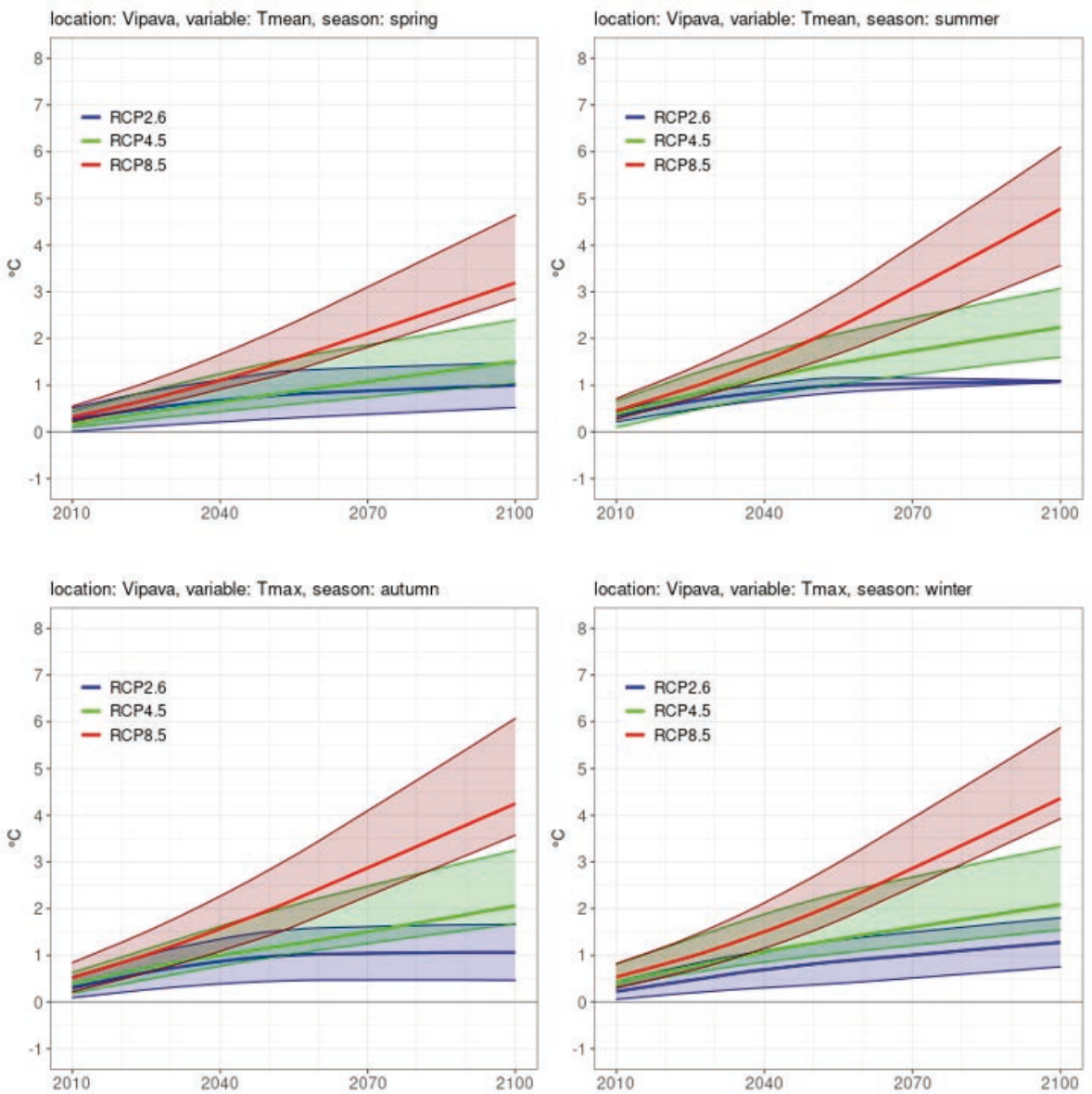


Slika 4: Levo: Letni časovni potek spremembe količine padavin (v %) z možnimi razponi do konca 21. stoletja za porečje Pesnice glede na referenčno obdobje 1981–2010 za tri scenarije izpustov. Srednje črte za posamezen scenarij prikazujejo glajeno mediano modelskih projekcij, zgornji in spodnji rob pa največjo in najmanjšo vrednost; Desno: Povprečni razponi (minimalni, srednji, maksimalni) sprememb količine padavin po meteoroloških letnih časih in letno za tri scenarije. Vodoravna črta v stolpcu prikazuje mediano ansambla modelskih rezultatov, stolpec pa razpon vseh simulacij modelskega ansambla

Figure 4: Left: Annual precipitation change (%) projections with possible ranges for the basin of Pesnica until the end of the 21st century relative to 1981–2010 for three RCP scenarios. The middle lines for each scenario show the smoothed median of the model projections, and the upper and lower bounds show the maximum and minimum value; Right: Average ranges (minimum, medium, maximum) of precipitation changes by meteorological seasons and annually for three scenarios. The horizontal line in the column shows the ensemble median, and the column shows the range of all simulations of the ensemble models

3,4 °C višje poletne Tmax. Povečanje števila ekstremno toplih dni se kaže tudi za zimo, do konca stoletja lahko pričakujemo tudi do 4,1 °C višje Tmax, če bi se uresničil scenarij RCP8.5, srednji scenarij RCP4.5 pa kaže 2,2 °C višje Tmax in najbolj optimističen scenarij povišanje za 1,5 °C. Zelo podobni kot za Tmax so tudi modelski rezultati za Tmin, stopnja zanesljivosti je visoka. V letnem povprečju se bodo v prvem obdobju Tmin povišale nekaj manj kot za 1 °C po vseh treh scenarijih (0,8 do 0,9 °C), do konca stoletja pa po RCP4.5 za 1,7 °C in po RCP8.5 za 3,4 °C. Pregled po sezonah pokaže, da bo sprememba Tmin najmanjša pomladi, od 1,1 °C po RCP2.6 do 2,8 °C po RCP8.5, izrazitejše povišanje pa kažejo projekcije za zimo, od 1,6 °C po RCP2.6 do 4,0 °C po RCP8.5. Jeseni in poleti so projekcije povišanja Tmin zelo podobne, od 1,2 °C po RCP2.6 do 3,4 °C po RCP8.5.

V prvem obdobju je za vse tri scenarije tako na letni skali kot tudi po sezonah stopnja zanesljivosti označena



Slika 5: Časovni potek odklona Tpov po meteoroloških letnih časih z možnimi razponi do konca 21. stoletja za porečje Vipave glede na referenčno obdobje 1981–2010 za tri scenarije izpustov. Srednje črte za posamezen scenarij prikazujejo glajeno mediano modelskih projekcij, zgornji in spodnji rob pa največjo in najmanjšo vrednost

Figure 5: Mean air temperature change projections with possible ranges for the basin of Vipava until the end of the 21st century relative to 1981–2010 for three RCP scenarios by meteorological seasons. The middle lines for each scenario show the smoothed median of the model projections, and the upper and lower bounds show the maximum and minimum value

kot 'ni spremembe', kar pomeni, da so projekcije sprememb količine padavin majhne in statistično neznačilne, v okviru naravne spremenljivosti. V 2. obdobju se kaže porast letnih padavin z visoko zanesljivostjo po RCP8.5 (12,8 %), v 3. obdobju pa prav tako z visoko zanesljivostjo porast padavin po RCP4.5 (12,1 %) in RCP8.5 (10 %). Sezonske spremembe v 2. obdobju so opazne le po scenariju RCP8.5, za jesen je projekcija povečanja količine padavin za okrog 14 %, za zimo 20 %, za poletje in pomlad pa ni statistično značilnih sprememb (Slika 4). Do konca stoletja projekcije kažejo le zanesljive spremembe v zimski količini padavin, ki bi se naj povečala po RCP4.5 scenariju za okrog 17 % in po RCP8.5 za 35,5 %, scenarij RCP4.5 pa za to obdobje z veliko zanesljivostjo kaže tudi, da bi se naj za okrog 10 % povečale poletne padavine.

3.3 PODNEBNE PROJEKCIJE ZA POREČJE REKE VIPAVE

Vsi trije scenariji do leta 2100 za porečje Vipave predvidevajo naraščanje Tpov, in sicer RCP2.6 za 1,2 °C (razpon 0,6–1,7 °C), RCP4.5 za 1,7 °C (razpon 1,4–2,5 °C) in RCP8.5 za 3,5 °C (razpon 2,9–4,9 °C). V prvem scenariju Tpov sprva narašča, a se do konca 21. stoletja nekoliko ustali, pri RCP 4.5 narašča tudi v zadnjem obdobju, pri RCP8.5 pa izrazito narašča tudi v zadnjem obdobju. V prvem in drugem obdobju so temperaturna odstopanja od primerjalnega obdobja (1981–2010) med posameznimi scenariji primerljiva, v zadnjem obdobju (2071–2100) se časovni poteki ločijo, še posebej pri scenariju RCP8.5. Medtem ko projekcije po RCP2.6 in RCP4.5 za to obdobje kažejo dvig Tpov za 1,2 °C in 1,7 °C, se izrazitejša sprememba pričakuje po scenariju RCP8.5, ki kaže dvig Tpov za 3,5 °C, z razponom od 2,9 °C do 4,9 °C.

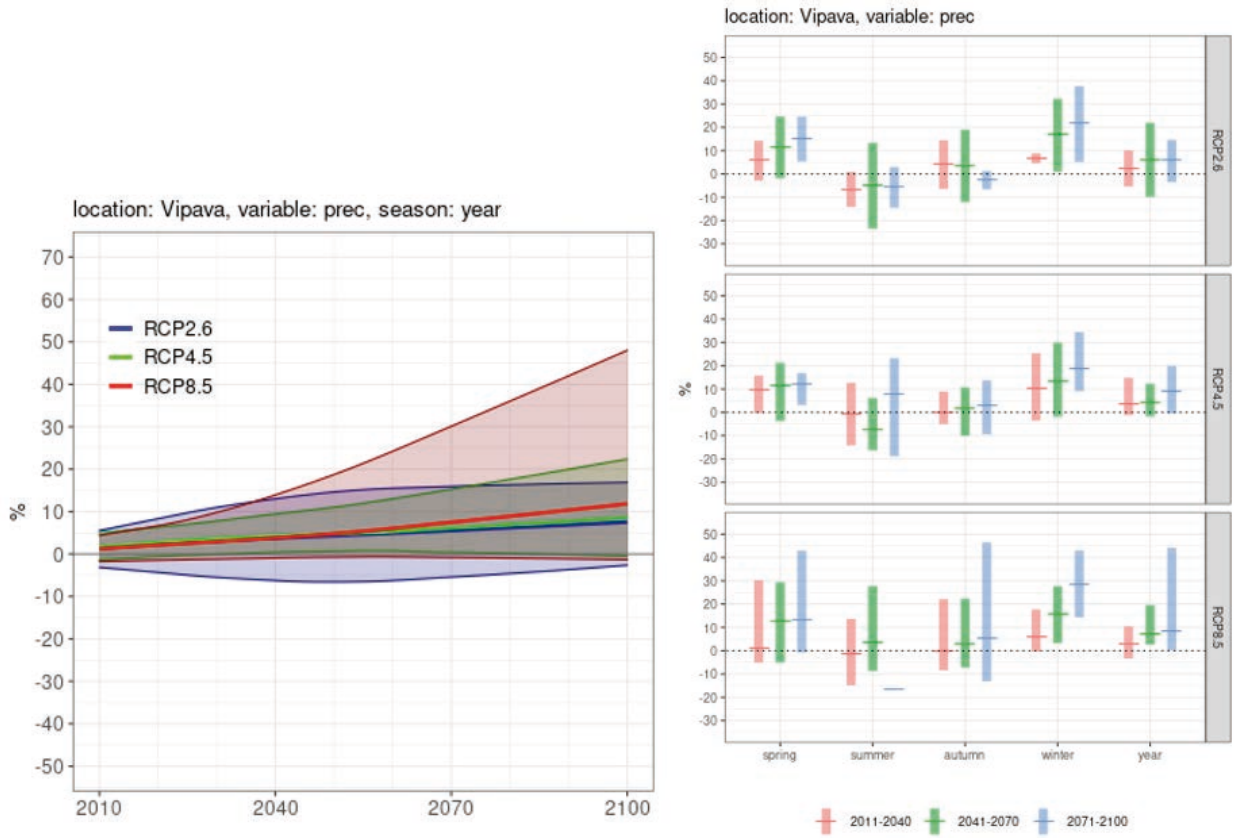
Do konca 21. stoletja naj bi se najbolj segrele zime, projekcija po RCP2.6 kaže povečanje za 1,3 °C, po RCP4.5 za 1,9 °C in po RCP8.5 za 3,7 °C (Slika 5). Odstopanje temperature pozimi bo ob koncu stoletja zelo podobno letnemu segrevanju, enako kažejo scenariji tudi za jesen. Poletja naj bi se v povprečju segrela podobno kot leto v povprečju po RCP2.6 scenariju, po RCP4.5 in RCP8.5 kažejo projekcije ob koncu stoletja 1,9 °C oziroma 3,8 °C višje poletne temperature. Za pomlad kažejo projekcije nekoliko manjše segrevanje od letnega povprečja, od 1,0 °C po RCP2.6 do 2,7 °C po RCP8.5. V prvem obdobju (2011–2040) znašajo projekcije dviga temperature za pomlad od 0,4 °C do 0,7 °C, za zimo od 0,6 °C do 0,8 °C, za poletje od 0,8 °C do 0,9 °C, še največje segrevanje, za 0,8 °C do 1,0 °C, kažejo projekcije za jesen. V drugem obdobju se po RCP2.6 najbolj segrevata poletje (za 1,2 °C) in zima (za 1,3 °C), po RCP4.5 poletje in zima enako, za 1,9 °C, prav tako po RCP8.5 projekcije kažejo največje spremembe za poletje (3,8 °C) in zimo (3,7 °C). Zanesljivost temperaturnih sprememb je visoka za vse scenarije in obdobja.

Projekcije tudi za Tmax kažejo z veliko zanesljivostjo, da se bodo do konca stoletja le-te višale, tako v letnem povprečju kot tudi po sezonah. Letne Tmax se bodo v prvem obdobju povišale za 0,7 °C po RCP2.6 scenariju in 0,8 °C po ostalih dveh scenarijih. Za drugo obdobje je pričakovano povišanje Tmax po RCP2.6 0,9 °C in po RCP8.5 1,9 °C. Večja razpršenost velja za tretje obdobje, ko projekcije Tmax po RCP2.6 kažejo povišanje za 1,1 °C, največje odstopanje po RCP8.5 pa znaša 3,6 °C. Vse navedeno so mediane modelskih rezultatov. Najmanjše povišanje Tmax kažejo projekcije za pomlad, v prvem obdobju ne predvidevajo večjega odklona od 0,6 °C, do konca stoletja pa bi lahko bile Tmax od 0,9 °C (RCP4.5) do 2,6 °C (RCP8.5) višje. Tudi za poletje in jesen se bodo Tmax povišale, vzorec za obe sezoni je precej podoben, poleti je npr. do konca stoletja po RCP2.6 sprememba 1,0 °C (jesen 1,1 °C), po RCP4.5 1,8 °C (jesen enako), najbolj pesimističen scenarij pa kaže kar za 4,0 °C višje poletne Tmax (za jesen 3,7 °C). Povečanje števila ekstremno toplih dni se kaže tudi za zimo, do konca stoletja lahko pričakujemo tudi do 3,7 °C višje Tmax, če bi se uresničil scenarij RCP8.5, srednji scenarij RCP4.5 pa kaže 1,9 °C višje Tmax in najbolj optimističen scenarij povišanje za 1,2 °C. V letnem povprečju projekcije v prvem obdobju kažejo povišanje Tmin manj kot za 1 °C po vseh treh scenarijih (0,7 do 0,8 °C), do konca stoletja pa po RCP2.6 za 1,2 °C, po RCP4.5 za 1,7 °C in po RCP8.5 za 3,5 °C. Pregled po sezonah pokaže, da bo sprememba Tmin najmanjša pomladi, od 1,0 °C po RCP2.6 do 2,7 °C po RCP8.5. Po scenariju RCP8.5 kažejo projekcije do konca stoletja zvišanje Tmin za poletje za 3,8 °C, nekoliko manj za jesen (3,6 °C) in zimo (3,5 °C). Ostala dva scenarija kažeta večje spremembe za zimo, projekcije po RCP2.6 kažejo do konca stoletja 1,3 °C višje in po RCP4.5 1,9 °C višje zimske Tmin.

Za padavine je v prvem obdobju za vse tri scenarije tako na letni skali kot tudi po sezonah stopnja zanesljivosti označena kot 'ni spremembe', kar pomeni, da so projekcije sprememb količine padavin majhne in statistično neznačilne, v okviru naravne spremenljivosti. Do konca stoletja projekcije kažejo z visoko zanesljivostjo porast letnih količin padavin po RCP8.5 (8,5 %) in po RCP4.5 (9,1 %).

Sezonske spremembe so opazne le po scenariju RCP8.5, za pomlad je v 2. obdobju projekcija povečanja količine padavin za okrog 13 %, za zimo v 3. obdobju 29 %, za poletje in jesen pa projekcije ne pokažejo statistično značilnih sprememb (Slika 6).

Med porečjema Ledave in Pesnice pri projekci-



Slika 6: Levo: Letni časovni potek spremembe količine padavin (v %) z možnimi razponi do konca 21. stoletja za porečje Vipave glede na referenčno obdobje 1981–2010 za tri scenarije izpustov. Srednje črte za posamezen scenarij prikazujejo glajeno mediano modelskih projekcij, zgornji in spodnji rob pa največjo in najmanjšo vrednost; Desno: Povprečni razponi (minimalni, srednji, maksimalni) sprememb količine padavin po meteoroloških letnih časih in letno za tri scenarije. Vodoravna črta v stolpcu prikazuje mediano ansambla modelskih rezultatov, stolpec pa razpon vseh simulacij modelskega ansambla

Figure 6: Left: Annual precipitation change (%) projections with possible ranges for the basin of Vipava until the end of the 21st century relative to 1981–2010 for three RCP scenarios. The middle lines for each scenario show the smoothed median of the model projections, and the upper and lower bounds show the maximum and minimum value; Right: Average ranges (minimum, medium, maximum) of precipitation changes by meteorological seasons and annually for three scenarios. The horizontal line in the column shows the ensemble median, and the column shows the range of all simulations of the ensemble models

jah sprememb povprečnih letnih temperatur ob koncu stoletja ni opaznih razlik, minimalne so le pri scenariju RCP8.5. Projekcije spremembe padavin na letni ravni kažejo nekoliko manjše spremembe v količini padavin za porečje Pesnice glede na porečje Ledave. Razlike niso statistično značilne, za scenarij RCP2.6 pa so spremembe majhne in v okviru naravne spremenljivosti. Slovenija je tako kot velik del Evrope v prihodnosti v prehodnem območju med bolj vlažnim severnim in bolj suhim južnim podnebjem, zato se podnebni modeli pogosto ne ujemajo glede znaka spremembe, zanesljivost projekcij pa je majhna (Sperna Weiland in sod., 2021). Nekoliko opaznejše so razlike projekcij sprememb temperature za porečje reke Vipave glede na ostali dve obravnavani porečji. Pri scenariju RCP8.5 so spremembe tako za Tpov kot tudi Tmin in Tmax v porečju Vipave nekoliko večje, po tem scenariju je sprememba Tmax ob koncu stoletja za 0,4 °C večja kot v porečjih Ledave in Pesnice. Tudi dosedanje raziskave so že pokazale, da so bile najizrazitejše spremembe podnebja v zadnjih desetletjih v poletnem času v delu južne in jugozahodne Slovenije (Bertalanič in sod., 2018). Količina padavin se bo po vseh treh scenarijih povečala na letni ravni v porečju Vipave nekoliko manj kot v porečjih Ledave in Pesnice, razlike so zelo majhne, spet pa velja, da so projekcije sprememb količine padavin glede na temperature manj zanesljive. Primerjava za vsa tri porečja kaže, da bi nekoliko višje spremembe temperature in manjše povečanje količine padavin v porečju Vipave lahko imele večji negativni vpliv na kmetijstvo v primerjavi z ostalima dvema porečjema.

Po usklajenosti podnebnih modelov in s tem zanesljivosti se izrazito ločijo projekcije temperature zraka in

Preglednica 2: Primerjava odklonov (mediana, v oklepajih razponi) letnih povprečnih (Tpov), najnižjih (Tmin), najvišjih (Tmax) temperatur zraka ter letnih količin padavin za obdobje 2071–2100 po porečjih
Table 2: Comparison of deviations (median, ranges in parentheses) of annual average (Tpov), minimum (Tmin), maximum (Tmax) air temperatures and annual precipitation amounts for the period 2071–2100 by the river basins

Spremenljivka/scenarij	porečje Ledave	porečje Pesnice	porečje Vipave
Tpov			
RCP2.6	1,3 °C (0,7–1,9)	1,3 °C (0,7–1,9)	1,2 °C (0,6–1,7)
RCP4.5	1,7 °C (1,4–2,7)	1,7 °C (1,4–2,7)	1,7 °C (1,4–2,5)
RCP8.5	3,3 °C (3,0–5,3)	3,3 °C (3,0–5,4)	3,5 °C (2,9–4,9)
Tmin			
RCP2.6	1,3 °C (0,7–1,9)	1,3 °C (0,7–1,9)	1,2 °C (0,6–1,7)
RCP4.5	1,7 °C (1,4–2,8)	1,7 °C (1,4–2,8)	1,7 °C (1,4–2,5)
RCP8.5	3,4 °C (3,1–5,4)	3,4 °C (3,1–5,5)	3,5 °C (2,9–4,9)
Tmax			
RCP2.6	1,2 °C (0,6–1,7)	1,2 °C (0,6–1,7)	1,1 °C (0,6–1,5)
RCP4.5	1,7 °C (1,4–2,6)	1,7 °C (1,3–2,6)	1,8 °C (1,4–2,4)
RCP8.5	3,2 °C (2,9–5,2)	3,2 °C (3,0–5,3)	3,6 °C (2,9–4,8)
Padavine			
RCP2.6	9,0 % (4,9–13,1)[1]	7,4 % (3,6–11,3)[1]	5,6 % (-3,5–14,6)[1]
RCP4.5	12,6 % (-1,9–21,6)	12,1 % (-1,1–20,1)	9,1 % (-0,4–19,9)
RCP8.5	10,4 % (4,6–45,0)	10,0 % (4,6–37,7)	8,5 % (0,2–44,2)

[1] stopnja zanesljivosti 'ni spremembe': spremembe so majhne in v okviru naravne spremenljivosti, za vse ostale projekcije je stopnja zanesljivosti visoka

padavin. Projekcije temperatur zraka so zelo zanesljive, modeli med seboj kažejo dobro ujemanje. Kot pri dosedanjih trendih temperature zraka v Sloveniji (Dolinar in sod., 2018; Berkely Earth, 2023), ki močno presegajo globalni trend (IPCC, 2023), velja enako tudi za projekcije za prihodnost. Pri padavinah dosedanji trendi v Sloveniji niso statistično značilni (ARSO, 2021), skladno z lego Slovenije na prehodnem območju med severnim in južnim delom Evrope (ICPDR, 2019). Tako je tudi veliko projekcij padavin nezanesljivih ali ne kažejo spremembe, prav tako v skladu s projekcijami za Evropo (Probst in Mauser, 2023; Sperna Weiland in sod., 2021).

4 SKLEPI

Analiza ansambla modelskih simulacij za RCP4.5 in RCP8.5 kaže zelo podobne rezultate za porečje rek Ledave, Pesnice in Vipave, in sicer naraščanje temperature (največ pozimi, najmanj spomladi) in naraščanje letne količine padavin zaradi dviga pozimi. Podobno lahko vidimo pri projekcijah za celotno Slovenijo ali posamezne regije, vendar pa za uporabo v različnih modelih potrebujemo specifične projekcije na manjši prostorski skali, kot so predstavljene za povodja. Pri projekcijah podnebnih sprememb je vedno prisotna negotovost, ki jo moramo upoštevati pri razlagi rezultatov. Nikoli ne govorimo o posamezni vrednosti spremembe, temveč o razponu, ki ga nakazuje nabor različnih podnebnih modelov, poleg tega uporabljamo oznake zanesljivosti. Previdni smo, da absolutne vrednosti za prihodnost uporabljamo le, ko imajo projekcije narejene popravke napak, v nasprotnem primeru pa govorimo o relativnih spremembah. Pomembno je, da se strokovna in splošna javnost zaveda, da je uporaba projekcij podnebnih sprememb s strokovno razlago nujna pri določanju ranljivosti nekega območja in grajenju odpornosti z uvajanjem ukrepov prilagajanja na podnebne spremembe ter da so projekcije v veliki meri na voljo na ARSO, mogoče pa je pripraviti tudi bolj specifične rezultate. Zaenkrat se za akcijske načrte prilagajanja na podnebne spremembe odločajo posamezne občine, slediti pa jim bodo morale vse ostale in Slovenija s celostno usmerjenostjo v odpornejšo družbo.

5 ZAHVALA

Raziskava je bila finančno podprta s strani ARRS,

javne agencije za raziskovalno dejavnost Republike Slovenije s pogodbo L4-2625. Zahvaljujemo se doc. dr. Matjažu Glavanu in prof. dr. Marini Pintar za vodenje projekta.

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doi:10.14720/aas.2024.120.2.17017 Original research article / izvirni znanstveni članek

Gas chromatography-tandem mass spectrometry multiresidual method for determination of pesticide residues in honey

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Received December 14, 2023; accepted April 19, 2024.
Delo je prispelo 12. decembra 2023, sprejeto 19. aprila 2024.

Gas chromatography-tandem mass spectrometry multiresidual method for determination of pesticide residues in honey

Abstract: In our laboratory we introduced and validated a new analytical method for determination of environmental pesticide residues in honey. The extraction was conducted using acetone, petroleum ether and dichlorometane. The determination was conducted using gas chromatography coupled with tandem mass spectrometry. Practical usage of method was analyses of 31 samples of Slovenian honey. 33 active substances (pesticides) were sought. The insecticide cypermethrin was the only active substance found in three samples. The active substances sought were not found in 90.3 % of the samples analysed. The risk assessment showed that no unacceptable risk is expected for consumers. The results were compared with those from the literature. We revealed that honey from Slovenia contained a lower portion of positive samples per active substance sought as in Italy, comparable as in Estonia and Spain, comparable to higher as in Poland and higher as in Egypt.

Key words: honey, GC-MS/MS, pesticide residues, multiresidual method

Multirezidualna metoda za določanje ostankov fitofarmacevtskih sredstev v medu s plinsko kromatografijo sklopljeno s tandemsko masno spektrometrijo

Izvleček: V našem laboratoriju smo uvedli in validirali novo analizno metodo za določanje ostankov fitofarmacevtskih sredstev iz okolja v medu. Ekstrakcijo smo izvedli z acetonom, petroletrom in diklorometanom, določitev pa s plinsko kromatografijo sklopljeno s tandemsko masno spektrometrijo. Praktična uporaba metode je bila analiza 31 vzorcev slovenskega medu. Določali smo 33 aktivnih spojin (pesticidov). Edina najdena aktivna snov je bil insekticid cipermetrin v treh vzorcih. Iskanih aktivnih snovi nismo določili v 90,3 % analiziranih vzorcev. Ocena tveganja je pokazala, da ni pričakovati nesprejemljivega tveganja za potrošnika. Rezultate smo primerjali z literaturnimi podatki. Odkrili smo, da je slovenski med vseboval manjši delež pozitivnih vzorcev na aktivno snov kot v Italiji, primerljiv kot v Estoniji in Španiji, primerljiv do večji kot na Poljskem in večji kot v Egiptu.

Ključne besede: med, GC-MS/MS, ostanki fitofarmacevtskih sredstev, multirezidualna metoda

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1 INTRODUCTION

Honey is produced from nectar collected by bees, which gets broken down into simple sugars stored inside the honeycomb. Therefore, honey is mainly composed of carbohydrates (approx. 80 %): glucose, fructose, sucrose and maltose, and water (approx. 20 %). It also contains minor compounds such as vitamins, minerals, amino acids, proteins and aroma compounds (Geană at al., 2020, Kahraman et al., 2010). Nutritional properties and therapeutic applications of honey are reason for its frequent use.

Honey bees can fly within a radius of 4.8 km in all directions from their apiary (Eckert, 1933). On their way they can come into contact with pesticide residues when they collect nectar and pollen on plants treated with plant protection products (PPPs) (Colin et al., 2004) and/or on the ground, in water, in the air, on melliferous in-field weeds and off-field plants where PPPs were carried by the drift after treatment (Bonmatin at al., 2015, Krupke et al., 2012, SANTE, 2023, Ward et al., 2022). Bees carry pesticide residues into the hive, from where they eventually end up in honey (Zhou et al., 2018).

Technical guidelines for determining the magnitude of pesticide residues in honey and setting Maximum Residue Levels in honey (SANTE/11956/2016 rev. 9) entered into force on 1 January 2020. With the introduction of this guideline, during PPPs authorisation of uses on plants with melliferous capacity, experiments are required to determine residues in honey. Therefore, monitoring of PPP residues in honey is recommended.

For extraction procedures of analytical methods for determination of PPP residues in honey nowadays mainly use modified Quick Easy Cheap Effective Rugged and Safe method also called QuEChERS method, where acetonitrile is used (Gawel et al., 2019, Karise et al., 2017, Shendy et al., 2016). In some laboratories extraction is performed with ethyl acetate (Panseri et al., 2014) or the mixture of ethyl acetate and cyclohexane (Brugnerotto et al., 2023). In our laboratory a mixture of acetone, dichloromethane and petroleum ether was used, to achieve the extraction of very polar (for instance, flonicamid) to non-polar (for instance, cyhalothrin-lambda) pesticides at the same time (Baša Česnik et al., 2019). Besides, when extracting materials containing high amount of sugar with acetone, no double layered extract is obtained like with acetonitrile (Luke et al., 1975).

Determination of pesticide residues is nowadays usually performed using gas chromatography coupled with mass spectrometry (GC-MS) (Brugnerotto et al., 2023, Karise et al., 2017, Mukiibi et al., 2021), gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) (Gawel et al., 2019, Lazarus et al., 2021, Panseri et al., 2014, Shendy et al., 2016, Sun et al., 2022) and/or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Gawel et al., 2019, Karise et al., 2017, Liu et al., 2022). The most sensitive is tandem mass spectrometry, which was also used by our laboratory.

Numerous authors have analysed pesticide residues in honey with GC-MS/MS. Gawel et al. (2019) analysed 53 active substances in honey from Poland. Panseri et al. (2014) tested honey samples from Italy for 28 active substances. Shendy et al. (2016) introduced a method for determining 200 active substances in honey samples from Egypt. Wang et al. (2022) used a method for determining 203 active substances in China honey. In our study up to 24 of active substances sought in literature studies were introduced. 97.0 % of active substances selected in this paper are authorised for use in Slovenia. The rest were authorised in previous years. Of those selected, 57.6 % were fungicides, 21.2 % were acaricides and/or insecticides and 21.2 % were herbicides.

Our paper is presenting a new GC-MS/MS multiresidual method for determination of 33 active substances (pesticides) in honey. The old extraction procedure using acetone, dichlorometane and petroleum ether was used, but new active substances were introduced and validated with the new, more sensitive instrument. Method was used in practice. 31 honey samples, collected from Slovenian beekeepers, were analysed. Results were compared with literature data and consumer risk assessment was calculated.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

The certified pesticide standards were obtained from Dr. Ehrenstorfer (Augsburg, Germany). For extraction procedure acetone - p.a. grade, dichlorometane – p.a. grade and petroleum ether – p.a. grade, were obtained from J.T.Baker (Deventer, Netherlands). Also acetone HPLC-grade, which was used for preparation of standards, was obtained from J.T.Baker (Deventer, Netherlands). All other chemicals used were supplied by Sigma-Aldrich (Steinheim, Germany). The water used was MilliQ deionised water.

2.1.2 Preparation of the solutions

Stock solutions of individual active substances were

prepared in acetone. Concentration of each active substance was 625 μg ml^{-1}. From 33 stock solutions, three mixed solutions of all 33 active substances were prepared with a concentration of 5 μg ml^{-1}, 1 μg ml^{-1} and 0.1 μg ml^{-1}.

2.2 EXTRACTION PROCEDURE

Extraction procedure was conducted with acetone, petroleum ether and dichlorometane. We used the same extraction procedure as the one for determination of chlorfenvinphos, coumaphos and thymol, described by Baša Česnik at al. (2019). The only difference was that the final dry extract was dissolved in acetone HPLC-grade.

2.3 DETERMINATION

The samples were analysed using a gas chromatograph (Agilent Technologies 8890, Shanghai, China) coupled with tandem mass spectrometer (Agilent Technologies 7010B, Santa Clara, USA), equipped with a Gerstel 20PRE0795 multipurpose sampler (Gerstel, Sursee, Switzerland) and a HP-5 MS UI column (Agilent Technologies, 30 m, 0.25 mm i. d., 0.25 μm film thickness) with a constant flow of helium at 1.2 ml min^{-1}. The GC oven was programmed as follows: 55 °C for 2 min, from 55 °C to 100 °C at 20 °C min^{-1}, from 100 °C to 280 °C at 4 °C min^{-1}, held at 280 °C for 19.75 min. The temperature of the ion source was 230 °C, the auxiliary temperature was 280 °C and the quadrupoles temperature was 150 °C. For qualitative and quantitative determination, the MRM transitions were used presented in Table 1. For each active substance two to four transitions were scaned. For calibration matrix match standards were used.

2.4 VALIDATION OF METHODS

2.4.1 LOQ and linearity

The linearity was tested with matrix match standards. F test was used to check linearity and determine linearity range. Each calibration curve had three to seven concentration levels with two repetitions at each level.

Estimation of LOQs was conducted using matrix match standards. S/N ratio had to be at least 10.

2.4.2 Precision

Blank honey was purchased in store. It was analysed on presence of pesticide residues sought. After proving that it does not contain pesticides of our choice, it was spiked in two parallel samples at LOQ within the period of 10 days. For the determination of precision (ISO 5725), i.e. repeatability and reproducibility, the standard deviation of the repeatability of the level and the standard deviation of reproducibility of the level were both calculated from results obtained.

2.4.3 Uncertainty of repeatability and uncertainty of reproducibility

The uncertainty of repeatability and the uncertainty of reproducibility were calculated by multiplying the standard deviation of repeatability and the standard deviation of reproducibility by the Student's t factor, for nine degrees of freedom and a 95 % confidence level ($t_{95;9}$ = 2.262).

$$U_r = t_{95;9} \times s_r;\ U_R = t_{95;9} \times s_R$$

The measurement uncertainty for PPP residues should be 50 %, as proposed in SANTE/11312/2021. The method is fit for purpose when during validation it is proven that measurement uncertainty is ≤ 50 %.

2.4.4 Accuracy

The accuracy was verified by checking the recoveries. We used recoveries obtained during test for precision. 20 results for each active substance (pesticide) were averaged and RSD was calculated. According to the requirements for method validation procedures (SANTE/11312/2021), acceptable mean recoveries are those within the range of 70 % to 120 %, with an associated repeatability of RSDr ≤ 20 %.

The guidelines for single-laboratory validation (Alder et al. 2000) require mean recoveries at level > 0.001 mg kg^{-1} and ≤ 0.01 mg kg^{-1} from 60 % to 120 %, with an associated repeatability RSDr ≤ 30 %.

2.5 CONSUMER RISK ASSESSMENT

Long-term exposure was calculated using the EFSA PRIMo model revision 3.1. Chronic consumer exposure was expressed in % of the Acceptable Daily Intake (ADI). The acceptable limit for long-term exposure is 100 % of the ADI.

Short-term exposure was calculated using the EFSA PRIMo model revision 3.1. Acute consumer exposure

Table 1: Active substances sought, their activity type, MRM transitions, dwell time and collision energy

Active substance	Activity type[a]	MRM transitions (Q1, Q2, Q3)[b]	Dwell (ms)	CE (V)[c]
8-hydroxyquinoline	F	145->117.1, 145->89, 117->90	77.5	10, 40, 10
benthiavalicarb-isopropyl	F	181->180, 181->126.9, 181->83.1	20.3, 17.6	20, 40, 40
boscalid	F	140->112, 140->76	45.7	10, 30
clomazone	H	204->107, 125->99	87.2	20, 20
cypermethrin	A, I	181->152.1, 181->126.9, 181->76.9	24.2, 19.7, 19.1, 22.1	30, 40, 40
cyprodinil	F	225->223.7, 224->208.1	17.3	20, 20
deltamethrin	I	253->171.9, 253->93.1, 253->77	26.9	10, 20, 40
fenhexamid	F	301->176.9, 301->97, 301->54.8	13.5	10, 10, 40
flonicamid	I	174->146, 174->126, 174->69	77.6	10, 20, 40
fluazifop-p-butyl	H	383->282.1, 254->146	8.2	10, 20
fludioxonil	F	248->182.1, 248->154.1, 248->127.1	9.7	10, 20, 30
flufenacet	H	151->136.1, 151->95.1	30.2	10, 30
fluopicolide	F	347->172, 209->182, 173->145	14.5	30, 20, 10
fluopyram	F	173->145, 173->95.1	15.3	20, 30
flutolanil	F	172.8->145, 172.8->95, 172.8->75	12.6	15, 35, 55
iprovalicarb	F	158->98, 158->72.1, 158->55.1	8.6, 8.1	10, 10, 20
kresoxim-methyl	F	206->131.1, 206->116.1	12.7	10, 10
lambda-cyhalothrin	I	181->152.1, 181->127.1, 181->77.1	18.6	20, 30, 40
metazachlor	H	209->132.1, 209->117.1, 133->131.7	14	20, 40, 20
myclobutanil	F	179->125, 179->90, 179->63	8.6	10, 40, 40
napropamide	H	271->72, 128->100.1, 128->72.1	17.7	20, 10, 10
penconazole	F	248->206.1, 248->192.1, 248->157.1	12.7	10, 10, 30
pendimethalin	H	252->191.1, 252->162.1, 252->106.1	12.2	10, 10, 40
pirimicarb	I	238->166.1, 166->96.1	33.4	10, 10
proquinazid	F	288->245, 288->217, 272->216	13.5	10, 30, 20
prosulfocarb	H	251->128.1, 162->91.1, 162->65	32.5	10, 10, 40
pyraclostrobin	F	164->132.1, 164->104, 132->104	34.1	10, 30, 10
pyrimethanil	F	198->183.1, 198->118	63.4	20, 40
tebuconazole	F	250->153, 250->125, 250->70	10.2	10, 30, 10
tebufenpyrad	A	335->319.9, 333->318.2, 333->276.1	21.3	10, 10, 10
tefluthrin	I	177->137, 177->127, 177->87.1	36.6	20, 20, 40
tetraconazole	F	336->218.1, 336->164	24.7	20, 30
trifloxystrobin	F	222->162.1, 222->130, 131->116	11.1	10, 10, 20

[a] A = acaricide, I = insecticide, F = fungicide, H = herbicide
[b] Q = qualifier ion, bold qualifier was used for integration
[c] CE = collision energy

was expressed in % of the Acute Reference Dose (ARfD). The acceptable limit for short-term exposure is 100 % of the ARfD.

2.6 SAMPLING

31 honey samples were collected from Slovenian beekeepers from 11 statistical regions in Slovenia in 2023. The sampling distribution is presented in Table 2.

3 RESULTS AND DISCUSSION

3.1 VALIDATION OF METHOD

3.1.1 LOQ and linearity

The linear model is valid for all active substances presented in Table 3. Linearity was proven in the range of 0.005 mg kg^{-1} to 0.02 mg kg^{-1} for pendimethalin, in the range of 0.005 mg kg^{-1} to 0.04 mg kg^{-1} for 8-hydroxyquinoline and prosulfocarb, in the range of 0.005 mg kg^{-1} to 0.05 mg kg^{-1} for flonicamid and in the range of 0.005 mg kg^{-1} to 0.03 mg kg^{-1} for all other active substances. R^2 ranged from 0.987 to 1.000. Results are presented in Table 3.

3.1.2 Accuracy

The recoveries at LOQs for the active substances scanned with GC-MS/MS are in the range of 92.8 % to 98.9 %, with RSDs of 6.0 % to 11.3 %. The results are presented in Table 3.

All recoveries and RSDs are within the required ranges from the literature (Alder et al., 2000; SANTE/11813/2017).

3.1.3 Uncertainty of repeatability and uncertainty of reproducibility

The uncertainty of repeatability and uncertainty of reproducibility were determined at concentrations equal to the LOQs. Uncertainty of repeatability ranged from 0.0004 mg kg^{-1} to 0.0009 mg kg^{-1}, which is 7.6 % to 18.3 % of LOQ. Uncertainty of reproducibility ranged from 0.0007 mg kg^{-1} to 0.0013 mg kg^{-1}, which is 13.3 % to 25.2 % of LOQ. The results are presented in Table 3.

Table 2: Sampling distribution according to statistical regions of Slovenian honey samples collected in 2023

	No of samples		
Region	Pouring in 2022	Pouring in 2023	sum
Goriška	5	0	5
Jugovzhodna Slovenija	1	1	2
Koroška	1	3	4
Obalno Kraška	1	0	1
Osrednja Slovenija	3	2	5
Podravska	5	1	6
Pomurska	1	1	2
Posavska	0	1	1
Primorsko-Notranjska	1	0	1
Savinjska	3	0	3
Zasavska	1	0	1
sum	22	9	31

3.2 SURVEY OF PESTICIDE RESIDUES IN HONEY SAMPLES

Of the 31 honey samples analysed, only 3 contained one active substance: cypermethrin in concentrations 0.006 (honey poured in 2022, Osrednja Slovenija), 0.015 (honey poured in 2023, Koroška) and 0.048 mg kg^{-1} (honey poured in 2023, Koroška). This means that in 90.3 % of all samples analysed, were free of pesticides sought. In Slovenia, cypermethrin is authorised as insecticide for seed treatment of cereals (formulation ES, Emulsion for seed treatment), and for use on soil at planting of melliferous crops like oilseed rape, pumpkin and aubergines and on non-melliferous crops like onion, garlic, head cabbage, horseradish, chinese cabbage, carrot, potatoes, kale, tomatoes, parsnips, parsley, beetroots, radishes, sugar beet, shallots, tobacco, celery and grass (formulation GR, Granule). Cypermethrin is a non-systemic and cannot be translocated in plants. But granules of PPPs contain 10 % dust (SANTE, 2023). Dust from treated seeds and/or granules of PPPs can be deposited on melliferous in-field weeds and off-field plants like clover or dandelion (Bonmatin at al., 2015, SANTE, 2023). The consequence is that residues of all active substances used in the field near the hive can be present in honey up to 0.05 mg kg^{-1}, which is MRL for cypermethrin in honey. Value of 0.05 mg kg^{-1} is calculated as a default value for all active sub-

Table 3: Validation parameters for honey

Active substance	Linearity range (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	Recovery (%)	RSD[a] (%)	U_r[b] (mg kg^{-1})	U_r[c] (%)	U_R[d] (mg kg^{-1})	U_R[e] (%)
8-hydroxyquinoline	0.005-0.04	0.995	0.005	95.5	8.2	0.0007	13.8	0.0009	17.9
benthiavalicarb-isopropyl	0.005-0.03	0.999	0.005	97.3	7.4	0.0004	7.6	0.0008	16.6
boscalid	0.005-0.03	0.997	0.005	95.1	7.4	0.0006	11.4	0.0008	16.2
clomazone	0.005-0.03	0.999	0.005	96.3	7.3	0.0005	10.9	0.0008	16.2
cypermethrin	0.005-0.03	0.997	0.005	93.3	11.0	0.0009	18.3	0.0012	23.4
cyprodinil	0.005-0.03	0.999	0.005	95.0	6.1	0.0005	10.8	0.0007	13.3
deltamethrin	0.005-0.03	0.997	0.005	92.8	9.8	0.0008	16.5	0.0010	20.8
fenhexamid	0.005-0.03	0.999	0.005	96.4	11.2	0.0005	9.8	0.0012	24.9
flonicamid	0.005-0.05	0.987	0.005	98.3	7.0	0.0006	11.9	0.0008	15.7
fluazifop-p-butyl	0.005-0.03	0.999	0.005	96.9	8.6	0.0008	15.6	0.0009	18.9
fludioxonil	0.005-0.03	0.998	0.005	95.7	8.0	0.0007	13.3	0.0009	17.5
flufenacet	0.005-0.03	0.999	0.005	96.5	7.6	0.0006	12.5	0.0008	16.8
fluopicolide	0.005-0.03	0.998	0.005	97.0	7.6	0.0007	13.2	0.0008	16.9
fluopyram	0.005-0.03	0.999	0.005	97.3	6.4	0.0004	8.5	0.0007	14.2
flutolanil	0.005-0.03	0.999	0.005	95.6	8.2	0.0007	14.9	0.0009	17.9
iprovalicarb	0.005-0.03	0.999	0.005	96.1	8.1	0.0008	15.9	0.0009	17.8
kresoxim-methyl	0.005-0.03	0.999	0.005	97.0	7.4	0.0006	11.5	0.0008	16.4
lambda-cyhalothrin	0.005-0.03	0.999	0.005	98.7	7.8	0.0009	18.0	0.0009	18.0
metazachlor	0.005-0.03	0.999	0.005	96.2	6.8	0.0005	9.7	0.0007	15.0
myclobutanil	0.005-0.03	0.998	0.005	97.1	7.0	0.0005	10.8	0.0008	15.7
napropamide	0.005-0.03	0.999	0.005	95.9	6.0	0.0007	14.0	0.0007	14.0
penconazole	0.005-0.03	1.000	0.005	96.8	8.0	0.0006	11.3	0.0009	17.8
pendimethalin	0.005-0.02	1.000	0.005	93.7	7.3	0.0007	13.8	0.0008	15.6
pirimicarb	0.005-0.03	0.997	0.005	96.7	8.0	0.0007	13.7	0.0009	17.6
proquinazid	0.005-0.03	0.999	0.005	96.4	7.0	0.0005	10.5	0.0008	15.6
prosulfocarb	0.005-0.04	1.000	0.005	93.7	8.1	0.0008	15.7	0.0009	17.2
pyraclostrobin	0.005-0.03	0.993	0.005	96.9	11.3	0.0006	12.7	0.0013	25.2
pyrimethanil	0.005-0.03	1.000	0.005	95.1	7.7	0.0007	13.8	0.0008	16.8
tebuconazole	0.005-0.03	0.999	0.005	96.7	8.5	0.0007	14.1	0.0009	18.9
tebufenpyrad	0.005-0.03	0.998	0.005	95.9	7.2	0.0004	8.7	0.0008	15.9
tefluthrin	0.005-0.03	0.999	0.005	95.9	6.8	0.0005	9.9	0.0008	15.0
tetraconazole	0.005-0.03	0.999	0.005	94.1	8.7	0.0006	11.4	0.0009	18.9
trifloxystrobin	0.005-0.03	0.998	0.005	97.7	10.2	0.0008	15.5	0.0011	22.9

[a] RSD was obtained during recovery analyses
[b,c] U_r = uncertainty of repeatability
[d,e] U_R = uncertainty of reproducibility

Table 4: Literature data for active substances analysed by our laboratory, but not found in Slovenian honey samples

Active substance	Max content (mg kg $^{-1}$)	Ratio of positive samples (%)	Country of origin	Reference
boscalid	not reported	27.8	Italy	Panseri et al., 2014
boscalid	0.005	5	Poland	Gawel et al., 2019
cyhalothrin	0.0073	6.0	Egypt	Malhat et al., 2015
tebuconazole	0.012	10	Poland	Gawel et al., 2019
tebuconazole	0.005	9.1	Estonia	Karise et al., 2017
tebuconazole	0.004	9.1	Spain	Juan-Borrás et al., 2016
tetraconazole	0.005	3	Poland	Gawel et al., 2019
trifloxystrobin	not reported	20.8	Italy	Panseri et al., 2014

stances and presumes that the lowest ARfD is 1.5 x 10^{-4} mg (kg bw)$^{-1}$ d^{-1} (for active substance carbofuran) and the highest portion of consumed honey is 3.58 g (kg bw)$^{-1}$ (children consumption) (SANTE/11956/2016, rev. 9), meaning that residue of 0.05 mg kg^{-1} does not present acute risk for consumer. When residues are < 0.05 mg kg^{-1} it is not suspected that violation of PPPs happened. We do not have data about exact location of hives where Slovenian honey with cypermethrin residues was produced. Cypermethrin was probably found in Slovenian honey as a consequence of its use in vicinity of agricultural fields with melliferous off-field plants. We assume that in-field weeds were not present at application of PPPs and cereal seeds, containing cypermethrin, on soil. Farmers probably removed in-field weeds before sowing/planting. Therefore it is recommended that before PPPs are used, off-field plants near hives are mowed, to prevent presence of pesticide residues in honey.

A consumer risk assessment was performed using the EFSA PRIMo model rev. 3.1, which includes 36 national diets from EU countries. Slovenia did not create its own model, therefore EU model was used. The same model is also used during authorisation of PPPs in Slovenia and EU. For chronic exposure ADI of 0.005 mg (kg bw)$^{-1}$ d^{-1} and Supervised Trial Median Residue (STMR) of 0.015 mg kg^{-1} were used. The calculations of chronic exposure showed that the highest was observed in the German diet for children. It represented 0.03 % of ADI. For acute exposure ARfD of 0.005 mg (kg bw)$^{-1}$ d^{-1} and the Highest Residue (HR) of 0.048 mg kg^{-1} were used. The calculations of acute exposure showed that the highest was observed for children. It represented 3 % of ARfD. Based on these calculations, the conclusion was that the analysed honey samples do not represent unacceptable risk for consumers.

Our results were compared with the results from other scientific papers. Cypermethrin was not found in literature by our knowledge. Panseri et al. (2014), Malhat et al. (2015) and Juan-Borrás et al. (2016) did not measure presence of cypermethrin in Italy, Egypt and Spain. Cypermethrin was measured only by Gawel et al. (2019), but was not found in honey samples from Poland. The reason is probably that PPPs containing cypermethrin were not used in vicinity of locations of Polish hives. Other active substances (pesticides) analysed in our laboratory, namely boscalid, lambda-cyhalothrin, tebuconazole, tetraconazole and trifloxystrobin, were not found in Slovenian honey, but were found in samples analysed in Egypt, Estonia, Italy, Poland and Spain. Literature data for these active substances are presented in Table 4.

4 CONCLUSIONS

A method for determining pesticide residues originating from the environment in honey was introduced and validated by our laboratory. The limit of quantification was 0.005 mg kg^{-1} for all active substances. The calibration curves gave a linear response with R^2 0.987 to 1.000. The recoveries ranged from 92.8 % to 98.7 % with RSDs from 6.0 % to 11.3 %. The measurement uncertainty of repeatability ranged from 7.6 to 18.3 % and the measurement uncertainty of reproducibility from 13.3 to 25.2 %. The method was found to be fit for purpose for analysing 33 active substances and for determination of possible MRL exceedances.

In practice method was tested by analysing 31 honey samples gathered from Slovenian beekeepers, all from conventional production. A total of 33 active substances were sought, but only the insecticide cypermethrin was found in three of these samples, below valid MRL. In 90.3 % of the samples analysed, the active substances sought were not found. A risk assessment revealed that the analysed Slovenian honey samples are safe for consumers.

5 ACKNOWLEDGEMENTS

The authors expresses thanks to Janja Debevc for her help with the preparation of the extracts. For financial support we express our thanks to the Ministry of the Republic of Slovenia for Agriculture, Forestry and Food (MAFF), Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection (AFSVPP). We also acknowledge the financial support of the Slovenian Research Agency (research core funding No. P4-0133).

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doi:10.14720/aas.2024.120.2.17110 Original research article / izvirni znanstveni članek

Soil water dynamics and olive yield (*Olea europaea* L.) under different surface drip irrigation treatments in northern Mediterranean

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Received December 13, 2023; accepted April 25, 2024.
Delo je prispelo 13. decembra 2023, sprejeto 25. aprila 2024.

Soil water dynamics and olive yield (*Olea europaea* L.) under different surface drip irrigation treatments in northern Mediterranean

Abstract: The use of modern irrigation systems and monitoring of soil water status can help improve crop performance and water use efficiency. The influence of different irrigation treatments on soil water content dynamics and olive oil yield was studied over two growing seasons using a surface drip irrigation system in an olive grove in northern Mediterranean climate. Irrigation treatments included optimal irrigation, sustained deficit irrigation (33 % of optimal irrigation), and rainfed treatment. Based on the water applied, we calculated the percentage of replenished estimated evapotranspiration (ET_c*) for each treatment using the Penman-Monteith method. Soil water content dynamics were monitored with capacitive probes at five depths (10 to 50 cm). The increase in soil water content at a depth of 30 to 50 cm, which was only achieved with optimal irrigation, resulted in a significantly higher olive oil yield. In contrast, deficit irrigation, despite the addition of water, did not lead to an increase in soil water in the layers below 30 cm, so that the yield was equal to that of rainfed treatment. In irrigated olive groves, it is beneficial to monitor the water content of the soil at several depths to ensure that a sufficient amount of water has been applied.

Key words: diviner, evapotranspiration, irrigation management, olive, soil depths, volumetric soil water content

Dinamika vode v tleh in pridelek oljk (*Olea europaea* L.) pri različnih načinih površinskega kapljičnega namakanja v severnem Sredozemlju

Izvleček: Uporaba sodobnih namakalnih sistemov ter spremljanje stanja vode v tleh lahko pripomore k izboljšanju učinkovitosti rastlinske pridelave in rabe vode. Vpliv različnih načinov namakanja na dinamiko vsebnosti vode v tleh in pridelek oljčnega olja smo preučevali v dveh rastnih dobah z uporabo površinskega kapljičnega namakalnega sistema v oljčnem nasadu v severnem sredozemskem podnebju. Obravnavanja so vključevala optimalno namakanje, trajno namakanje s primanjkljajem (33 % optimalnega namakanja) in brez namakanja. Na podlagi porabljene vode smo z uporabo metode Penman-Monteith izračunali odstotek nadomeščene ocenjene evapotranspiracije (ET_c*) za vsako obravnavo. Dinamiko vsebnosti vode v tleh smo spremljali s kapacitivnimi merilniki na petih globinah (od 10 do 50 cm). Povečanje vsebnosti vode v tleh na globini od 30 do 50 cm, ki je bilo doseženo le z optimalnim namakanjem, je povzročilo večji pridelek oljčnega olja. Nasprotno pa se pri namakanju s primanjkljajem kljub dodajanju vode ni povečala količina vode v tleh v plasteh pod 30 cm, zato je bil pridelek enak pridelku brez namakanja. V namakanih oljčnih nasadih je koristno spremljati vsebnost vode v tleh na več globinah, da se zagotovi, da je bila priskrbljena zadostna količina vode.

Ključne besede: diviner, evapotranspiracija, upravljanje namakanja, oljke, globine tal, volumska vsebnost vode v tleh

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1 INTRODUCTION

Olive (*Olea europaea* L.) is traditionally cultivated in regions with water scarcity (Rufat et al., 2014). The vulnerability of the Mediterranean region to climate change has been highlighted by the increasing occurrence and intensity of agricultural droughts (Tramblay et al., 2020). In recent years, Slovenian olive growers and producers have struggled to achieve consistent yields and olive oil quality due to extreme weather conditions, particularly the more frequent occurrence of droughts (Podgornik et al., 2018; Valenčič et. al., 2018).

Olive irrigation is a well-known agrotechnical measure to improve olive oil yield and quality (Rufat et al., 2018; Santos, 2018). Regulated deficit irrigation is a commonly studied management practice in water-scarce environments, however the optimal irrigation regime is not easy to define because it is a complex interaction of different factors, such as tree age, size, health, nutrition, weed cover, and others (Arampatzis et al., 2018; Carr, 2013). In northern Mediterranean climate, Podgornik et al. (2017) showed that the olive oil yield of the cultivar 'Istrska Belica' can still be significantly improved by irrigation. However, out of a total area of 2571 ha of olive groves in Slovenia, only 47 ha were irrigated in 2023 (MKGP, 2024). Since 2008, most irrigation systems have been based on drip irrigation using public water as the main water source (Podgornik et al., 2022).

The use of modern irrigation systems and monitoring of soil and crop water status can contribute to improved crop performance and water use efficiency in the face of a changing climate. Automated or decision-supported systems for irrigation scheduling based on soil water content (θ) measurement are commonly used to optimize water use in agriculture (Cvejić et al., 2020; Navarro-Hellín et al., 2016; Vera et al., 2021). The use of profile capacitance sensors inserted into an access tube has the added advantage that θ can be measured at multiple depths simultaneously (Arampatzis et al., 2018; Egea et al., 2016). In micro-irrigated heterogeneous crop system, such as Mediterranean tree crops, the variability of soil water content in the field depends on the spatial distribution of roots and local water supply. Consequently, such heterogeneity affects crop water status and management strategies (Rallo et al., 2018).

Despite predictions that olive growing areas will expand to higher elevations and northward in the future (Tanasijević et al., 2014), there are currently few studies on the effects of different water regimes on olive trees in sub-humid and/or northern Mediterranean regions. Studies on the response of olive trees to water availability in sub-humid regions often focus on the aboveground part of the plant (D'andria et al., 2009; Podgornik et al., 2017; Tognetti et al., 2008) and the water balance of the olive grove (Zupanc et al., 2018). Despite the fact that crop yields are more closely related to soil water availability than to any other soil or meteorological variable (de Jong and Bootsma, 1996), few studies have been conducted on the dynamics of soil water content in irrigated olive groves in the northern Mediterranean region.

The objective of this study was to investigate how different amounts of water used in surface drip-irrigation (optimal irrigation, sustained deficit irrigation, and rainfed) affect the dynamics of soil water content in the soil profile and how they influence olive oil yield.

2 MATERIALS AND METHODS

2.1 SITE DESCRIPTION

The study was conducted during the 2016 and 2017 irrigation seasons in a 17-year-old olive grove (*Olea europaea* 'Istrska Belica') located in Slovenian Istria (Dekani: 45°33.541′N, 13°47.637′E; 96 m above sea level) (Fig. 1), a typical olive-growing area in southwestern Slovenia. The olive variety 'Istrska Belica' is the most widespread variety in the northern part of the Adriatic region and is intensively propagated in Slovenian Istria and in the Friuli-Venezia Giulia region in Italy. This is due to its excellent adaptability to pedoclimatic conditions, its very good and regular fertility and its high oil content (Bandelj et al., 2004). This olive oil has a high phenol content, which gives the oil a special flavour characterised by bitterness and pungency. These sensory characteristics are very intense in oil from drought-stressed trees and are generally perceived as unpleasant by consumers. Irrigation can influence the content of phenols in olive oil and thus its sensory characteristics (Dag et al., 2008; Gómez-Rico et al., 2007; Romero et al., 2002).

Southwestern Slovenia has a sub-mediterranean climate with an average annual precipitation of 969 mm (20-year mean, 1999-2019), although seasonal precipitation varies greatly from year to year, especially in monthly distribution (Sušnik and Matajc, 2013). The daily mean temperature varied from −2 to 7 °C in winter (December/January) and 20 to 28 °C in summer (July/August). The mean annual reference evapotranspiration (ET_0) is 1035 mm. Mean precipitation data for the experimental olive grove were obtained from the local meteorological station (ARSO, 2022). Olive trees are spaced 6 m × 5 m apart, with an overall plantation density of 300 plants ha^{-1}. The olive grove is covered with natural greenery and no tillage was used during the experiment.

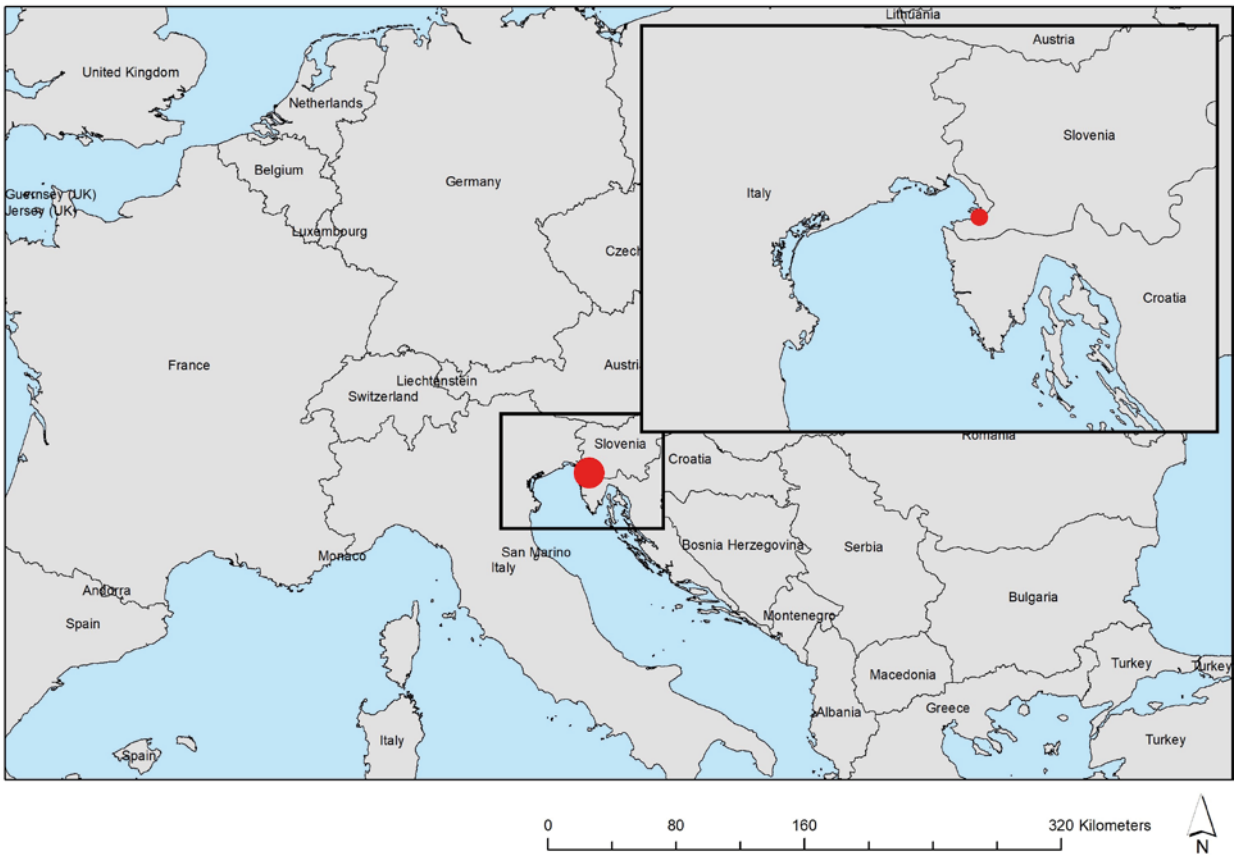

Figure 1: Location of experimental olive grove in the region

The soil characteristics for the experimental olive grove are given in Table 1. The soil type is clay loam with a mean depth of 0.74 m. Soil water content (θ) at field capacity (FC) and permanent wilting point (PWP) were determined for the 25 cm to 30 cm soil layer in the laboratory using a pressure plate extractor. The θ at FC at a soil matric potential of −0.033 MPa is 0.32 m^3 m^{-3}. The θ at PWP (−1.5 MPa) is 0.19 m^3 m^{-3}. Ratliff et al. (1983) suggested that if absolute accuracy is necessary for water-balance calculations, laboratory-estimated soil water limits (e.g., field capacity, wilting point) should be used with caution, and field-measured limits are preferred, if available.

The phenological growth stages of the olive variety 'Istrska Belica' observed in the experiment in 2016 and 2017 growing seasons are listed in Table 2.

Table 1: Soil texture and organic matter content (OM) of the soil horizons of the olive grove in Dekani (Slovenia) (Podgornik et al., 2017)

Soil horizon	Depth (cm)	Sand (%)	Loam (%)	Clay (%)	Texture	OM (%)
Ah	0-2	31.7	43.5	24.8	Loam	18.0
P1	2-24	29.3	42.1	28.6	Clay loam	3.1
P2	24-51	28.7	43.4	27.9	Clay loam	2.2
P3	51-74	32.3	38.2	29.5	Clay loam	1.6

Table 2: Phenological growth stages (Sanz-Cortés et al., 2002) of the olive variety 'Istrska Belica' in 2016 and 2017

BBCH	Description	2016	2017
11	First leaves completely separated	10/04	08/04
31	Shoots reach 10 % of final length	14/04	15/04
51	Inflorescence buds start to swell	21/04	21/04
60	First flowers open	22/05	22/05
65	Full flowering: at least 50 % of flowers open	29/05	29/05
69	End of flowering, fruit set, non-fertilised ovaries fallen	04/06	05/06
71	Fruit about 10 % of final size	11/06	13/06
81	Beginning of fruit colouring	25/09	20/09
89	Harvest maturity: fruits are suitable for oil extraction	01/11	01/11
92	Overripe: fruits lose turgidity and start to fall	10/11	06/11

2.2 IRRIGATION REGIMES

The surface drip irrigation system was established in April 2009 to provide different amounts of water throughout the season (i.e., June–October). Trees were surface drip-irrigated with different combinations of 2 l h^{-1} pressure-compensating drippers placed around the trees. They provided different irrigation treatments with distinct water regimes: optimal irrigation, in which seasonal irrigation attempted to compensate for all water loss so that the water content at 25 cm depth was maintained near FC; sustained deficit irrigation, in which irrigation volume was 33 % of optimal irrigation; and rainfed, in which the trees were not irrigated. The amount of water for deficit irrigation (33 % optimal) was chosen based on relatively high long-term annual precipitation (about 1000 mm). Optimal irrigation was achieved with 15 drippers spaced 0.47 m apart on the dripline around the tree at a distance of 1.5 m from tree trunk. Sustained deficit irrigation was achieved with 5 drippers placed 1.41 m apart. Timing and amount of irrigation were automated based on continuous measurement of θ with two TRIME-Pico 32 sensors (IMKO micromodultechnik GmbH, Ettlingen, Germany) installed horizontally at a depth of 25 cm between two drippers under the drip line. Irrigation was triggered so that the θ at optimal irrigation in 2016 ranged from 0.25 m^3 m^{-3} (start of irrigation) to 0.31 m^3 m^{-3}. Due to high water use in 2016, the irrigation regime was changed in 2017 and optimal irrigation was maintained only in the range of 0.23 m^3 m^{-3} to 0.30 m^3 m^{-3}, resulting in less frequent irrigation events compared to 2016.

Estimated crop evapotranspiration (ET_c^*) for olive grove was calculated based on Penman-Monteith calculations with a single crop coefficient (K_c) (FAO-56 approach). The reference evapotranspiration ET_0 was obtained from the local meteorological station (ARSO, 2022), and $K_c = 0.7$ ($K_{c\,mid}$) was used for olive groves with 40-60 % ground cover through the canopy (Allen et al., 1998). However, some authors have calculated lower values of $K_{c\,mid} = 0.45$ (Pastor and Orgaz, 1994). The ratio of water applied by precipitation and/or irrigation ($P + I$) to calculated ET_c was calculated for each treatment on a weekly basis.

2.3 STUDY DESIGN AND MEASUREMENTS

The study design included four rows of trees. In each row, blocks of four trees were randomly selected for each irrigation treatment (total 16 trees per treatment). θ was measured near two randomly selected trees for each irrigation treatment, weekly during the irrigation season (from June to September) using a Diviner 2000 soil moisture sensor (Sentek Pty Ltd., Stepney, Australia), previously calibrated for the experimental soil. The Diviner 2000 is a portable device with a hand-held logger and a capacitance sensor inserted into an access tube (Sentek, 2009). The measurement of θ was technically repeated three times, and the mean value was used for further analysis. Measurements of θ were taken at five different soil depths (10 cm, 20 cm, 30 cm, 40 cm, 50 cm) at a distance of 1.5 m from the tree trunk. Diviner access tubes were installed near two TRIME-Pico 32 sensors, which triggered irrigation at a threshold θ (Fig. 2).

Olive oil yield was measured in the 2016 season on eight randomly selected trees per treatment (2 per row). In 2017, yield was measured on the same trees as in the previous season. In both experimental years 2016 and 2017, harvesting was carried out in November (November 7 and 9, respectively). Trees were harvested individually by hand. The fruit mass of each tree was measured after harvest, and samples of 700 g of olives per treatment were taken for each year to determine the oil content. Oil

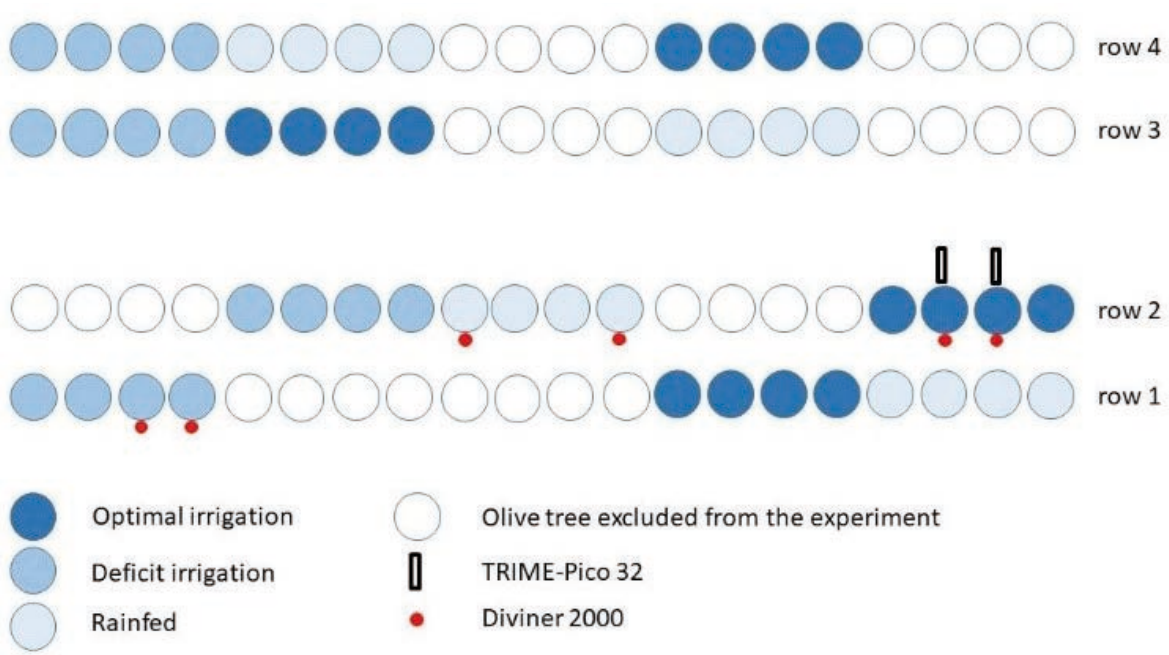

Figure 2: Experimental design

extraction was performed using a laboratory olive mill (Abencor, MC2 Ingeniería y Sistemas SL, Seville, Spain). The fruits were crushed with a hammer mill, the resulting olive pulp was malaxed at 25 °C for 20 min, and the oil was separated by centrifugation. The oil was then filtered and the oil yield and content were determined.

2.4 STATISTICAL ANALYSIS

All statistical analyses were performed using R statistical software version 4.2.1. To evaluate the effects of the three irrigation treatments: rainfed, deficit and optimal irrigation on soil water content during two growing seasons, a linear-mixed model (mixed model ANOVA) function *lmer()* (package "lme4") was used for each of the two seasons (2016 and 2017) separately. A random effect of date (random intercept), a random effect of six Diviner 2000 access tube locations that have been repeatedly sampled over time (random intercept), and an interaction of two fixed factors - irrigation treatment (rainfed, deficit irrigation, optimal irrigation) and depth (10 cm, 20 cm, 30 cm, 40 cm, 50 cm) were included in the model. Homogeneity of variances was checked using residual plots for each treatment and depth. The normality assumption was checked using the Q-Q plot.

For olive oil yield analysis, a linear model was used to analyze the data for each of the two seasons (2016, 2017) separately, using the generalized least squares ("*gls() function*") and accounting for the different variances for each irrigation treatment. Post-hoc analysis was performed for both variables using the package "emmeans" with "mvt" adjustment (multivariate *t*-distribution) for pairwise comparisons. Statistical significance was assumed at the = 0.05 level.

3 RESULTS

3.1 ACTUAL IRRIGATION TREATMENTS

Total precipitation (P), optimal irrigation (I), reference evapotranspiration (ET_0), estimated crop evapotranspiration (ET_c^*; from single crop K_c), and estimated daily mean ratio of total $P + I$ to ET_c^* for periods between consecutive Diviner measurements are shown for each irrigation treatment for the 2016 and 2017 growing seasons in Tables 3 and 4, respectively. Estimated mean daily ET_c^* ranged from 2.0 mm (September) to 4.4 mm (early August) in the 2016 season, and from 1.3 mm (late September) to 4.8 mm (July) in 2017.

The monthly ratio of $P + I$ to ET_c^* for each irrigation treatment is shown in Table 5. In August 2016, well over 100 % of the estimated ET_c^* was applied (234.1 % from 02/08/2016 to 29/08/2016), while in August 2017, slightly more than 100 % of the calculated ET_c^* was applied (127.2 % from 01/08/2016 to 28/08/2016) under optimal irrigation. In July 2016, applied water under optimal irri-

Table 3: Precipitation (*P*) and irrigation (*I*) amount for optimal irrigation treatment with sum of reference ET_0 and estimated evapotranspiration (ET_c*), estimated mean daily ET_c*, and ratio of sum of irrigation + precipitation to ET_c* for all treatments. Data is shown for the 2016 growing season for periods between two consecutive Diviner 2000 soil water content measurements. ND is number of days

Year 2016	ND	*P* (mm)	I optimal (mm)	ET_0 (mm)	ET_c* (mm) (K_c = 0.7)	Daily mean ET_c* (mm)	*P* + *I* (mm)		Ratio *P* + *I* / ET_c* (%)		
							Optimal	Deficit	Optimal	Deficit	Rainfed
08/06-13/06	6	45.9	0.0	20.8	14.6	2.4	45.9	45.9	315.2	315.2	315.2
14/06-20/06	7	45.9	21.5	28.7	20.1	2.9	67.4	53.0	335.4	263.8	228.5
21/06-27/06	7	0.1	71.3	40.7	28.5	4.1	71.4	23.6	250.6	82.9	0.4
28/06-05/07	8	0.5	62.7	46.1	32.3	4.0	63.2	21.2	195.9	65.7	1.5
06/07-15/07	10	10.1	3.4	60.4	42.3	4.2	13.5	11.2	32.0	26.6	23.9
16/07-18/07	3	0.0	0.0	15.3	10.7	3.6	0.0	0.0	0.0	0.0	0.0
19/07-26/07	8	5.6	3.0	45.2	31.6	4.0	8.6	6.6	27.1	20.8	17.7
27/07 - 01/08	6	1.7	35.4	33.2	23.2	3.9	37.1	13.4	159.7	57.6	7.3
02/08-09/08	8	1.0	53.8	50.0	35.0	4.4	54.8	18.7	156.5	53.6	2.9
10/08-16/08	7	7.3	58.9	35.3	24.7	3.5	66.2	26.7	267.7	108.2	29.5
17/08-22/08	6	31.3	50.8	27.9	19.5	3.3	82.1	48.1	420.2	246.1	160.3
23/08-29/08	7	0.0	43.9	37.5	26.3	3.8	43.9	14.5	167.4	55.2	0.0
30/08 - 05/09	7	2.2	47.6	32.2	22.5	3.2	49.8	17.9	220.8	79.4	9.8
06/09-12/09	7	9.1	37.2	30.5	21.4	3.1	46.3	21.4	217.1	100.2	42.6
13/09-19/09	7	53.5	7.6	20.4	14.3	2.0	51.1	46.0	357.5	322.1	374.6
20/09-26/09	7	0.0	0.0	23.7	16.6	3.4	0.0	0.0	0.0	0.0	0.0

gation was lower (73.0 % from 28/06/2016 to 26/07/2016) due to problems with the automated system. The results show that the ET_c* calculation based on a single K_c approach does not account for the additional evaporative losses at the surface, because more water than estimated ET_c* was applied to increase θ.

Deficit irrigation replenished approximately 100 % of calculated ET_c* in August 2016 (102.4 % from 02/08/2016 to 29/08/2016) and 66.2 % in August 2017 (from 01/08/2017 to 28/08/2017). Comparison of the three-month mean water balance from June to August in 2016 and 2017 shows that more water was applied for both irrigation treatments in 2016. Optimal irrigation (179.4 % of calculated ET_c from 08/06/2016 to 29/08/2016) and deficit irrigation (91.6 % from 08/06/2016 to 29/08/2016) in 2016, while in 2017 optimal irrigation reached 116.2 % of calculated ET_c* from 30/05/2017 to 28/08/2017 and deficit irrigation reached 60.5 % from 30/05/2017 to 28/08/2017.

3.2 EFFECT OF IRRIGATION TREATMENTS ON VOLUMETRIC SOIL WATER CONTENT

Figures 3, 4, and 5 show the temporal dynamics of the θ measured during the 2016 and 2017 irrigation seasons (mean and standard error of two access tubes θ measurements for each depth at 34 time points), as well as the irrigation and precipitation events that occurred during the periods studied. Additional secondary axis for (*I* + *P*) to ET_c * ratios was added, showing only ratios below 350 % ET_c*. The dashed lines indicate the 100 % and 33 % ET_c* ratios. The black dots represent the mean ratios *I* + P / $\underline{ET_c}$* during the selected period between two consecutive Diviner 2000 measurements and are scaled on the secondary axis. From 04/07/2016 to 20/07/2016 and from 05/07/2017 to 18/07/2017, the automatic irrigation did not work properly, so the irrigation was applied manually, causing the θ to decrease at all depths.

Soil water content increased after precipitation events. Optimal irrigation treatment resulted in higher θ

Table 4: Precipitation (*P*) and irrigation (*I*) amount for optimal irrigation treatment with sum of reference ET_0 and estimated evapotranspiration (ET_c*), estimated mean daily ET_c*, and ratio of sum of irrigation + precipitation to ET_c* for all treatments. Data is shown for the 2017 growing season for periods between two consecutive Diviner 2000 soil water content measurements. ND is number of days

							$P + I$ (mm)		Ratio $P + I$ / ET_c* (%)		
Year 2017	ND	*P* (mm)	I optimal (mm)	ET_0 (mm)	ET_c* (mm) (K_c = 0.7)	Daily mean ET_c* (mm)	Optimal	Deficit	Optimal	Deficit	Rainfed
23/05-29/05	7	0.3	0.1	37.5	26.3	3.8	0.4	0.3	1.4	1.2	1.1
30/05-05/06	7	0.0	21.2	40.6	28.4	4.1	21.2	7.0	74.7	24.6	0.0
06/06-12/06	7	3.3	16.8	40.7	28.5	4.1	20.1	8.9	70.7	31.1	11.6
13/06-19/06	7	0.1	26.7	42.3	29.6	4.2	26.8	8.9	90.5	30.1	0.3
20/06-26/06	7	9.5	28.8	41.4	29.0	4.1	38.3	19.0	132.1	65.6	32.8
27/06-03/07	7	65.7	0.0	35.4	24.8	3.5	65.7	65.7	265.1	265.1	265.1
04/07-10/07	7	0.8	20.4	43.8	30.7	4.4	21.2	7.5	69.1	24.6	2.6
11/07 - 17/07	7	0.0	25.4	47.5	33.3	4.8	25.4	8.4	76.4	25.2	0.0
18/07-24/07	7	0.0	25.2	40.9	28.6	4.1	25.2	8.3	87.9	29.0	0.0
25/07-31/07	7	3.5	43.9	39.4	27.6	3.9	47.4	18.0	171.8	65.2	12.7
01/08-07/08	7	15.9	0.0	43.9	30.7	4.4	15.9	15.9	51.7	51.7	51.7
08/08 - 14/08	7	4.1	43.5	34.0	23.8	3.4	47.6	18.4	199.8	77.5	17.2
15/08-21/08	7	16.9	15.5	36.6	25.6	3.7	32.4	22.0	126.4	85.9	66.0
22/08-28/08	7	0.0	33.9	31.2	21.8	3.1	33.9	11.2	155.2	51.2	0.0
29/08-04/09	7	21.5	27.3	26.5	18.6	2.7	48.8	30.5	262.9	164.4	115.9
05/09-11/09	7	84	15.8	16.7	11.7	1.7	99.8	89.2	853.5	763.1	718.6
12/09-18/09	7	86.6	9.4	15.7	11.0	1.6	96.0	89.7	873.6	816.2	788.0
19/09-25/09	7	56.2	0.0	13.3	9.3	1.3	56.2	56.2	603.9	603.7	603.7

Table 5: Approximate monthly irrigation + precipitation ($I + P$) to ET_c ratios for each irrigation treatment

	Mean ratio $I + P$ / ET_c* and amount of water ($I + P$) applied (mm)		
Year and month	Optimal irrigation	Deficit irrigation	Rainfed
June 2016 (08/06-27/06)	292.5% (184.7 mm)	194.0 % (122.5 mm)	145.5 % (91.9 mm)
July 2016 (28/06-26/07)	73.0 % (85.3 mm)	33.4 % (39.0 mm)	13.9 % (16.2 mm)
August 2016 (02/08-29/08)	234.1 % (246.9 mm)	102.4 % (108.8 mm)	37.5 % (39.6 mm)
June – August 2016 (08/06-29/08)	179.4 % (554.0 mm)	91.6 % (282.9 mm)	48.4 % (149.4 mm)
June 2017 (30/05-26/06)	92.2 % (106.4 mm)	37.9 % (43.8 mm)	11.2 % (12.9 mm)
July 2017 (04/07-31/07)	99.2 % (119.1 mm)	35.1 % (42.2 mm)	3.6 % (4.3 mm)
August 2017 (01/08-28/08)	127.2 % (129.7 mm)	66.2 % (67.5 mm)	36.2 % (36.9 mm)
June – August 2017 (30/05-28/08)	116.2 % (421.0 mm)	60.5 % (219.2 mm)	33.1 % (199.8 mm)

at deeper layers - 30 cm, 40 cm, and 50 cm compared to the rainfed treatment. In August 2016, more than 100 % of the estimated (single K_c) ET_c* was applied during most periods (dots of ratios above 100 % ET_c* line) to compensate for surface evaporative losses. In 2017, however, the ratios are closer to 100 % estimated ET_c*. Interestingly, although deficit irrigation in August 2016 and 2017 replenished more than 33 % of estimated ET_c*, θ at 20 cm depth did not increase but remained low. It is also interesting to note that under deficit irrigation, similar amounts of water were applied (18 mm *I* and 3.5 mm *P*; 27 mm ET_c*) during the rainless period (25/7/2017 - 31/7/2017) as during the following rainy week (1/8/2017-7/8/2017; 0 mm *I*, 15.9 mm *P*; 30.7 mm ET_c*), but θ at depths from 10 cm to 50 cm increased only during the second week (mainly rain), but not during the first week (mainly irrigation). A similar situation can be observed during 2/8/2016-9/8/2016 and 8/8/2017-14/8/2017.

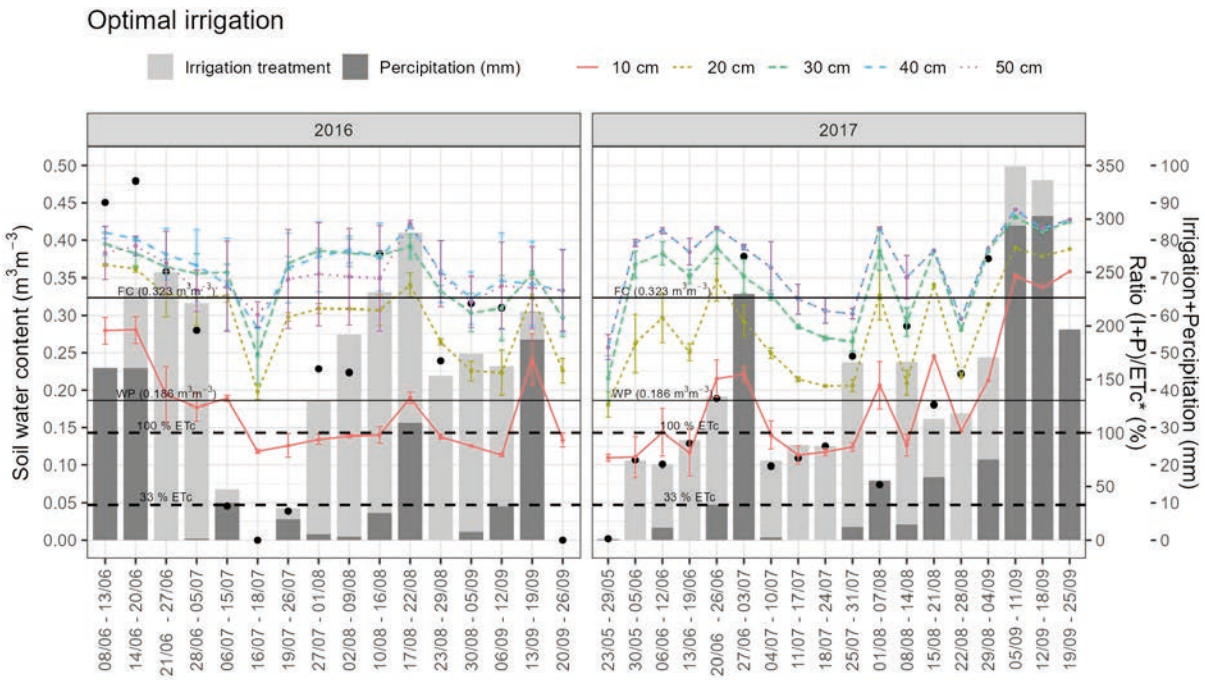

Figure 3: Temporal dynamics of mean volumetric soil water content with standard error under optimal irrigation at different soil depths (10 cm, 20 cm, 30 cm, 40 cm, 50 cm) and weekly precipitation and irrigation during the 2016 and 2017 growing seasons. Black dots represent the ratio of rainfall to estimated ET_c* (secondary axis). Field capacity and wilting point are also indicated, along with 100 % ET_c and 33 % ET_c

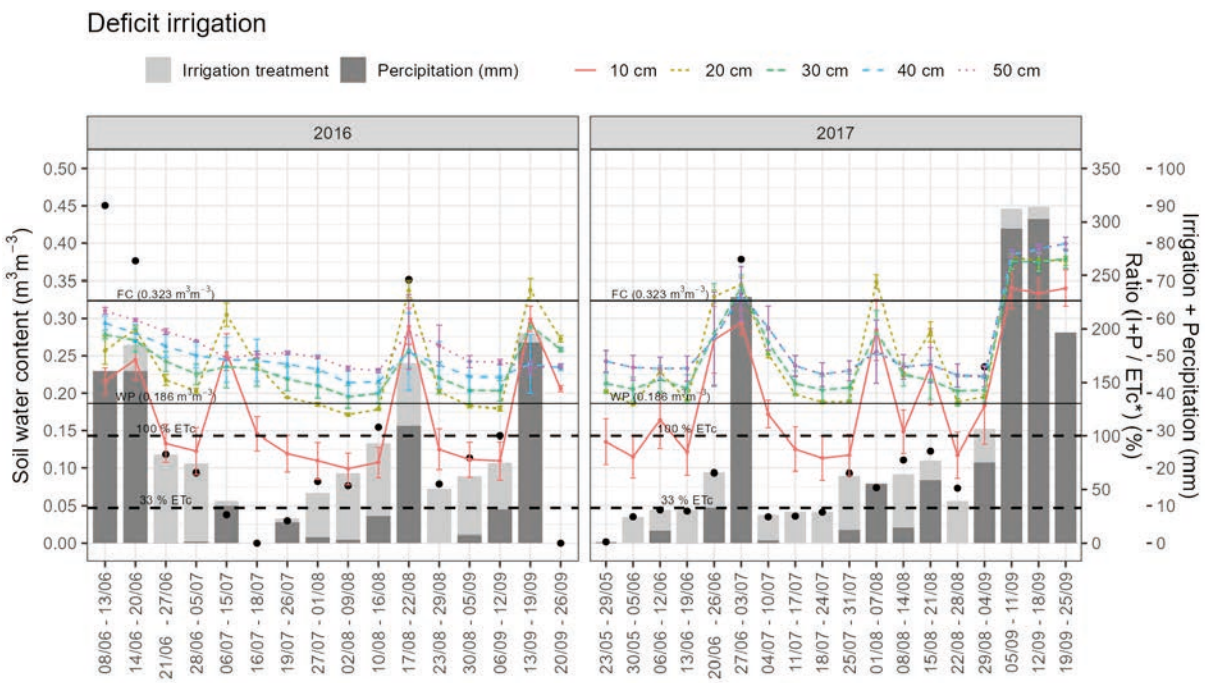

Figure 4: Temporal dynamics of mean volumetric soil water content with standard error under deficit irrigation at different soil depths (10 cm, 20 cm, 30 cm, 40 cm, 50 cm) and weekly precipitation and irrigation during the 2016 and 2017 growing seasons. Black dots represent the ratio of rainfall to estimated *ET* * (secondary axis). Field capacity and wilting point are also indicated, along with 100 % ET_c and 33 % ET_c

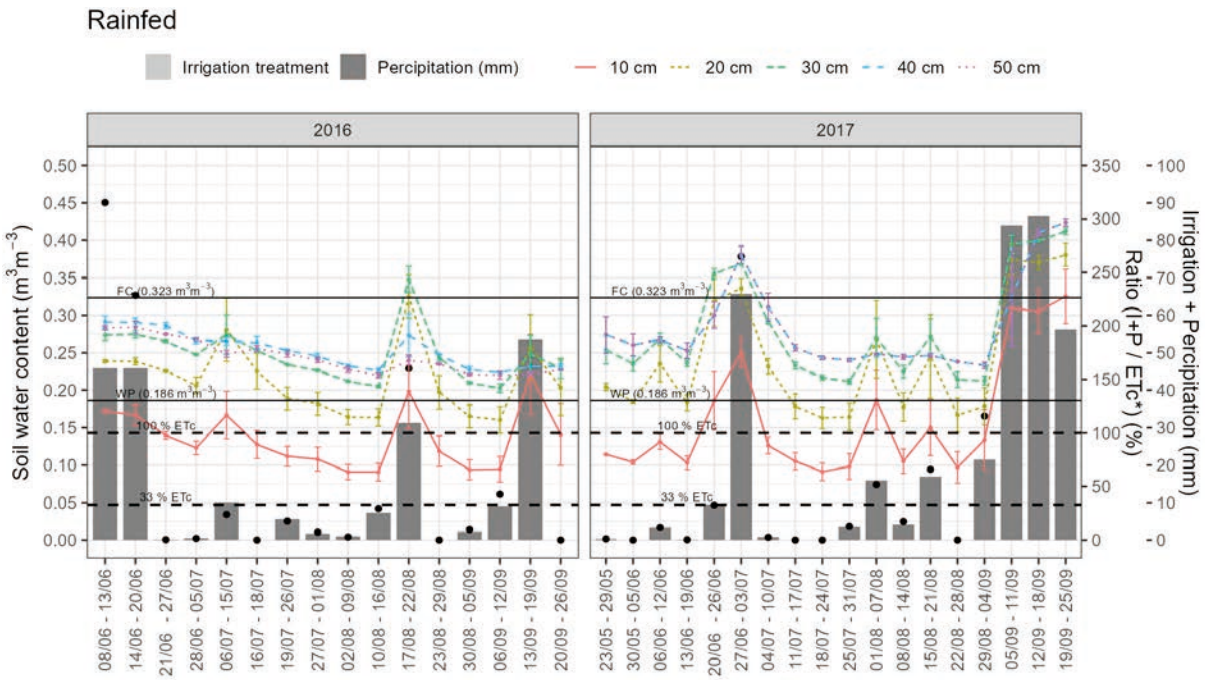

Figure 5: Temporal dynamics of mean volumetric soil water content with standard error under rainfed treatment at different soil depths (10 cm, 20 cm, 30 cm, 40 cm, 50 cm) and weekly precipitation during the 2016 and 2017 growing seasons. Black dots represent the ratio of rainfall to estimated ET_c^* (secondary axis). Field capacity and wilting point are also indicated, along with 100 % ET_c and 33 % ET_c

Fig. 6 shows the combined temporal dynamics of θ under rainfed, deficit irrigation, and optimal irrigation for each of the five depth layers. The black line represents the mean θ measurements from TRIME-Pico 32 under optimal irrigation. θ measurements made with two different sensor types agree well within the standard errors of the Diviner measurements during most of the growing season. From 23/05/2017 to 10/07/2017, TRIME-Pico 32 measurements were not successfully transmitted (data was lost), although the irrigation regime was maintained throughout the 2017 growing season. There is a similar θ pattern between the different irrigation treatments in both growing seasons, however differences in mean θ are less obvious in 2017 due to the lower amount of water applied. Mean θ was higher under optimal irrigation than under deficit irrigation and rainfed treatment at 30 cm, 40 cm, and 50 cm, but not at 10 and 20 cm. No clear differences were found between rainfed and deficit irrigation at any of the five depths.

The interaction between treatment and depth was statistically significant ($p < 0.05$), as were the main effects of treatment ($p < 0.05$) and depth ($p < 0.001$) in both growing seasons. Model prediction-means and 95% confidence intervals (CI) of soil water content measurements for each of the three treatments at different soil depths (10 cm, 20 cm, 30 cm, 40 cm, 50 cm) during the 2016 and 2017 growing seasons are shown in Fig. 7. Mean model prediction data are shown in Table 8 in the Appendix.

Differences in mean θ over two growing seasons between different irrigation treatments are shown by measurement depth for each growing season (Table 6). At 30 cm, mean θ was 0.12 $m^3\ m^{-3}$ higher under optimal irrigation compared with deficit irrigation in 2016 (95 % *CI* from 0.03 $m^3\ m^{-3}$ to 0.20 $m^3\ m^{-3}$) and 0.09 $m^3\ m^{-3}$ higher in 2017 (95 % *CI* from 0.09 $m^3\ m^{-3}$ to 0.17 $m^3\ m^{-3}$). At 30 cm, the difference in mean θ between optimal irrigation and rainfed treatment was statistically significant ($p = 0.023$) only in the 2016 growing season, with a higher mean θ under optimal irrigation, 0.11 $m^3\ m^{-3}$ (95 % *CI* from 0.03 $m^3\ m^{-3}$ to 0.19 $m^3\ m^{-3}$). At 40 cm, the difference in mean θ between optimal and deficit irrigation was statistically significant in both growing seasons ($p < 0.05$), with optimal irrigation having 0.12 $m^3\ m^{-3}$ higher mean θ in 2016 (95% *CI* from 0.04 $m^3\ m^{-3}$ to 0.20 $m^3\ m^{-3}$) and 0.10 $m^3\ m^{-3}$ higher mean θ in 2017 (95% *CI* from 0.02 $m^3\ m^{-3}$ to 0.19 $m^3\ m^{-3}$). At 40 cm, mean θ was 0.11 $m^3\ m^{-3}$ higher under optimal irrigation than under rainfed treatment (95 % *CI* from 0.03 $m^3\ m^{-3}$ to 0.19 $m^3\ m^{-3}$) in 2016 and 0.09 $m^3\ m^{-3}$ higher in 2017 (95 % *CI* from 0.00 $m^3\ m^{-3}$ to 0.17 $m^3\ m^{-3}$). At 50 cm, mean θ was 0.09

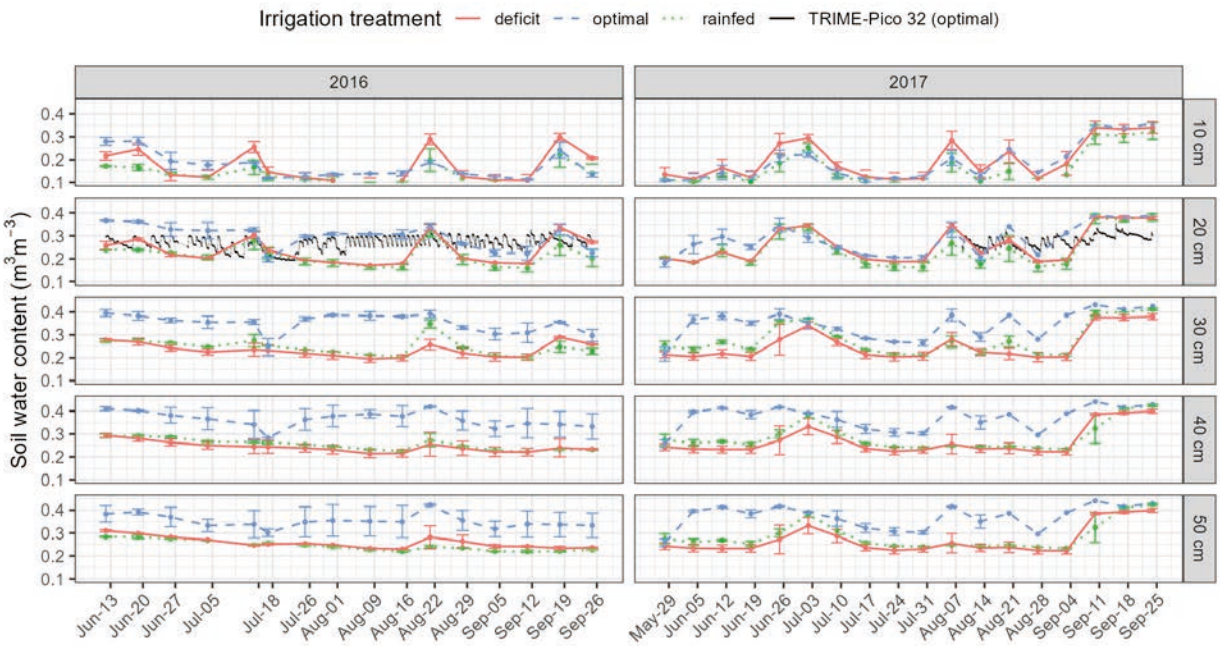

Figure 6: Temporal dynamics of the mean soil water content with standard error under three treatments at different soil depths (10 cm, 20 cm, 30 cm, 40 cm, and 50 cm) measured weekly with Diviner and continuous measurement of soil water content with TRIME-Pico 32

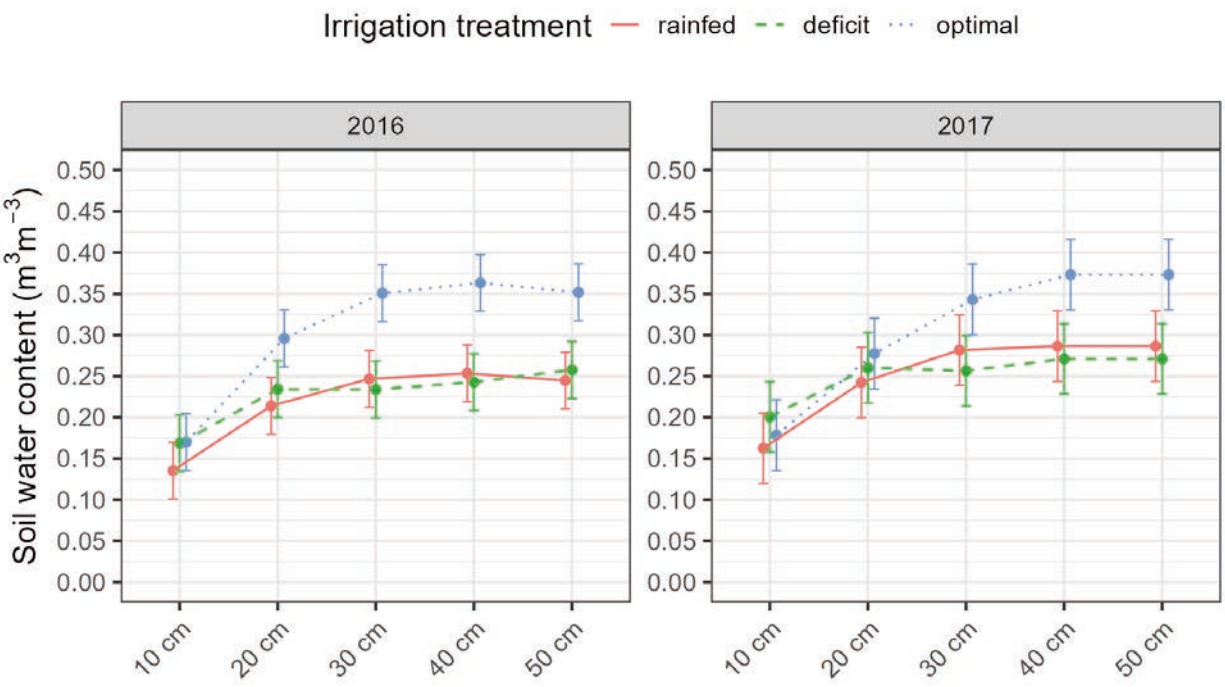

Figure 7: Model predictions of mean values and 95% confidence intervals of volumetric soil water content measurements for each of three treatments at different soil depths (10 cm, 20 cm, 30 cm, 40 cm, 50 cm) for the 2016 and 2017 growing seasons

$m^3\ m^{-3}$ higher under optimal irrigation than under deficit irrigation (95% *CI* from 0.01 $m^3\ m^{-3}$ to 0.18 $m^3\ m^{-3}$) in 2016 and 0.10 $m^3\ m^{-3}$ higher in 2017 (95 % *CI* from 0.02 $m^3\ m^{-3}$ to 0.19 $m^3\ m^{-3}$). At 50 cm in both growing seasons, mean θ was higher under optimal irrigation than under rainfed treatment.

Thus, the θ under the optimal irrigation treatment was higher compared to deficit irrigation and rainfed treatments in both growing seasons. Mean differences are higher in growing season 2016. The level of soil water content under the optimal irrigation treatment reflected the amount of water applied in each growing season. However, this was not the case in the deficit irrigation and rainfed treatments, between which no significant differences in θ were found at any depth, although more water was applied in the deficit irrigation treatment (Figures 4 and 5).

Table 6: Pairwise comparisons of differences in mean soil water content between irrigation treatments for each depth of monitoring for the 2016 and 2017 growing seasons

Year	Depth	Pairwise comparison	2.5 % percentile ($m^3\ m^{-3}$)	Mean differences ($m^3\ m^{-3}$)	97.5 % percentile ($m^3\ m^{-3}$)	*p*-value
2016	10 cm	optimal - deficit	−0.08	0.00	0.08	1.000
		optimal - rainfed	−0.05	0.03	0.12	0.455
		deficit - rainfed	−0.05	0.03	0.12	0.493
	20 cm	optimal - deficit	−0.02	0.06	0.14	0.122
		optimal - rainfed	−0.00	0.08	0.16	0.051
		deficit - rainfed	−0.06	0.02	0.10	0.826
	30 cm	optimal - deficit	0.03	0.12	0.20	0.015
		optimal - rainfed	0.02	0.10	0.19	0.023
		deficit - rainfed	−0.10	−0.01	0.07	0.968
	40 cm	optimal - deficit	0.04	0.12	0.20	0.013
		optimal - rainfed	0.03	0.11	0.19	0.019
		deficit - rainfed	−0.09	−0.01	0.07	0.987
	50 cm	optimal - deficit	0.01	0.09	0.18	0.032
		optimal - rainfed	0.02	0.11	0.19	0.021
		deficit - rainfed	−0.07	0.01	0.10	0.966
2017	10 cm	optimal - deficit	−0.11	−0.02	0.06	0.770
		optimal - rainfed	−0.07	0.02	0.10	0.918
		deficit - rainfed	−0.05	0.04	0.12	0.384
	20 cm	optimal - deficit	−0.07	0.02	0.10	0.892
		optimal - rainfed	−0.05	0.04	0.12	0.455
		deficit - rainfed	−0.07	0.02	0.10	0.876
	30 cm	optimal - deficit	0.00	0.09	0.17	0.045
		optimal - rainfed	−0.02	0.06	0.15	0.131
		deficit - rainfed	−0.11	−0.03	0.06	0.692
	40 cm	optimal - deficit	0.02	0.10	0.19	0.026
		optimal - rainfed	0.00	0.09	0.17	0.045
		deficit - rainfed	−0.10	−0.02	0.07	0.923
	50 cm	optimal - deficit	0.02	0.10	0.19	0.026
		optimal - rainfed	0.00	0.09	0.17	0.045
		deficit - rainfed	−0.10	−0.02	0.07	0.924

3.3 EFFECT OF IRRIGATION TREATMENTS ON OLIVE OIL YIELD

Mean fruit yield, oil content and olive oil yield are shown in Fig. 8. Fruit yield and olive oil yield of the different irrigation treatments in each of the two growing seasons reflect the observed differences in θ. However, mean oil content is the highest under deficit irrigation treatment in 2016. In 2017 mean values of oil content appear higher under rainfed and deficit than under optimal irrigation treatment.

The mean olive oil yield with 95 % percentiles for the studied trees is shown in Table 9 in the Appendix. Pairwise comparisons of differences in mean olive oil yield between different irrigation treatments for two growing seasons are shown in Table 7. A linear model accounting for different variances for each treatment was used for each growing season, and statistically significant differences in olive oil yield between treatments were observed (p = 0.022). Pairwise comparisons between treatments in the 2016 season showed statistically significant differences in mean yield between optimal and deficit irrigation treatment (p = 0.045) with a 2.24 l tree^{-1} higher olive oil yield under optimal irrigation compared to deficit (95 % *CI* from 0.06 l tree^{-1} to 4.43 l tree^{-1}). Differences between optimal and rainfed treatment in 2016 season (p = 0.084) were not statistically significant, although olive oil yield has been 1.95 l tree^{-1} 0.53 l tree^{-1} higher under optimal irrigation (Table 7).

A similar pattern was observed in the 2017 growing season. Differences in mean olive oil yield between optimal irrigation and rainfed treatment were statistically significant (p = 0.048), with mean olive oil yield under optimal irrigation being 1.56 l tree^{-1} higher (95 % *CI* from 0.01 l tree^{-1} to 3.31 l tree^{-1}). Differences in mean olive oil yield between optimal and deficit irrigation were nearly statistically significant (p = 0.058), with mean olive oil yield higher under optimal irrigation by 1.50 l tree^{-1} (±0.57 l tree^{-1}).

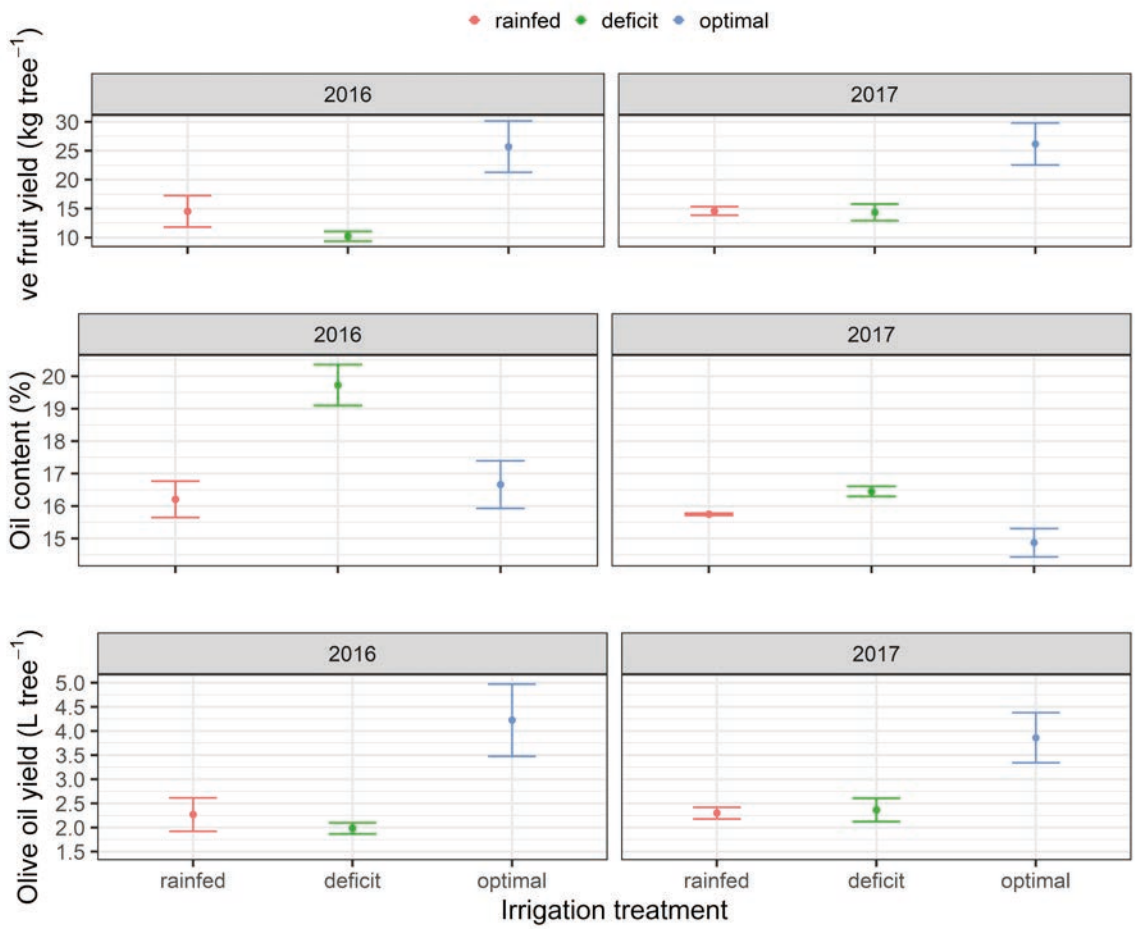

Figure 8: Mean olive fruit yield, oil content and olive oil yield per tree with standard errors for eight olive trees per treatment for the 2016 and 2017 growing seasons

Table 7: Pairwise comparisons of yield (litres of olive oil) between rainfed, deficit irrigation, and optimal irrigation treatment for the 2016 and 2017 growing seasons

Growing season	Contrast	2.5 % percentile (l tree^{-1})	Mean differences (l tree^{-1})	97.5 % percentile (l tree^{-1})	*p*-value
2016	optimal - deficit	0.1	2.2	4.4	0.045
	optimal - rainfed	−0.3	2.0	4.2	0.084
	deficit - rainfed	−1.3	−0.3	0.7	0.709
2017	optimal - deficit	−0.1	1.5	3.1	0.058
	optimal - rainfed	0.01	1.6	3.1	0.048
	deficit - rainfed	−0.7	0.06	0.8	0.967

4 DISCUSSION

Although deficit irrigation is often advantageous compared to rainfed olive groves (Fereres and Soriano, 2007; Fernandes-Silva et al., 2010), it was not superior to rainfed treatment in terms of θ and olive oil yield in the present study. However, a surface drip irrigation system was used in the present study, which, according to Martínez and Reca (2014), results in lower olive oil yields compared to the subsurface irrigation system due to water loss through soil evaporation. A similar observation regarding water evaporation was made for citrus irrigation in Mediterranean climate (Martínez-Gimeno et al., 2018). Caruso et al. (2013), using subsurface drip irrigation, obtained 82 % of olive oil yield with deficit irrigated olives (46–52 % of water supply) compared to optimal irrigation. Potential water savings from switching from surface to subsurface drip irrigation were also described by Bonachela et al. (2001).

Since θ did not differ at any depth under rainfed treatment and deficit irrigation in either growing season (Fig. 4), this raises the question of the effectiveness of such sustained deficit irrigation with a surface drip system. Similar soil water content values between rainfed and deficit irrigation can be explained by advective heat transfer from the dry soil surface surrounding the small wet surface around the surface emitters (Matthias et al., 1986). Bonachela et al. (2001) measured evaporation with microlysimeters and found that it can be as high as 8 mm day^{-1} near the wetter surface (0.2 m from the emitter) and 6 mm day^{-1} at a distance of 0.2 to 0.35 m from the emitter. This is much higher than our maximum estimated daily ET_c^* calculated from the reference ET_0 using Penman-Monteith method and single crop coefficient $K_{c\,mid}$ for olive orchard, a method that assumes complete and uniform soil wetting. An irrigation study conducted on a 9-year-old olive orchard ('Coregiolo') in Australia showed that evapotranspiration during the irrigation was higher in irrigated than in rainfed trees because evapotranspiration was limited in rainfed trees due to low water content in the soil during summer (Zeleke, 2014).

Measured olive oil yields and θ at depths of 10 to 50 cm in two growing seasons, indicate that it is important to measure θ at different depths to assess whether the irrigation system achieves an increase in θ at the root depth (Datta et al., 2017). In our case, it was critical to increase the water content at a depth of 30 to 50 cm to increase the olive oil yield. Relying only on replenishing the estimated ET_c^* with a single crop coefficient and the reference ET_0 value of the previous day or week does not necessarily guarantee an increase in soil water content and thus yield. Estimation of the true ET_c value may be erroneous due to non-uniform soil wetting during surface drip irrigation (Matthias et al., 1986; Bonachela et al., 2001), errors in estimating K_c values when calculating ET_c (Allen et al., 2005), and the distance between the weather station and the location of the irrigated area (Fernández García et al., 2020). The irrigation water used could be wasted, as in our case of surface deficit irrigation. A better estimate of ET_c could be obtained with the double crop coefficient approach, which includes a separate prediction of soil evaporation (Allen et al., 1998). However, this approach could not be used in the present study because daily irrigation data were not available. Dual crop coefficient approach is also more complicated and more computationally intensive, especially because of the determination of daily K_e values for surface evaporation. The total K_c for non-uniformly wetted surfaces can be as high as $K_c = 1.3$ (Allen et al., 1998), which in our case would better correspond to evapotranspiration losses.

Conesa et al. (2021) compared an automated surface drip irrigation system, based on management allowed depletion threshold to trigger irrigation using θ values obtained with multi-depth capacitance sensors, with a conventional irrigation scheduling using estimated ET_c for nectarine trees grown in the Mediterranean region under two water availability scenarios. Similar to our study, irrigation dose based on the 100 % ET_c method did not necessarily increase θ close to FC at a depth of 0.5

m from May to July (unlike an automated system with a threshold trigger). The 100 % ET_c method supplied water only to the upper soil layer.

By measuring soil water content at relevant depths (the main root water uptake zone) with properly installed θ sensors to maintain adequate soil water content during the critical period, we can ensure that the irrigation system replenishes sufficient water, even without knowing and calculating the estimation of the true ET_c values.

5 CONCLUSIONS

This research addresses the influence of different irrigation treatments on the dynamics of soil water content and olive oil yield. A surface drip irrigation system was used in an olive grove in a northern Mediterranean climate, an olive growing area that has not been yet well studied. An increase in soil water content at a depth of 30 to 50 cm, achieved only with optimal irrigation, resulted in significantly higher olive oil yield. In contrast, sustained deficit irrigation did not increase soil water in the layers below 30 cm, despite the addition of water, so the yield was equal to that of rainfed treatment. Therefore it is advisable for olive oil producers to monitor soil water content in layers deeper than 30 cm to verify that enough water was applied to compensate for evapotranspiration losses. Policymakers and legislators should also be aware of the benefits of monitoring soil water content in a given soil layer, especially when deficit surface irrigation is used, as water is wasted if it does not reach the roots at the desired depth. Irrigation scheduling based on estimated ET_c using a single K_c approach can be problematic when using surface drip irrigation systems. In addition, the placement of drip emitters can also be an important contributor to water allocation. The shortcomings of this study are that the experiment was conducted in a single olive grove, with a single olive tree variety, with a specific soil type and a specific configuration of the surface drip irrigation system. Therefore, it is not necessarily transferable to sites with other characteristics. Under different growing conditions, further studies are needed to more accurately determine best irrigation practices, including irrigation system, timing, frequency, water quantity, and to evaluate the effects of different deficit irrigation strategies on olive tree growth, olive oil quantity and quality. Future work should also investigate deficit subsurface drip irrigation in olive groves in the northern Mediterranean climate.

5.1 ACKNOWLEDGMENTS

We would like to thank the Hlaj family for allowing us to perform our study in their olive grove in Dekani. We are grateful to Peter Korpar and our colleagues from the Science and Research Centre Koper, the Institute for Oliveculture for their technical assistance. We would like to thank dr. Miha Curk for his help in creating the map of the experimental site.

5.2 FUNDING

This study was supported by the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia and the Slovenian Research Agency, Project No. V4-1609 and Research Programme P40085.

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7 APPENDIX

Table 8: Model predictions of mean values and 95 % confidence intervals of soil water content of irrigated treatments at different depths for the 2016 and 2017 growing seasons

Growing season	Depth	Treatment	2.5 % percentile ($m^3\ m^{-3}$)	Mean θ ($m^3\ m^{-3}$)	97.5 % percentile ($m^3\ m^{-3}$)
2016	10 cm	rainfed	0.10	0.14	0.17
		deficit	0.13	0.17	0.20
		optimal	0.14	0.17	0.20
	20 cm	rainfed	0.18	0.21	0.25
		deficit	0.20	0.23	0.27
		optimal	0.26	0.30	0.33
	30 cm	rainfed	0.21	0.25	0.28
		deficit	0.20	0.23	0.27
		optimal	0.32	0.35	0.39
	40 cm	rainfed	0.22	0.25	0.29
		deficit	0.21	0.24	0.28
		optimal	0.33	0.36	0.40
	50 cm	rainfed	0.21	0.24	0.28
		deficit	0.22	0.26	0.29
		optimal	0.32	0.35	0.39
2017	10 cm	rainfed	0.16	0.12	0.21
		deficit	0.20	0.16	0.24
		optimal	0.18	0.14	0.22
	20 cm	rainfed	0.24	0.20	0.29
		deficit	0.26	0.22	0.30
		optimal	0.28	0.23	0.32
	30 cm	rainfed	0.28	0.24	0.32
		deficit	0.26	0.21	0.30
		optimal	0.34	0.30	0.39
	40 cm	rainfed	0.29	0.24	0.33
		deficit	0.27	0.23	0.31
		optimal	0.37	0.33	0.42
	50 cm	rainfed	0.29	0.24	0.33
		deficit	0.27	0.23	0.31
		optimal	0.37	0.33	0.42

Table 9: Mean olive oil yield with 95 % percentiles for eight olive trees per treatment for the 2016 and 2017 growing seasons

Growing season	Treatment	2.5% percentile (l $tree^{-1}$)	Mean (l $tree^{-1}$)	97.5 % percentile (l $tree^{-1}$)
2016	optimal	2.5	4.2	6.0
	deficit	1.7	2.0	2.3
	rainfed	1.5	2.3	3.1
2017	optimal	2.6	3.9	5.1
	deficit	1.8	2.4	2.9
	rainfed	2.0	2.3	2.6

doi:10.14720/aas.2024.120.2.17995 Original research article / izvirni znanstveni članek

Global assessment of Algerian honeys quality by palynological, physicochemical analyses, trace elements and potentially toxic elements screening

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Received January 16, 2024; accepted April 04, 2024.
Delo je prispelo 16. januarja 2024, sprejeto 4. aprila 2024.

Global assessment of Algerian honeys quality by palynological, physicochemical analyses, trace elements and potentially toxic elements screening

Abstract: The quality of twenty Algerian honeys was assessed based on their palynological and physicochemical properties, and their trace and toxic elements composition. A qualitative pollen analysis was conducted to estimate the botanical origin. The physicochemical analyses included moisture content, pH, electrical conductivity, 5-hydroxymethylfurfural (HMF), colour, and the content of 3 sugars (fructose, glucose, and sucrose). The analysis of mineral and heavy metals included Zn, Mn, Fe, Cu, Cr, Ni, Pb, Cd, and As. The pollen spectrum showed a great diversity with 60 taxa identified. The palynological analyses revealed the presence of 15 honeys with pollen dominance (unifloral): *Citrus* sp., *Eucalyptus* sp., *Ziziphus* lotus (L.) Lam., *Sinapis arvensis* L., *Dorycnium* sp., *Bupleurum* sp., *Echium* sp., *Lotus* sp., and 5 honeys without pollen dominance (polyfloral). The physicochemical results showed that the samples conform to international quality standards, with few exceptions related to HMF, mainly due to beekeeping practices. The colour was from water white to dark amber. Pb and Cd concentrations were found to be below the maximum residue limits set by the European Directive with which the toxic elements were compared. These results would contribute to the assessment of Algerian honey and provide a database for the regulation of honey trade and consumer protection.

Key words: honey, Algeria, mellisopalynology, quality, toxic elements

Celokupna ocena kakovosti alžirskih medov s palinološkimi in fizikalno-kemijskimi analizami ter pregledom vsebnosti elementov v sledeh in potencialno strupenih elementov

Izvleček: Sestava in kakovost dvajsetih vzorcev alžirskega medu sta bili ocenjeni na osnovi palinoloških in kemijsko-fizikalnih analiz ter vsebnosti elementov sledeh in potencialno strupenih elementov. Kvalitativna analiza peloda v medu je bila narejena z namenom ugotoviti njegovo botanično poreklo. Fizikalno kemijske analize so obsegale določanje vsebnosti vode, pH, električno prevodnost, vsebnost HMF (hidroksimetilfurfural), barve in vsebnost treh sladkorjev (fruktoze, glukoze in saharoze). Analiza elementov in težkih kovin je obsegala analizo vsebnosti Zn, Mn, Fe, Cu, Cr, Ni, Pb, Cd in As. Pelodni spekter je pokazal veliko raznolikost s šestdesetimi ugotovljenimi taksoni. Pri petnajstih vzorcih medu je bila ugotovljena dominanca posameznih rastlinskih vrst oziroma rodov (unifloralni med) kot so: *Citrus* sp., *Eucalyptus* sp., *Ziziphus lotus* (L.) Lam., *Sinapis arvensis* L., *Dorycnium* sp., *Bupleurum* sp., *Echium* sp.in *Lotus* sp., pri petih vzorcih dominance posameznih vrst ni bilo (polifloralni med). Fizikalno-kemijske anlize so pokazale, da vzorci medu ustrezajo mednarodnim standardom kakovosti, z nekaj izjemami, ki se nanašajo na HMF, v glavnem zaradi razlik v čebelarjenju. Barva medu je bila od prozorne do jantrne. Vsebnosti Pb in Cd so bile znantno pod maksimalnimi vrednostmi, ki jih predpisuje Evropska direktiva o vsebnosti strupenih elementov v medu. Rezultati te raziskave prispevajo k oceni alžirskega medu in dajejo osnovne podatke za trgovanje z medom in zaščito potrošnikov.

Ključne besede: med, Alžirija, melisopalinologija, kakovost, strupeni elementi

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1 INTRODUCTION

Honey is a natural sweet substance mainly composed of carbohydrates at around 80–85 % (w/w). Its moisture content is about 15–20 % (w/w), and to a minor extent of about 1 % other components are represented such as organic compounds, inorganic ions, enzymes, vitamins, hormones, flavonoids, proteins, and amino acids. Several nutritional and therapeutic properties like antioxidant, antibacterial and anti-inflammatory activities are recognized for honey due to this composition (Aljohar et al., 2018).

In general, honey quality and its composition are related to several factors such as geographical and botanical origins with their characteristics, and the climatic and seasonal conditions. The Codex Alimentarius (Codex Alimentarius, 2001) as well as the European Community (Council Directive, 2002) have both adopted standards to assess honey quality. These standards established a maximum moisture content of 20 %, and hydroxymethylfurfural (HMF/ a result of fructose degradation) at 40 mg kg^{-1}. These two parameters are considered among the most widely used quality parameters to determine honey stability and freshness.

Certain metals are recognized to be essentials for the different metabolism needs. The following trace elements: iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), chromium (Cr) and nickel (Ni) have several physiological and biochemical activities making them indispensable for good cellular metabolism (APVMA, 2015). However, the presence of these elements in high amounts can have an opposite effect and be harmful instead of.

Nonessential elements, such as lead (Pb), cadmium (Cd) and arsenic (As) are of no use or interest for biological functioning. Moreover, they can be very toxic even at low rates. The contamination of honey by these elements may be due to mining and industrial pollution, or due to the use of Cd- or As-based fertilizers that can be spread into the soil and water and consequently can contaminate plants harvested by bees.

Metal contamination of food is a problem of major concern. In order to protect public health, a limit of 1 and 0.1 mg kg^{-1} for Pb and Cd respectively in honey has been proposed to the European Community (Bal-Prylypko et al., 2018; Bogdanov et al., 2003). On 2015, the European Community, set a limit of 0.1 mg kg^{-1} for Pb for honey consumption by children and persons with specific dietary needs (Commission Regulation (EU), 2015), while there is no specification about the maximum acceptable limit for Arsenic (Bal-Prylypko et al., 2018).

The determination of heavy metals in honey is therefore of great interest, mainly for quality control and nutritional purposes. It is important to note that there are no specific limits established by the Algerian regulation concerning the heavy metals in honey. Consequently, we are required to adhere to the standards of the codex alimentarius or those of the countries where our products are being received.

Honey in Algeria has an excellent reputation and a notable place among Algerian consumers, as it is consumed for both nutritional and therapeutic purposes. The main feature of Algerian honey is its organoleptic quality which is also related to the botanical and geographical origins. Algerian honey possesses comparable and competing advantages, such as a diverse array of flora (cultivated crops, wild plants, forests, and mountains), a range of climates (Mediterranean and Saharan climates) for production, and expansive unpolluted fields. Lots of research have been carried out to evaluate the physicochemical characteristics but a little has been conducted on minerals and toxic elements aspect of Algerian honey. It is therefore crucial to define its quality parameters to establish standards for specific Algerian honey and to protect the consumer from frauds and health risk. The present study aims to assess the quality of Algerian honey according to its botanical origin and to verify its compliance with the standards set by the Codex and the European Community.

Twenty samples of different botanical and geographical origins were analysed. We first carried out a melissopalynological analysis to determine the pollen spectrum and botanical origin, a physicochemical analysis to determine the corresponding quality: colour, moisture, acidity, pH, electrical conductivity, and HMF. Sugars (fructose, glucose, and sucrose) were also determined. Finally, certain trace elements and heavy metals (Zn, Mn, Fe, Cu, Cr, Ni, Pb, Cd, and As) were analysed to certify the safety of these honeys according to the European Directive.

2 MATERIALS AND METHODS

2.1 HONEY SAMPLES

Twenty honey samples (250 g each); produced in Algeria; were collected from beekeepers over two campaigns in 2020 and 2021. Collected samples were stored at a temperature of +4 °C in the refrigerator throughout the analysis process. The location of samples according to their geographical origin is shown in Figure 1.

Samples Nbr	Locality	Samples Nbr	Locality
E1/E32	Tessala El Mardja/ Beni Messous/ Alger	E39	Chahbounia/Médéa
E2/E41	Boufarik/ Blida	E40	Tébessa
E31	El Bayadh	E42	Naama
E33	Kolea/ Tipaza	E44/E46	Chlef
E34/E38/E51/E69	Aflou /Laghouat	E71	El Taref
E35	Guelma	E74	Mostaganem
E36	Djelfa	E78	Jijel

Figure 1: Location of honey samples (presented in map and list)

2.2 METHODS OF ANALYSIS

2.2.1 Qualitative pollen analysis

Pollen grains were identified and counted according to the harmonized methods of melissopalynology described by Louveaux et al. (1977) and Von Der Ohe et al. (2004). This method establishes all the pollen types present by determining their pollen frequencies expressed as a percentage relative to the total number of pollen grains counted (500 grains in our case). The pollens are then divided into four pollen frequency classes: predominant pollens (+ 45 %); secondary pollens (16-45 %); important minor pollens (3-15 %); and minor pollens (< 3 %). The pollen grains were observed using an optical microscope, and pollen types were identified by comparing them with the pollen reference established by (Ricciardelli d'Albore, 1998). We have also used our reference collection of pollen from plants with recognized scientific and local names. The pollen grains were classified as pollen type, as a genus, or as a single species whenever it was possible (Louveaux et al., 1977).

Taxa distribution frequencies have been calculated according to the number of honey samples in which they are found. As explained by Feller-Demalsy et al. (1989), they are classified as very frequent taxa (+ 50 %); frequent taxa (20-50 %); rare taxa (10-20 %); sporadic taxa (-10 %).

2.2.2 Physicochemical analysis

Except for colour, all physicochemical parameters (water content, HMF, pH, free acidity, and electrical conductivity) were analysed following the analysis techniques recommended by the International Honey Commission (IHC) published by Bogdanov et al. (2004) and updated by Bogdanov (2009) .

Water content was measured by refractive index using an ATAGO NAR-3T refractometer. Electrical conductivity was determined by using a conductivity meter (CORNING). pH and free acidity were measured by using a HANNA pH meter. HMF was measured by a spectral method using a CECIL CS-3041 UV-Vis spectrophotometer. Colour measurement was performed according to Bianchi method as described by Lacerda et al. (2010) and Ferreira et al. (2009), a 50 % (w/v) honey solution was prepared with warm water between 45 ° and 50 °C, then filtered to remove any coarse particles and measured by absorbance reading at 635 nm. Colour intensity was determined using the Pfund scale according to the equation (1):

$$Pfund = - 38.70 + 371.39\ x\ Abs \quad (1)$$

The carbohydrate profile was determined using an Agilent 1260 Infinity II HPLC equipped with a DAD detector and Open Lab CDS data processing software, sugars were separated using an Ammonia (NH_2) USP L8 analytical column (25 cm _ 4.6 mm, 5 mm i. d.). Standard solutions of fructose (2 g %), glucose (2 g %), and sucrose (0.5 g %) were prepared in ultrapure water, and sample preparation was carried out by dissolving 2 g of honey in 20 ml distilled water. The analysis procedure was conducted following Aljohar et al. (2018). Identification of sugars, their peaks and their concentrations was made possible by comparing their chromatograms, retention times, and the surfaces of their peaks to those obtained from standard sugar solutions.

2.2.3 Trace and toxic elements analysis

Six minerals and three heavy metals were the subject of our study. 200 mg of previously homogenized sample to which 7 ml HNO_3 (65 %) and 1 ml H_2O_2 (30 %) were added, the mixture was placed in the digestion microwave (Ethos Easy - Milestone Connect microwave). The concentration of each analyte was determined in the sample and blank solutions using an ICP-MS inductively coupled plasma mass spectrometer (ICAP-RQ Thermo Scientific). The results were expressed as mg kg^{-1} of honey for Zn, Mn, Fe, Cu, Cr, and Ni and per µg kg^{-1} of honey for Cd, Pb, and As.

2.2.4 Statistical analysis

The results were reported as a mean ± standard deviation. To investigate correlations among several variables, including physicochemical parameters, mineral concentrations, and toxic metal levels, a Spearman correlation analysis was performed and a graphic was generated using a corrplot package. Multiple factor analysis (MFA) was performed using the FactoMineR on a data frame containing several sets of qualitative and quantitative variables structured into three groups; physical, mineral (trace elements), and heavy metal (toxic elements). This analysis aims to describe the characteristics of the honey samples spread over two groups: honey with pollen dominance (unifloral) and honey without pollen dominance (polyfloral). All statistical analyses were carried out using R software (version 4.2.2).

3 RESULTS AND DISCUSSION

3.1 QUALITATIVE POLLEN ANALYSIS

Sixty taxa were identified for the twenty honey samples analysed, of which 55 were nectariferous, five were nectarless, and of which 20 were sporadic, 22 were rare, 13 were frequent, and 5 were very frequent (Table 1).

From the distribution of identified taxa, the presence of 18 pollen types; corresponding to the classes frequent and very frequent; are represented in the four pollen frequency classes (Figure 2).

The pollen analysis (Tab. 2 and 3) highlighted the presence of 15 unifloral honeys: *Sinapis arvensis* L., *Citrus s*p., *Eucalyptus* sp., *Ziziphus lotus* (L.) Lam., *Echium* sp., *Bupleurum* sp., Fabaceae honey with the genera of *Dorycnium* sp. and *Lotus* sp. and one unifloral honey of *Peganum harmala* L.. The 05 remaining samples were polyfloral honey composed of secondary pollens from 1 to 3 taxa maximum.

Table 1: Distribution frequencies of taxa in the 20 samples of honey

Relative frequency classes (%)	Taxa
Sporadic < 10%	Anthylis (5 %), Araceae (5 %), *Bubplerum* sp. (5 %), Buxaceae (5 %), *Castanea* sp. (5 %), Chenopodiaceae (5 %), Cucurbitaceae (5 %), *Daucus carota* L. (5 %), Dipsacaceae (5 %), *Dorycnium* sp. (5 %), Ephedraceae (5 %), Geraniaceae (5 %), *Melilotus* sp. (5 %), Orobanchaceae (5 %), Palmeae (5 %), Ranunculaceae (5 %), Raffeciaceae (5 %), Rubiaceae (5 %), *Salix* sp. (5 %), *Sophora* sp. (5 %)
Rare 10-20 %	Annacardiaceae (10 %), Annonaceae (10 %), Betulaceae (10 %), *Borago officinalis* L. (10 %), Cupressaceae (10 %), *Echium* sp. (10 %), *Erica* sp. (10 %) Liliaceae (10 %), *Lotus* sp. (10 %), Malvaceae (10 %), *Muscari* sp. (10 %), *Ononis* sp. (10 %), *Sinapis arvensis* L. (10 %), Smilacaceae (10 %), *Taraxacum* sp. (10 %), Fagaceae (15 %), *Hedysarum coronarium* L. (15 %), *Peganum harmala* L. (15 %), *Senecio vulgaris* L. (15 %),Verbenaceae (15 %), *Citrus* sp. (20 %), Salicaceae (20 %)
Frequent 20-50 %	*Diplotaxis erucoides* (L.) DC. (25 %), *Ziziphus lotus* (L.) Lam. (25 %), Boraginaceae (30 %), Mimosaceae (30), Myrtaceae (30 %), *Oxalis* sp. (30 %), Brassicaceae (35 %), Ericaceae (35 %), Oleaceae (35 %), Polygonaceae (35 %), Poaceae (40 %), *Eucalyptus* sp. (50 %), *Euphorbia* sp. (50 %)
Very frequent > 50 %	Fabaceae (60 %), Lamiaceae (60 %), Asteraceae (70 %), Rosaceae (75 %), Apiaceae (90 %)

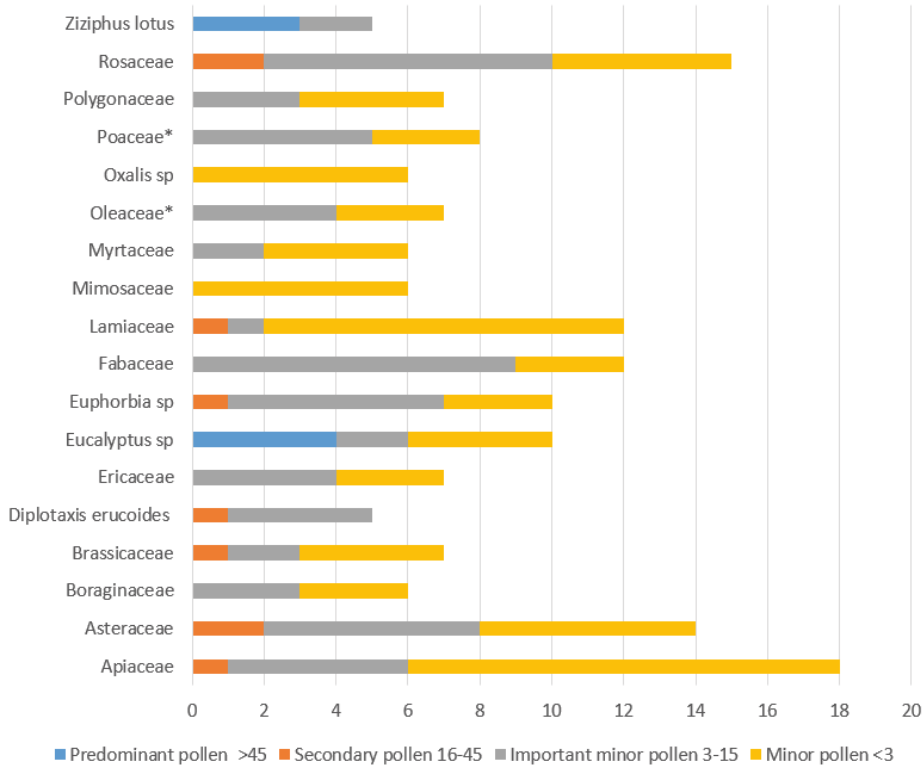

Figure 2: Presence of 18 pollen types; corresponding to frequent and very frequent classes; represented in four classes of pollen frequency: predominant pollen > 45 %, secondary pollen (16–45 %), important minor pollen (3–15 %) and minor pollen < 3 %. *Nectarless species

Samples E2 and E41 showed *Citrus* sp. frequencies of 30 % and 21 % respectively, Persano Oddo et al. (2004) reported that citrus pollen (*Citrus* sp.) is under-represented to a degree more or less important according to the different species or cultivars. A minimum frequency of citrus pollen varying from 10 to 20 % is accepted to be considered as citrus honey (Louveaux et al., 1977; Reyes, 2017; Seraglio et al., 2021). In addition to that, the physicochemical and sensory properties of these samples are in agreement with those described by Persano Oddo & Piro (2004) for citrus honey. In a study covering Mitidja zone, Benaziza-Bouchema et al. (2010) presented values ranging from 21 to 69 % for citrus honey, these values corroborate very well with our aforementioned values.

The eucalyptus pollen content ranged between 70 to 73 %. According to Persano Oddo & Piro (2004) eucalyptus pollen is overrepresented in honeys. Our results corroborate very well with those of (Benaziza-Bouchema & Schweitzer, 2010) and (Makhloufi et al., 2010) reporting both values of eucalyptus pollen more than 70 %.

The *Ziziphus lotus* pollen content ranged between 52 and 89 % of the pollen spectra. These values are consistent with those reported by Mekious et al. (2015); Zerrouk et al. (2017) for 45.75 to 97.12 % and 45.3 to 93.7% respectively.

3.2 PHYSICOCHEMICAL ANALYSES

The results of the physicochemical analyses are summarized in Table 4. They are presented according to the results of the pollen analysis. Hence, we distinguish two groups, the unifloral honey group (whose pollen spectrum includes dominant pollen) and the polyfloral honey group (whose pollen spectrum does not include any dominant pollen).

Electrical conductivity values; for all samples; were between 0.12-0.68 mS cm^{-1}. A maximum of 0.8 mS cm^{-1} is established by the Codex Alimentarius for nectar honey, which is the case for all our samples.

The highest pH mean value was registered for unifloral honey group with 4.09. According to Gonnet (1986), nectar honeys, or a mixture of nectar and honeydew honeys have a pH between 3.5 and 4.5, honeydew honeys have a pH of 4.5 and 5.5. Our samples were therefore nectar honey. The range of pH values for all samples was 3.41-5.07. The high pH values obtained were due to

Table 2: Predominant and secondary pollen types in 20 honey samples

Sample Number	Predominant pollen (>45 %)	Secondary pollen (16-45 %)
E2	-----	*Citrus* sp. (30 %), *Sinapis arvensis* L. (18 %)
E41	-----	*Citrus* sp. (21 %), *Diplotaxis erucoides* (L.) DC. (20 %), *Lotus* sp. (17 %)
E32	*Eucalyptus* sp. (71 %)	-----
E44	*Eucalyptus* sp. (70 %)	-----
E74	*Eucalyptus* sp. (73 %)	-----
E78	*Eucalyptus* sp. (71 %)	-----
E36	*Ziziphus lotus* (L) Lam. (52 %)	-----
E46	*Zyziphus Lotus* (L) Lam. (81 %)	-----
E69	*Zyziphus Lotus* (L.) Lam (89 %)	-----
E1	*Sinapis arvensis* L. (83%)	-----
E31	*Dorycnium* sp. (72 %)	-----
E33	*Echium* sp. (85 %)	-----
E35	*Bupleurum* sp. (62 %)	-----
E38	*Lotus* sp. (46 %)	-----
E39	*Peganum harmala* L. (81 %)	-----
E34	-----	*Peganum harmala* L. (33 %), Rosaceae (21 %), *Euphorbia* sp. (19 %)
E40	-----	*Melilotus* sp. (28 %)
E42	-----	*Anthylis* sp. (38 %), *Senecio vulgaris* L. (27 %)
E51	-----	Apiaceae (23 %), Rosaceae (21 %), Brassicaceae (20 %)
E71	-----	Lamiaceae (38 %), *Erica* sp. (33 %)

the presence of jujube honey which is characterized by a high pH value (5.1 for E69) such a value is also reported by Mekious et al. (2015) and Zerrouk et al. (2017) with 5.17 ± 0.48 and 5.5 ± 0.6 for jujube honey respectively.

The highest free acidity mean value was registered for the polyfloral honey group with 25.03 meq kg^{-1}. Whereas, free acidity values for all samples ranged from 10.25-34 meq kg^{-1}. The (Codex Alimentarius, 2001; Council Directive, 2002) set a maximum limit of 50 meq kg^{-1} of honey. Our samples, therefore, complied with international standards showing an absence of undesirable fermentations.

Moisture content for all samples was in the range of 13.6 to 18.25 %. A high value was registered for unifloral honey group with 16.25 %. The water content provides information on the maturity of the honey and also determines its conservation (Terrab et al., 2003). A low water content preserves the honey against microbial development (Bogdanov, 2009). Whereas, with a high water content, honey tends to ferment easily (El Sohaimy et al., 2015).

The HMF values for all honey samples ranged between 0-184.7 mg kg^{-1}. Unifloral honey showed the highest mean value with 27.67 mg kg^{-1}. Indeed, three samples from this group showed values above the limit set by the European and international standards (40 mg kg^{-1} of honey), namely E1 and E46 for 59.0 and 50.5 mg kg^{-1} respectively, and even more than the recommended limit set for honeys from tropical climate and blends of these honeys (80 mg kg^{-1} of honey) (Council Directive, 2002) for E44 with 184.7 mg kg^{-1}. The HMF does not occur in newly harvested honey but its content rises through conditioning and storage. To prevent the granulation of honey and also to decrease its viscosity, beekeepers usually, tend to warm it during the harvesting process. The quality of honey is not affected at temperatures of 32–40 °C. However, the application of higher temperatures tends to increase the HMF levels in honey (Anklam, 1998) . HMF is therefore considered an indicator of freshness and/or overheating of honey.

In general, and for high-quality honey, it is recommended a maximum moisture content of 18 % and an HMF rate of no more than 15 mg kg^{-1} of honey. At these rates, the risk of fermentation is avoided and the honey remains fresh until its final consumption (Schweitzer, 1998). The results of these two parameters for our samples showed a maximum water content of 18.3 % which indicates respect for the honey maturity process before harvesting and compliance with international standards. Whereas, the HMF values were above the level for three samples reaching a maximum value of 184.7 mg kg^{-1} of honey. Knowing that consumption of HMF may create certain health problems such as irritation of the mucous membranes of the upper respiratory tract, eyes, skin, etc… if it is consumed beyond the recommended limits (Pastoriza de la Cueva et al., 2017; Shapla et al., 2018). HMF can also be metabolized to 5-sulfooxymethylfurfural (SMF) by sulfotransferases (Pastoriza de la Cueva et al., 2017), the SMF is an intermediate molecule that can bind to DNA and induce mutagenic effects. Svendsen et

Table 3: Important minor and minor pollen types in 20 honey samples

Sample Number	Important minor pollen (3-15 %)	Minor pollen (< 3 %)
E2	Oleaceae (13 %), Polygonaceae (11 %), Dipsacaceae and Rosaceae (7 %), Fabaceae (4 %), Apiaceae and Asteraceae (3 %)	*Salix* sp., Poaceae, Boraginaceae, Ericaceae, Lamiaceae, Liliaceae, Malvaceae, Myrtaceae, Orobanchaceae, *Oxalis* sp.,
E41	Boraginaceae (7 %), Oleaceae (6 %), *Senecio vulgaris* L. and Fabaceae (5 %), Rosaceae (4 %), *Eucalyptus* sp. and Polygonaceae (3 %)	*Oxalis* sp., Rubiaceae, Smilacaceae, Annonaceae, Apiaceae, Malavaceae, Geraniaceae,
E32	*Hedysarum coronarium* L. (10 %), Ericaceae (7 %), *Euphorbia* sp. (5 %), Asteraceae (4 %)	Brassicaceae, Apiaceae, Lamiaceae, Mimosaceae
E44	Oleaceae (10 %) Salicaceae and Poaceae (5%), *Hedysarum coronarium* L. (4 %), Brassicaceae (3 %)	Apiaceae, Mimosaceae, Asteraceae, Betulaceae, Ephedraceae, Fagaceae, Lamiaceae, *Oxalis* sp., Rosaceae, Verbenaceae,
E74	Oleaceae (13 %), *Erica* sp. (8 %), Fabaceae (5 %)	Apiaceae, Asteraceae
E78	Fabaceae (13 %), Apiaceae (6 %), Rosaceae (5 %), Ericaceae (3 %)	*Taraxacum* sp., *Castanea* sp., Lamiaceae
E36	Rosaceae (14 %), Poaceae (13 %), Fabaceae (6 %), Asteraceae and Myrtaceae (3 %)	Apiaceae, Araceae, Brassicaceae, *Euphorbia* sp., Boraginaceae, Lamiaceae, Mimosaceae
E46	Apiaceae (8 %), *Hedysarum coronarium* L. (6 %), Asteraceae (5 %)	------
E69	*Ononis* sp. (5 %), *Euphorbia* sp. (3 %)	Apiaceae, Fagaceae, Rosaceae, Asteraceae, *Borago officinalis* L.
E1	*Citrus* sp. (11 %), Fabaceae (4 %)	Apiaceae, Oleaceae, Rosaceae, Myrtaceae, Poaceae, *Taraxacum* sp.
E31	*Echium* sp. and Polygonaceae (8 %), Palmeae and Rhamnaceae (3 %)	*Euphorbia* sp., Poaceae, Apiaceae, Asteraceae, Brassicaceae, Lamiaceae
E33	Fabaceae, Salicaceae (4 %), Asteraceae (3 %)	Ericaceae, *Eucalyptus* sp., Lamiaceae, O*xalis* sp., Apiaceae, Brassicaceae
E35	Rosaceae (13 %), *Eucalyptus* sp. (11 %), Asteraceae (5 %), Brassicaceae, Ericaceae (3 %)	Lamiaceae, *Oxalis* sp., Boraginaceae, Buxaceae, Mimosaceae
E38	Poaceae (13 %), *Diplotaxis erucoides* (L) DC. (9 %), *Sophora* sp. (8%), *Muscari sp.*, Rosaceae (5 %), Apiaceae, Asteraceae and Boraginaceae (4 %)	Annonaceae, Fabaceae, *Euphorbia* sp., Betulaceae, Lamiaceae, Myrtaceae
E39	*Diplotaxis erucoides* (L) DC. (6 %), Annacardiaceae (5 %), *Euphorbia* sp. (4 %)	Oleaceae, Smilacaceae, Asteraceae, Fabaceae, Apiaceae, Rosaceae
E34	*Ziziphus lotus* (L) Lam. (6 %), *Diplotaxis erucoides* (L) DC. (5 %), Poaceae (4%), Ericaceae (3 %)	Asteraceae, Fabaceae, Apiaceae, Cucurbitaceae, Polygonaceae, Cupressaceae, Liliaceae, Salicaceae, Verbinaceae
E40	Fabaceae, Myrtaceae (13 %), Apiaceae, Boraginaceae (9 %), Anacardiaceae (7 %), Lamiaceae (5 %), *Diplotaxis erucoides* (L) DC., Rosaceae (4 %), *Euphorbia* sp., Ranunculaceae (3 %)	Asteraceae, Polygonaceae, Ericaceae, Fagaceae, Mimosaceae
E42	*Euphorbia sp.*, Rosaceae (8 %), Brassicaceae (5 %), Chenopodiaceae, Poaceae (4 %)	*Borago officinalis*, Apiaceae, Cupressaceae, *Oxalis* sp., Polygonaceae
E51	Salicaceae (10 %), *Euphorbia* sp., *Peganum harmala* (L). (6 %), *Senecio vulgaris* L.(5 %), *Muscari* sp. (3 %)	Lamiaceae, Myrtaceae, Oleaceae, Rafflesciaceae, *Citrus* sp., *Ononis sp.*, Polygonaceae, Verbenaceae
E71	*Eucalyptus* sp. (12 %), Asteraceae, Fabaceae (5 %), *Daucus carota* L. (3 %)	Rosaceae, Mimosaceae

Table 4: Results of physicochemical parameters and sugar profile

Physicochemical Parameters	Unifloral honey (n = 15)	Polyfloral honey (n = 5)	Min-Max
EC (mS cm^{-1})	0.30 ± 0.16	0.26 ± 0.14	0.12 - 0.68
pH	4.09 ± 0.44	3.81 ± 0.31	3.41 - 5.07
Free acidity (meq kg^{-1})	22.88 ± 7.16	25.03 ± 8.45	10.25 - 34.40
Moisture (%)	16.25 ± 1.36	15.42 ± 1.41	13.60 - 18.25
HMF (mg kg^{-1})	27.67 ± 46.42	7.37 ± 4.5	0.00 - 184.70
Color (PFund)	86.78 ± 67.34	95.07 ± 86.1	5.68 - 210.13
	(Amber)	(Amber)	(Water white - dark amber)
Fructose (%)	34.21 ± 2.49	33.28 ± 1.34	28.90 - 39.51
Glucose (%)	32.24 ± 2.99	33.40± 2.21	27.63 - 37.49
F+G (%)	66.45 ± 3.87	66.69 ± 4.58	61.38 - 76.06
Sucrose (%)	3.15 ± 2.26	2.44 ± 2.2	0.68 - 6.39

al. (2009) reported in their study on rats that HMF and SMF could be initiators of colon cancer.

Hence the interest in drawing beekeepers' attention to this parameter and the importance of respecting the levels proposed for those who want to distinguish their honey by a qualitative approach.

Color values are presented in Pfund values (mm) and classified in a scale going from water white to dark amber. The unifloral and polyfloral honey groups showed mean values of 86.78 and 95.07 mm, corresponding both of them to amber. The extreme values of color ranged from 5.68–210.13 mm corresponding to water white to dark amber. The variation in color for the different honey samples is due to several factors including among others: the variation in the sources of nectar (Kuś et al., 2014), the electrical conductivity, the richness of the honey in minerals, and the storage conditions (González-Miret et al., 2005; Naab et al., 2008), as well as the composition of honey in phenolic compounds and their antioxidant power (Bertoncelj et al., 2007).

The study of the carbohydrate profile provides several information, such as possible fraud attempts through adulteration, which results in an increase in HMF, the classification of monofloral honeys (Persano Oddo & Piro, 2004), or the tendency of honey to crystallize; in fact, the ratios F/G (Fructose/Glucose) and G/E (Glucose / Water) are considered criteria for predicting the tendency of honeys to crystallize. High F/G levels suggest that honeys are more likely to remain liquid. For the G/E ratio, results equal to or less than 1.7 indicate liquid honey, while values equal to or greater than 2.1 predict rapid granulation (Doner, 1977).

The extreme values of fructose were 28.90–39.51 % with a high value registered for unifloral honey group (34.21 %), whereas glucose extreme values obtained ranged from 27.63-37.49 % with a high value registered for polyfloral honey group (33.4 %). In general, our data for glucose corroborate with those quoted by Makhloufi et al. (2010), Haderbache et al. (2013); Ouchemoukh et al. (2010), Mekious et al. (2015) and Zerrouk et al. (2017) who mentioned values ranging from 25.47-33.89 %. Contrariwise, the mean values of fructose are lower compared to those of the aforementioned authors who provided values ranging from 35.50-42.10 %, regardless of the type of honey studied. Nevertheless, fructose and glucose values obtained in our study corroborate with those of Gonnet (1971) who specifies that the sugar content of honey varies from 32-46 % for fructose and from 26-41 % for glucose. Molan (1996) reported that the nectar composition of plants influences the proportions of these two major sugars. Also, Mateo et al. (1998) reported that the sugar profile of honey depends greatly on the types of flora foraged by the bees, by regional and climatic conditions. In general and according to White et al. (1979), fructose predominates over glucose. This finding is confirmed in our study.

The total sugar content ranged from 61.38 %-76.06 %, with similar mean values to both groups (66.45 and 66.69 %). These values are in agreement with the standards of Codex Alimentarius (2001); Council Directive (2002) requiring a rate of more than 60 % for nectar honey.

Sucrose content oscillated between 0.68 %-6.39 %. The highest value was recorded for unifloral honey group with 3.15 %. (Anklam, 1998) explained that honeys of the same floral source can vary due to seasonal climatic variations or to a different geographical origin. The Codex alimentarius standard specifies 5 % of sucrose for all varieties of honey, with the exception of 10 % for *Banskia*, *Citrus*, *Hedysarum, Medicago*, and honeys, and of 15% for *Lavandula* honey. However, the high sucrose content (6.39 %) found in our study corresponds to *Ziziphus* honey. This latter is not among the honeys mentioned as exempted. Indeed, its high value could be due to different reasons such as overfeeding bees with sucrose syrup, adulteration, or harvesting honey early, where the sucrose has not been fully transformed into glucose and fructose (Anklam, 1998; Azeredo et al., 2003; Guler et al., 2007). However, all samples showed values corroborating to those of (Benaziza-Bouchema & Schweitzer, 2010) (between 0-7.6 %) and also remain below 10 %, a limit mentioned by Bocquet (1997) for sucrose.

3.3 TRACE AND TOXIC ELEMENTS ANALYSIS

All the minerals and heavy metals identified in honey samples are listed in Table 5. The mean mineral concentrations in the different honey groups were expressed by mg kg^{-1} for Cr, Mn, Fe, Ni, Cu, and Zn and by µg kg^{-1} for As, Pb, and Cd, the concentrations of the two later were compared to the maximum allowable contaminant levels established by the Commission Regulation (EU) (2015) and proposed by Bogdanov et al. (2003).

Considering the average value of all samples, the most abundant trace elements were Fe followed by Zn, Ni, Cu, Cr, and Mn. To the best of our knowledge, few studies were conducted on minerals and toxic elements in honeys in Algeria. (Haderbache et al. in 2013, Yaich Achour and Khali (2014) and Zerrouk et al. (2017).

Yaiche Achour et al. (2014) and Zerrouk et al. (2017) reported higher values than ours for Fe (6.37 and 6.3 mg kg^{-1}) respectively for jujube honey. Whereas, (Haderbache et al., 2013) reported values of (0.923 and 0.969 mg kg^{-1}) for jujube and multifloral honeys. These results are quite lower than our results.

Regarding Zn, Yaiche Achour & Khali (2014) and Zerrouk et al. (2017) reported values of 11.04 mg kg^{-1} for all types of honey and 1.8 mg kg^{-1} for jujube honey respectively. Although, unifloral honey group; in our study; showed a mean value quite similar to that of jujube honey.

The values of Ni reported by Haderbache et al. (2013) and Yaiche Achour & Khali (2014) are (0.0234, 0.0307 mg kg^{-1}) and (0.32 mg kg^{-1}) for jujube and multifloral honeys and for all types of honeys respectively. The Ni concentration value obtained in our study for unifloral honey group was quite similar to that reported by Yaiche Achour & Khali (2014). Whereas, polyfloral honey group exhibited a higher value than the previous studies.

Cu was studied only by Yaiche Achour & Khali (2014), their obtained values were in the range of 2.72-3.22 mg kg^{-1}, these values are higher than our results for both honey groups.

The obtained concentration values of Cr were higher than the reported one by Yaiche Achour and Khali (2014) 0.023 mg kg^{-1} for all types of honey. Whereas, for the Mn, the observed values were lower than the values of Haderbache et al. (2013) and Yaiche Achour & Khali (2014) (0.077, 0.069 mg kg^{-1}) for jujube and multifloral honeys and (3.06 mg kg^{-1}) for all types of honey respectively.

Regarding the toxic elements Haderbache et al. (2013) reported for Pb values lower than ours. While, Yaiche Achour and Khali (2014) reported higher values. (9.2, 16.3 mg kg^{-1}) for jujube and multifloral honeys and (0.22 mg kg^{-1}) for all types of honey respectively.

The Cd concentration values were much lower than those observed for Haderbache et al. (2013) and Yaiche Achour and Khali (2014) with (10.7, 13.9 mg kg^{-1}) for jujube and multifloral honeys and (0.018-0.019 mg kg^{-1}) for all types of honey respectively.

As concentration mean values; obtained in our study; were different from the values reported by Yaiche Achour and Khali (2014), (0.020-0.024 mg kg^{-1}) as a mean range for all type of honeys.

Considering previous investigations on honeys conducted in different country in Europe and China, the average value of Fe was 1.89 mg kg^{-1} (ranging from 0.25-21.54 mg kg^{-1}). Quite similar values were observed in Italy with 1.265 and 1.75 mg kg^{-1} for polyfloral and sweet chestnut honey (Buldini et al., 2001). Bilandžić et al. (2014); Hernández et al. (2005) reported higher values of Fe (4.85 and 3.61 mg kg^{-1}) comparatively to our results for honey produced in Spain and Croatia respectively.

Zn was the second most abundant trace element, with an average of 0.90 mg kg^{-1} (ranging from 0 - 4.46 mg kg^{-1}). The mean value of Zn was lower than those found in previous investigations in Croatia (1.69 and 1.17mg kg^{-1}) (Bilandžić et al., 2014; Lachman et al., 2007), Italy (2.64 and 3.205 mg kg^{-1}) (Buldini et al., 2001), Spain (1.57 and 1.441–4.496 mg kg^{-1}) (Fernandez-Torres et al., 2005; Hernández et al., 2005) respectively and China (1329.5 µg kg^{-1}) (Ru et al., 2013).

The Ni mean concentration for all honeys was 0.54 mg kg^{-1} (ranging from 0-3.57 mg kg^{-1}). The observed value was higher than the reported values in Italy (0.10-03.22 mg kg^{-1}) (Squadrone et al., 2020).

The Cu mean concentration for all honeys was 0.17 mg kg^{-1} (ranging from 0-2.92 mg kg^{-1}). This mean level was much lower than those observed in Italy (890 µg kg^{-1} and 0.30-0.95 mg kg^{-1}) (Buldini et al., 2001; Squadrone et al., 2020) respectively, Spain (0.37, < 0.531–0.693 mg kg^{-1}) (Fernandez-Torres et al., 2005; Hernández et al., 2005) respectively, Croatia (0.42 and 14.4 mg kg^{-1}) (Bilandžić et al., 2014; Lachman et al., 2007) respectively. The mean

Table 5: Results of minerals and heavy metals analyses

	Unifloral honey (n = 15)	Polyfloral honey (n = 5)	Min-Max
Cr (mg kg^{-1})	0.07 ± 0.06	0.13 ± 0.11	0.00 - 0.33
Mn (mg kg^{-1})	0.04 ± 0.06	0.02 ± 0.01	0.01 - 0.24
Fe (mg kg^{-1})	2.15 ± 5.38	1.09 ± 0.65	0.25 - 21.54
Ni (mg kg^{-1})	0.31 ± 0.47	1.22 ± 1.39	0.00 - 3.57
Cu (mg kg^{-1})	0.22 ± 0.75	0.03 ± 0.01	0.00 - 2.92
Zn (mg kg^{-1})	1.14 ± 1.38	0.19 ± 0.16	0.00 - 4.46
As (µg kg^{-1})	16.48 ± 37.05	191.16 ± 303.45	0.00 - 718.09
Cd (µg kg^{-1})	0.99 ± 2.82	3.48 ± 3.64	0.00 - 11.00
Pb (µg kg^{-1})	75.11 ± 98.46	42.69 ± 22.33	3.17 - 357.19

Cu content obtained was within the range of that found in honey from Croatia (0.14-1.39 mg kg^{-1}) (Bilandzic et al., 2017).

The Cr mean concentration for all honeys was 0.09 mg kg^{-1} (ranging from 0-0.33 mg kg^{-1}). This mean level fell within the range reported from Italy (0.068-0.093mg kg^{-1}) (Squadrone et al., 2020) but was higher than the range reported for honey from Croatia (4.97-27.6 µg kg^{-1}) (Bilandzic et al., 2017).

The Mn mean concentration for all honeys was 0.04 mg kg^{-1} (ranging from 0.01-0.24 mg kg^{-1}). This mean level was much lower than the ranges reported from Croatia and Italy (0.19-1.98 and 0.61-3.2 mg kg^{-1}) (Bilandzic et al., 2017; Squadrone et al., 2020) respectively.

In general, mineral elements come from the soil and end up in honey through plant nectar (Solayman et al., 2016). The variability in mineral content can be attributed to environmental, botanical, and geographical factors, or even beekeeping practices (Bogdanov, 2006; Sixto et al., 2019). The mean values of the total Cr, Mn, Fe, Ni, Cu and Zn concentrations were in the range of 2.7 mg kg^{-1} (polyfloral)-3.93 mg kg^{-1} (unifloral). These results were also in the range reported by (Squadrone et al., 2020) who mentioned values of 3.4 mg kg^{-1} (acacia)–7.0 mg kg^{-1} (multifloral). Such values indicate the contribution of these essential elements in the nutritional aspects of honey.

It is well known that lead is the most widespread heavy metal in the environment with potential toxicity. It has the potential to induce gradual poisoning and health complications like exhaustion, insomnia and body mass loss. Based on our study, the lead concentrations were high compared to As and Cd. The Pb mean concentration for all honey was 77.54 µg kg^{-1} (ranging from 3.17-357 µg kg^{-1}). The obtained Pb value in our study was lower than those found in honey from Italy (620 µg kg^{-1} for polyfloral honey) (Buldini et al., 2001), Croatia (530 µg kg^{-1}). While Bilandzic et al. (2017); Ru et al. (2013); Squadrone et al. (2020) reported values lower than ours with 33.98, 5.03–66.3, and 12.71 µg kg^{-1} for honeys from China, Croatia and Italy respectively.

The Cd mean concentration for all honeys was 1.02 µg kg^{-1} (ranging from 0.0-11 µg kg^{-1}). This obtained Cd value was almost similar to those obtained from China and Croatia (1.34 and 1.84 µg kg^{-1}) (Bilandžić et al., 2014; Ru et al., 2013) respectively. The Cd content found in this study was lower than those found in honey from Italy, (305 µg kg^{-1}) (Buldini et al., 2001).

The As mean concentration for all honeys was 60.15 µg kg^{-1} (ranging from 0.0-718.09 µg kg^{-1}). The obtained As concentration was much higher than those reported for honeys from Italy (7.7-17 µg kg^{-1})(Squadrone et al., 2020), China (13.44 µg kg^{-1}) (Ru et al., 2013) and Croatia (0.62-6.95 µg kg^{-1}) (Bilandzic et al., 2017) and lower than those reported from Croatia (140.7 µg kg^{-1}) (Bilandžić et al., 2014).

Pb and Cd are considered the most toxic heavy metals. The Codex Alimentarius (2001) stipulates that "honey should only contain heavy metals at levels that do not pose a risk to human health". The European Union proposed limits of 1.0 and 0.1 mg kg^{-1} for Pb and Cd respectively (Bogdanov et al., 2003).

High heavy metal values have several causes. Lead, as the most widespread metal, is mainly released into the air and then found in many products after being mixed with soil and thus penetrates plants, but in general, Pb is not transported by plants.

Cadmium and due to its use in a wide different industrial processes; notably the metallurgical industry and incinerators (Yao et al., 2019); is released into the environment, and through its absorption by plants from contaminated soil or water reaches the food chain. That said, several parameters influence the concentration of Cd in different locations, and consequently its concentration in honey. However, only a small proportion of Cd can reach honey by air, mainly in the proximity of incinerators.

Arsenic can also come from non-ferrous metallurgy and factories, but it can also be present in the environment through the use of agrochemicals such as arsenic-based fertilizers and pesticides. As a result, arsenic is found in water, soil, and air, and as it is absorbed by all plants, it finds its way into the food chain, including honey. Hence the importance of limiting the use of arsenic-based pesticides and introducing quality control measures for honey.

Poor beekeeping practices applied in the extraction and storage of honey can also cause a significant source of contamination in toxic elements, the acidic characteristic of honey also helps to release certain metals such as Pb from metal containers.

These results indicate that Algerian honey is not far from European and Chinese honeys in terms of quality and food safety. Even with regard to the European regulations, the levels of Pb and Cd are below the maximum limit, which suggests studying the possibility of establishing a national standard specific to Algerian honeys and also encouraging beekeepers to export their honeys without the risk of rejection due to non-compliance with heavy metals. But attention should be drawn to the specific limit of 0.1 mg kg^{-1}, beekeepers may think of introducing a variety of honey for children and persons with particular dietary needs.

3.4 STATISTICAL ANALYSIS

Several physicochemical parameters exhibited notable correlations. Specifically, Pb demonstrated strong positive associations with Cd (r = 0.708, $p < 0.01$), Cu (r = 0.62, $p < 0.05$), and Fructose (r = 0.52, $p < 0.05$). Similarly, Fe and Cr displayed a significant positive correlation (r = 0.7, $p < 0.001$). Cd and Ni also, showed a significant positive correlation (r = 0.6, $p < 0.01$). On the other hand, negative correlations were identified between Cr and pH (r = -0.52, $p < 0.05$), as well as between Cd and Zn (r = -0.52, $p < 0.05$).

Figure 3 shows a representation of the samples on the first two components which represent 36.4 % of the variability, with 19.8 % explained by the first axis and 16.6 % explained by the second axis.

The multiple factor analysis (Axes 1 and 2) distinct patterns within the two groups of honey samples. The polyfloral honey samples were characterized by a high concentration of Cr, As, and Ni. On the other hand, the unifloral group showed a high concentration of pH, Zn, and HMF.

The multiple factor analysis showed that the sample (E39) of *P. harmala* L. was characterized by a particularly high concentration of Cu and Cd. A study on the germination characteristics of *P. harmala* seeds exposed to heavy metals and their impact on rehabilitating polluted arid lands showed that *P. harmala* had a high germination ability even in highly contaminated soils (Schweitzer, 2001). Another study also showed the effectiveness of *P. harmala* seeds to remove Pb^{2+}, Zn^{2+} and Cd^{2+} ions from aqueous solutions (EIC, 2015). These findings suggest that *P. harmala* is well-suited to growing in polluted environments and may be an effective adsorbent for removing heavy metals (Schweitzer, 2001) and thus may explain the high levels of Cu and Cd in *P. harmala* honey.

The multiple-factor analysis also showed that eucalyptus samples (E32, E44, E78) had a high moisture content and high levels of free acidity. Eucalyptus honey is known for its high electrical conductivity. This latter re-

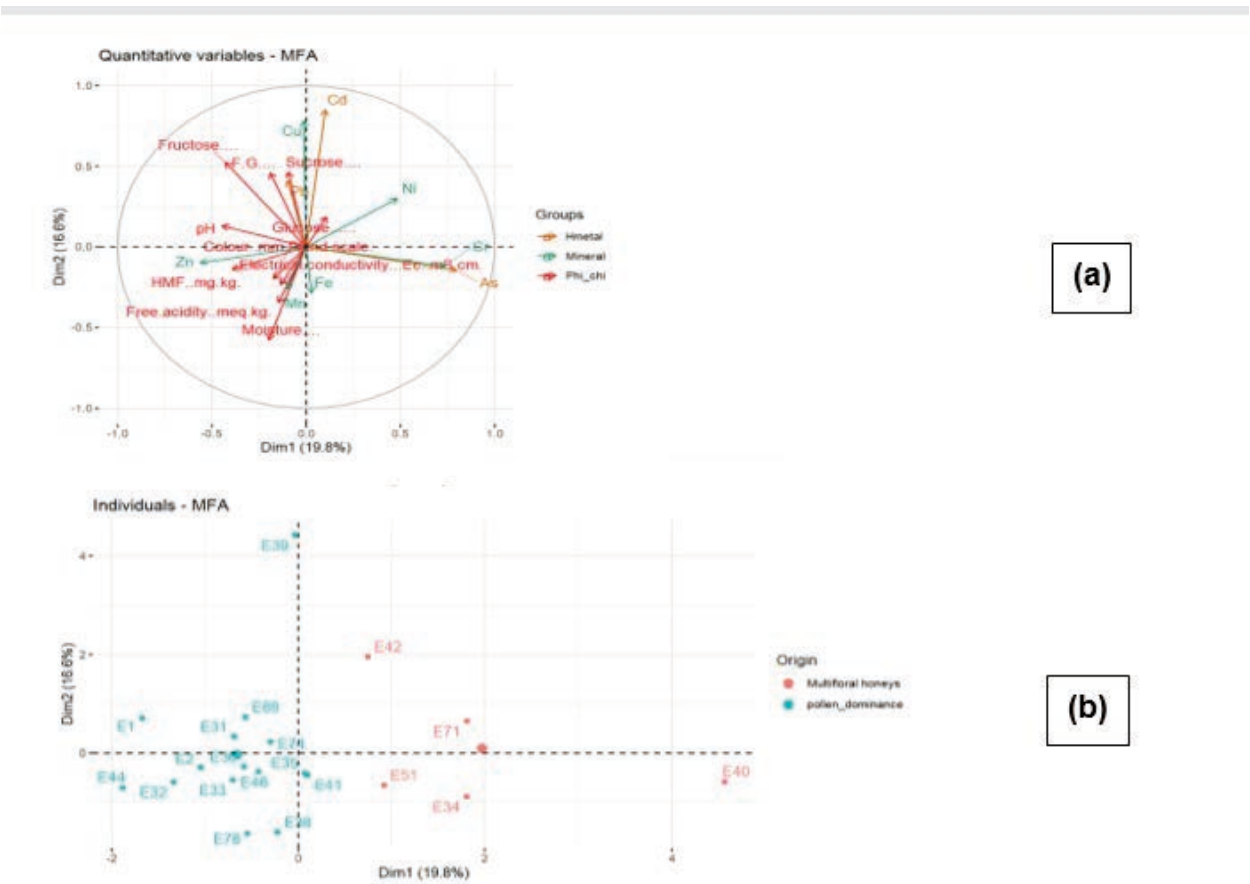

Figure 3: Multiple factor analysis (a) Loading biplot of the variables included in the analysis, (b) Score biplot of the samples regarding component 1 and 2

flects the richness of the honey in mineral elements and organic acids. Hence, a high content of organic acid and salts increases the free acidity present in honey (Ghorab et al., 2021). Moreover, Bogdanov et al. (2004) reported that honey's acidity is the result of the transformation of glucose into gluconic acid, and this transformation is more favored by high water content.

4 CONCLUSIONS

This study was carried out to analyze the pollen characteristics, physicochemical properties, and mineral and heavy metal composition of 20 types of honey from 13 different locations throughout Algeria. The study revealed the presence of several honey types, with the predominant pollen of *Citrus* sp., *Eucalyptus* sp., *Ziziphus lotus* (L.) Lam., *Sinapis arvensis* L., *Dorycnium* sp., *Echium* sp., *Bupleurum* sp., *Lotus* sp. and *Peganum harmala* L., and polyfloral honey. All types of honey meet the quality standards required by the Codex Alimentarius, the European Directive. Except HMF, for which we have noted non-conformity for three samples. Thus, it is noteworthy to mention that improving beekeepers' knowledge of honey harvesting techniques, processing, and storage is essential to produce high-quality honey that meets both national and international market standards.

The analyzed honey also complied with the standards of the European Directive for heavy metals. The concentrations of Pb and Cd in the honey samples were found to be below the maximum residue levels.

The obtained results are highly relevant to programs aimed at enhancing the value of honey and protecting it with a sign of quality or a geographical indication. This approach provides a real opportunity to maintain and improve the quality of local honey. The identified characteristics contribute to the creation of a reference database in order to establish regulations in Algeria regarding the physicochemical and heavy metals composition of honey.

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doi:10.14720/aas.2024.120.2.18157 Original research article / izvirni znanstveni članek

Spraying macro and micro fertilizers affects positively fruit yield and quality of 'Page' mandarin

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Received February 06, 2024; accepted April 16, 2024.
Delo je prispelo 6. februarja 2024, sprejeto 16. aprila 2024.

Spraying macro and micro fertilizers affects positively fruit yield and quality of 'Page' mandarin

Abstract: In the current work, the effects of foliar application of two commercial fertilizers [CalfalB (containing calcium and boron) and Rice (containing macro- and micronutrients)] on leaf minerals, chlorophyll content, yield, and fruit quality, as well as some phytochemical characteristics of mandarins 'Page' was investigated for two consecutive years. The solutions were applied three times: mid-June and two more sprays at intervals of 18 days. Based on the results, leaves of fertilized mandarin plants with Rice and CalfalB accumulated higher N, P K, Ca, Mg, Zn, Mn, and Fe concentrations than unfertilized plants. Application of fertilizers, especially Rice, increased significantly the content of chlorophyll a ($p < 0.001$) and total chlorophyll ($p = 0.0013$) in the leaves. Trees fertilized with Rice showed a higher percentage of fruit yield, juice, pulp, and rind. Moreover, mandarins treated with fertilizers, especially Rice, had a higher level of TSS (total soluble solids), TSS/TA (titratable acidity), color parameters of the rind [L^* (lightness), a^* (redness), and b^* (yellowness)], vitamin C, phenol compounds, carotenoid, and antioxidant activity. The results of our research work showed that an application of fertilizers containing macro- and micro-elements by spraying can considerably improve fruit yield and quality of the mandarin 'Page', especially in areas with poor soils.

Key words: Iran, mandarin, spraying fertilizers, foliar fertilization, fruit yield and quality, biochemical attributes of the fruita

Škropljenje z makro in mikro hranili vpliva pozitivno na pridelek in kakovost mandarin 'Page'

Izvleček: V raziskavi so bili v dveh zaporednih rastnih sezonah preučevani učinki dveh foliarnih komercialnih gnojil, CalfalB-a (vsebuje kalcij in bor) in Rice-a (vsebuje makro in mikro hranila), na vsebnost hranil v listih in klorofila, na pridelek in kakovost plodov, kot tudi na nekatere fitokemične lastnosti mandarin 'Page'. Raztopina s hranili je bila nanešena trikrat in sicer v sredini junija in nato še v dveh intervalih z razmikom 18 dni. Rezultati so pokazali, da so listi mandarinovca pognojeni z obema gnojilama (Rice ind CalfalB) vsebovali več N, P K, Ca, Mg, Zn, Mn in Fe kot nepognojeni. Uporaba gnojil, še posebej gnojila Rice, je zančilno povečala vsebnost klorofila a ($p < 0.001$) in celokupnega klorofila ($p = 0.0013$) v listih. Z gnojilom Rice pognojeni mandarinovci so imeli večji pridelek plodov kot tudi večji izplen soka, pulpe in olupkov v njih. Mandarine, ki so bile obravnavane z gnojili, še posebej z gnojilom Rice, so imele večjo vsebnost celokupne suhe snovi (TSS), večje razmerje TSS/TA (titratibilna kislost), večje vrednosti barvnih parametrov olupka (L^* - svetlost, a^* - rdečina in b^* - rumenost), večje vsebnosti vitamina C, fenolnih snovi in karotenoidov ter večjo antioksidacijsko aktivnost. Rezultati te raziskave kažejo, da foliarno gnojenje z gnojili, ki vsebujejo makro in mikro elemente znatno izboljša velikost pridelka in kakovost mandarin 'Page', še posebej na območjih z revnimi tlemi.

Ključne besede: Iran, mandarinovec, škropljenje z gnojili, foliarno gnojenje, pridelek plodov in njihova kakovost, biokemični parametri plodov

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1 INTRODUCTION

Citrus is produced in many countries and has the first rank of fruit production in the world (Liaquat et al., 2023). Citrus fruits are rich in vitamins, minerals, fiber, and various antioxidants such as carotenoids, flavonoids, and limuloids, which are beneficial for human health (Zou et al., 2016). Iran is one of the leading countries in citrus production, having different citrus fruits and an annual production of more than 3 million tons (FAOSTAT, 2021). In Iran, 'Page' mandarin is one of the most important citrus cultivars, and its cultivated area is increasing. However, fruit yield and quality of citrus in Iran are low due to different factors, mainly improper nutrition (Hosseini, 2018). Therefore, proper nutrition can be one of the strategies to improve fruit yield and quality of citrus.

Foliar spraying fertilizers, when properly planned and conducted, could be used to increase fruit yield and quality of citrus. Citrus trees respond well to foliar fertilization due to the presence of a great number of stomata on the lower surface of the leaf along with more cuticle pores (facilitating nutrient absorption) (Smoleń, 2012). In general, foliar application of essential nutrients is not practical enough to cover the entire nutritional needs of the plants. However, a significant part of the plant's need for essential elements (mostly micronutrients) could be provided by foliar fertilization (Smoleń, 2012). Foliar nutrition is applied to improve the nutritional status of the plants, to eliminate the deficiency of nutrients, and, as a result, to increase fruit yield and quality (Norozi et al., 2019; Van Dang et al., 2022). Furthermore, compared to soil fertilization, foliar feeding is the fastest way to introduce minerals into the aboveground parts of the plants (Fernández et al., 2013).

The roles of macronutrients in improving the quantitative and qualitative characteristics of citrus fruits have been proven (Srivastava, 2012; Reetika et al., 2018; Van Dang et al., 2022). Nitrogen, as a critical element for citrus, has more effect on the plant growth and on fruit yield and quality than any other nutrient (Liu et al., 2010). Phosphorus plays a vital role in enzyme activation, photosynthesis processes, cell division, metabolism, and sugars movement. Potassium has significant effects on cellular osmotic, stomatal opening and closing, electrochemical processes, enzyme activity, cell division, protein synthesis, sugars synthesis and translocation, and acid metabolism of fruit juice in citrus (Alva et al., 2006; Van Dang et al., 2022). Calcium has a significant effect on the improvement of fruit yield and quality of mandarin (Zaman et al., 2019). Magnesium plays a vital role in the production of chlorophyll, the absorption of phosphorus in the metabolism of carbohydrates, and its deficiency reduces the fruit yield and quality of citrus (Van Dang et al., 2022).

Sufficient supply of micronutrients leads to good fruit yield and quality of citrus (Bastakoti et al., 2022). Lack of use or limited use leads to a deficiency in these nutrients. Micronutrients can easily applied on leaves because they are needed in small amounts. Applying micronutrients by spraying reduces fruit drop and improves fruit yield and quality of citrus (Hosseini, 2019; Khalid et al., 2021; Bastakoti et al., 2022).

Although the role of foliar fertilization in citrus has been widely studied worldwide (Gerendás & Führs, 2013; Hosseini, 2019; Bastakoti et al., 2022; Van Dang et al., 2022), research on the effect of foliar fertilizing on nutritional status, fruit yield and quality of mandarin trees is scarce. In this study, for the first time, the impact of spraying two commercial fertilizers: CalfalB (containing calcium and boron) and Rice (containing macro- and micronutrients) on leaf nutrient contents, quality, yield, and phytochemical parameters of the fruit of 'Page' mandarin were investigated.

2 MATERIALS AND METHODS

2.1 SITE DESCRIPTION

A commercial 'Page' mandarin orchard located in Mazandaran province, Iran (latitude. 36° 50′; longitude. 53° 0′ E; altitude. 52 m above sea level) was used for nutritional treatments. The climatic conditions of the site of trials during the period of experiments are presented in Table 1. Studied plants were 10 years old and were planted in sandy a clay loam soil, which is presented in Table 2, and the distance of plantation is 2 meters on the row and 3 meters between the rows.

2.2 TREATMENTS AND EXPERIMENTAL DESIGN

A completely randomized block design with three nutrient treatments and six replications (trees) in each treatment was used in the experiment. The nutrient treatments included two commercial fertilizers: CalfalB (containing calcium and boron) and Rice (containing macro- and micronutrients). Distilled water was sprayed as the control. Table 3 shows the characteristics and concentrations of the fertilizers used. Fertilizers were purchased from a commercial company (Shahin Faraz Arian, Teheran, Iran; website: Falconagri.ir). The fertilizers were sprayed three times: mid-June and two more times at 18-day intervals. The fertilizers were sprayed in two years (2022-2023); however, the effect of fertilizers on

Table 1: Climatic conditions during the experiment period of the trials on spraying macro and micro fertilizers on 'Page' mandarin in Mazandaran province area, Iran

Months	Mean high temperature **(°C)**	Mean low temperature (°C)	Mean humidity (%)	Mean rainfall (mm)	Mean sunshine (h)
January	9.8	5.8	71	31.1	5.21
February	22.3	-1	72	89.2	4.28
March	14.6	10.2	70	47.8	3.31
April	39.2	5.4	70	15.7	3.91
May	35.6	11.8	78	63.4	6.16
June	37.2	12.2	67	4	9.15
July	36.2	19.2	66	7.6	6.80
August	36	21.5	74	50.7	9.10
September	25.4	25.2	66	159.4	5.76
October	37	14	76	101	4.43
November	18.6	13.2	76	51.9	4.94
December	27	2.5	80	59.6	3.42

Table 2: The content of minerals in the soil and physico chemical properties of the soil of the experimental orchard of 'Page' mandarin in Mazandaran province area, Iran

	Depth (cm)	
Parameters	0–30	30–60
Clay (%)	37	42
Silt (%)	24	23
Sand (%)	39	35
Soil texture	Clay loam	Clay
pH	7.03	7.21
Electrical conductivity (EC) (ds m^{-1})	0.53	0.56
Organic matter (OM) (%)	3.62	1.12
Organic Carbon (OC) (%)	2.11	0.65
N (%)	0.22	0.25
P (ppm)	92.9	5.8
K (ppm)	277	119
Fe (ppm)	4.3	14.02
Mn (ppm)	12.04	36.12
Zn (ppm)	1.00	9.18
Cu (ppm)	0.68	2.30

fruit yield and quality was not significant in the first year, and only the results of the second year are presented.

2.3 THE CONTENT OF MINERALS IN THE LEAVES

To measure the concentration of N, P, K, Ca, Mg, Zn, Mn, and Fe in the leaves, mandarin leaves were collected in early September. The samples of leaves were dried in the oven, and after being powdered, 0.2 g was used to determine the concentration of the minerals in the leaves. Kjeldahl method was used to determine N concentration. A spectrophotometer was used to determine the P concentration. K concentration was determined by Flame photometrically. An atomic absorption spectrophotometer (Varian, 220) was used to measure the concentrations of Ca, Mg, Zn, Mn, and Fe.

2.4 CHLOROPHYLL CONTENT IN THE LEAF

According to Lichtenthaler (1987), the chlorophyll content in leaves collected in early September was de-

Table 3: Active ingredient of compounds used in the study

Commercial name	Active Ingredient (w/w) %	Doses of foliar application (%)
CalfalB	Ca 8 %; B 0.5 %	0.3
Rice	N 15 %; P_2O_5 15 %; K_2O 30 %; MgO 1 %; Mn 1.1 %, Fe 0.1 %, Zn 0.52 %, B 0.25 %, Cu 0.21 %	0.5

termined. Chlorophyll concentrations were measured at wavelengths of 646.8 and 663.2 nm, and readings were recorded as mg g^{-1} FM (Fresh Mass) of the leaf.

2.5 FRUIT YIELD AND QUALITY

In early December, the fruits of each tree (replication) were picked and weighed to determine the yield (kg per tree). Fruit length and diameter, and skin thickness were determined by using a digital Vernier caliper. To determine the fruit size, the fruit was placed in a beaker filled with water, and the amount of overflowing water was considered equal to the fruit size (volume) (cm^3).

The percentage of fruit juice was calculated using the equation [A/B] x 100, where A and B are respectively the juice mass and the fruit mass. The percentage of fruit rind was determined using the equation [C/B] x 100, where C and B are respectively the rind mass and the fruit mass.

The firmness of the mandarin fruits was measured using a penetrometer (STEP SYSTEM, Germany), and results were recorded as kg cm^{-2}.

2.6 TOTAL SOLUBLE SOLIDS (TSS), TITRATABLE ACIDITY (TA) AND TSS/TA RATIO

A digital refractometer (Atago, PAL-1, Japan) was used to measure the total soluble solids (TSS) concentration of the fruit juice of mandarin, and results were given as % (Brix). By titration with 0.1 N NaOH up to a pH of 8.1, 1 ml of diluted juice in 25 ml distilled water, titratable acidity (TA) was determined, and results were given as a percentage of citric acid. The TSS/TA ratio was calculated by dividing TSS by TA.

2.7 THE FRUIT COLOR

The color parameters of the fruit rind [L^* (lightness), a^* (redness), and b^* (yellowness)] of 'Page' mandarins were measured using a colorimeter (CR 400-Minolta, Japan). The color was measured at three points of the fruit surface of each replicate, and the mean values were given.

2.8 BIOCHEMICAL ATTRIBUTES OF THE FRUITS

Vitamin C of the fruit juice was determined by oxidizing ascorbic acid with 2, 6-dichloro phenol-indo-phenol, and the results were determined in mg 100 ml^{-1} juice (Nielsen, 2017).

The Folin–Ciocalteau method was used to determine the content of total phenol in mandarin juice (Singleton and Rossi, 1965). Total phenol was measured at 520 nm spectrophotometrically (Cary Win UV 100, Varian, Australia). Total phenol values were determined by applying a calibration curve drawn for the gallic acid standard solution, and the results were determined in mg gallic acid ml^{-1} juice.

The content of carotenoid in the fruits was determined according to Lichtenthaler (1987). The carotenoid concentration in the fruit was measured using a Cary WinUV 100 spectrophotometer (Varian, Australia) at 470 nm, and results were determined in mg·g^{-1} FM.

The total antioxidant activity of the fruit juice was assessed based on the radical scavenging ability in reacting with DPPH (2, 2-diphenyl-1- picrylhydrazyl) according to Brand-Williams et al. (1995). Briefly, 100 µl of mandarin juice, 10 ml of methanol, and 1900 µl of DPPH solution (Sigma-Aldrich, USA) were mixed and stirred for 30 min. Then, at 517 nm against a blank (methanol), the absorbance was measured using a Cary WinUV 100 spectrophotometer (Varian, Australia). The percentage of antioxidant activity as the inhibition percentage of free radical DPPH was estimated using the following formula:

$$\text{Total antioxidant activity (\%)} = [(\text{blank absorbance} - \text{extract absorbance} / \text{blank absorbance}] \times 100$$

2.9 STATISTICAL ANALYSIS OF DATA

The data were analyzed using the GLM procedure of SAS (Statistical Analysis System) software (Version 9.1). Significant differences were assessed using Duncan's multiple range test at $p \leq 0.05$. To evaluate significant differences, Duncan's multiple range test at $p \leq 0.05$ was used.

3 RESULTS

3.1 THE CONTENT OF MINERALS IN THE LEAF

Foliar application of Rice and CalfalB fertilizers caused a significant increase in the concentrations of N ($p = 0.0002$), Mn ($p = 0.0010$), and Fe ($p < 0.0001$) in the mandarin leaves. The effect of Rice was statistically more remarkable than that of CalfalB. With Rice spray, the concentration of N, Mn, and Fe was respectively 26.41 %, 159.20 %, and 142.68 % greater than the unfertilized plants (Table 4).

Mandarin trees fertilized with Rice fertilizer showed the highest increase in the concentrations of P, K, and Mg in the leaves (196.00 %, 79.78 %, and 94.73 %, respectively). The effect of Rice was greater than that of CalfalB; nevertheless, there is no significant difference between CalfalB and the control (Table 4).

Compared to unfertilized trees, the Ca concentration of fertilized trees with CalfalB and Rice fertilizers was significantly (p <.0001) increased (12.42–37.85 %), and the effect of CalfalB was statistically more considerable than that of Rice (Table 4).

Plants sprayed with Rice and CalfalB fertilizers accumulated significantly a higher concentration of Zn than unsprayed plants. With the application of Rice and CalfalB, the concentration of Zn was respectively 180.19 % and 212.57 % higher than the control (Table 4).

3.2 THE CONTENT OF CHLOROPHYLL IN THE LEAVES

Spraying Rice and CalfalB fertilizers affect significantly the content of chlorophyll a (p < 0.0001) and total chlorophyll (p = 0.0013) in the leaves, but had no significant effect (p = 0.5158) on the content of chlorophyll b (Fig. 1). The influence of Rice was considerably more significant than that of CalfalB. With Rice application, the contents of chlorophyll a and total chlorophyll were

Table 4: Effect of spraying fertilizers on the content of minerals in the leaves of 'Page' mandarin in Mazandaran province area, Iran

Treatments	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Zn (ppm)	Mn (ppm)	Fe (ppm)
Control	2.12 c	0.25 b	0.94 b	1.77 c	0.19 b	16.06 b	25.10 c	49.08 c
Rice	2.68 a	0.74 a	1.69 a	1.99 b	0.37 a	50.20 a	65.06 a	119.11 a
CalfalB	2.45 b	0.35 b	1.00 b	2.44 a	0.22 b	45.00 a	40.13 b	82.16 b
P-value	0.0002	0.0043	0.0010	<.0001	0.0032	0.0018	0.0010	<.0001
CV (%)	1.70	18.77	7.52	1.21	11.10	12.75	10.33	2.06

Values in columns followed by the same letter are not significantly different at $p \leq 0.05$, Duncan's multiple range test. CalfalB: Ca 8 %, B 0.5 %; Rice: N 15 %, P_2O_5 15 %, K_2O 30 %, MgO 1 %, Mn 1.1 %, Fe 0.1 %, Zn 0.52 %, B 0.25 %, Cu 0.21 %

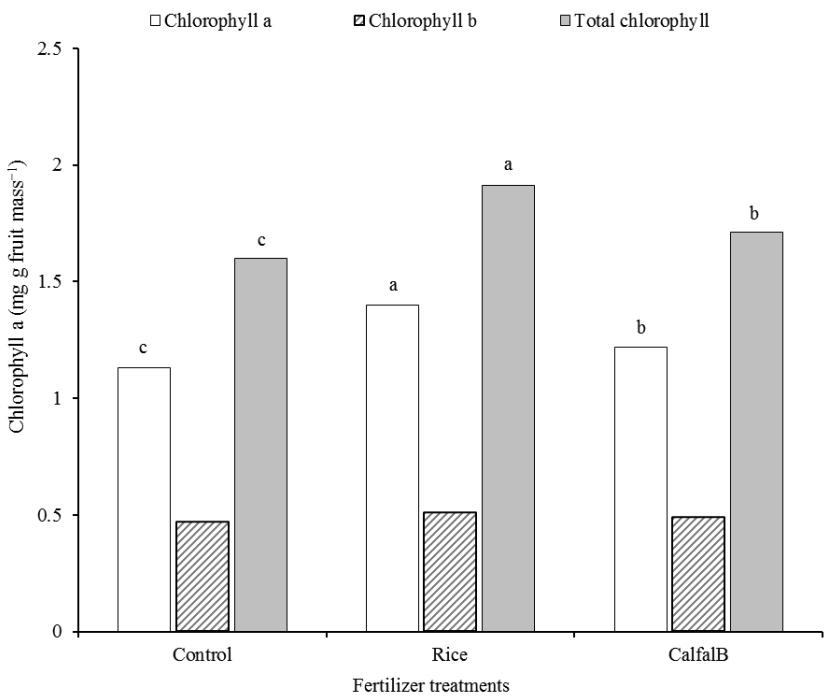

Fig. 1: Effect of spraying fertilizers on the content of chlorophyll in the leaves of 'Page' mandarin in Mazandaran province area, Iran. Different letters at the top of columns indicate significant differences ($p \leq 0.05$) among treatments. CalfalB: Ca 8 %, B 0.5 %; Rice: N 15 %, P_2O_5 15 %, K_2O 30 %, MgO 1 %, Mn 1.1 %, Fe 0.1 %, Zn 0.52 %, B 0.25 %, Cu 0.21 %

respectively 23.891 % and 19.37 % greater than the control (Fig. 1).

3.3 FRUIT YIELD AND QUALITY

The fertilizers affect significantly fruit yield (p = 0.0015), juice percentage (p = 0.0501), pulp percentage (p = 0.0031), and rind percentage (p = 0.0417) of mandarins, whereas there is no significant effect on fruit diameter (p = 0.5457), fruit length (p = 0.9545), fruit size (p = 0.2935), and firmness (p = 0.0909) (Table 5).

Compared to unfertilized plants, the yield of fertilized mandarins with CalfalB and Rice increased by 17.15 % and 45.59 %, respectively. The effect of Rice was considerably higher than that of CalfalB (Table 5).

The highest percentage of juice and rind was achieved with Rice fertilizer. With Rice application, the percentages of juice and rind were respectively 11.441 % and 15.87 % greater than the control (Table 5). Regarding the percentage of juice and rind, there is no significant difference between Rice and CalfalB and also between control and CalfalB (Table 5).

Mandarins fed with fertilizers exhibited a lesser pulp percentage than the control. The effect of Rice fertilizer was considerably superior to that of CalfalB. With Rice application, the fruit pulp percentage was 30.02 % less than the control (Table 5).

3.4 TSS, TA, AND TSS/TA

Spraying Rice and CalfalB fertilizers affect significantly TSS (p = 0.0011) and TSS/TA (p = 0.0500) of mandarins, but had no significant effect (p = 0.6621) on TA (Table 6). Compared to the unfertilized control, the TSS of fruits was 32.67 % and 20.09 % higher for Rice and CalfalB, respectively. The influence of Rice was considerably more significant than that of CalfalB (Table 6).

The highest TSS/TA (10.72) was obtained with the Rice application, which resulted in an increase of 25.67 % compared with the control. However, this influence was not superior to that of CalfalB. In addition, there is no significant difference between CalfalB and control (Table 6).

3.5 FRUIT COLOR

Foliar application of Rice and CalfalB fertilizers affect significantly the rind color parameters (L^*, a^*, and b^*) of the fruits of mandarin (Table 6). The influence of

Table 5: Effect of spraying fertilizers on fruit yield and quality of 'Page' mandarin in Mazandaran province area, Iran

Treatments	yield (kg tree^{-1})	Fruit length (mm)	Fruit diameter (mm)	Fruit size (cm^3)	Fruit juice (%)	Fruit pulp (%)	Fruit rind (%)	Fruit firmness (kg cm^{-2})
Control	58.60 c	58.37	66.98	124.16	47.02 b	30.04 c	22.93 b	10.06
Rice	85.32 a	58.75	67.43	131.66	52.40 a	21.02 a	26.57 a	11.10
CalfalB	68.65 b	58.50	68.20	132.06	48.31 ab	26.20 b	25.48 ab	11.46
P-value	0.0015	0.9545	0.5457	0.2935	0.0501	0.0031	0.0417	0.0909
CV (%)	4.70	2.67	1.88	4.58	4.02	5.20	4.63	5.36

Values in columns followed by the same letter are not significantly different at $p \leq 0.05$, Duncan's multiple range test.

Table 6: Effect of spraying fertilizers on TSS, TA, TSS/TA and rind color indices of 'Page' mandarin fruits in Mazandaran province area, Iran

Treatments	TSS (%)	TA (%)	TSS/TA	L^* (lightness)	a^* (redness)	b^* (yellowness)
Control	10.10 c	1.20	8.53 b	35.20 b	3.31 c	18.45 c
Rice	13.40 a	1.25	10.72 a	40.80 a	7.78 a	30.13 a
CalfalB	12.13 b	1.28	9.45 ab	35.93 b	4.76 b	24.32 b
P-value	0.0011	0.6621	0.0500	0.0039	0.0001	<.0001
CV (%)	3.19	8.62	7.77	2.57	5.85	2.31

Values in columns followed by the same letter are not significantly different at $p \leq 0.05$, Duncan's multiple range test. CalfalB: Ca 8 %, B 0.5 %; Rice: N 15 %, P_2O_5 15 %, K_2O 30 %, MgO 1 %, Mn 1.1 %, Fe 0.1 %, Zn 0.52 %, B 0.25 %, Cu 0.21 %

Rice was considerably superior to CalfalB. Application of Rice improved the values of L^*, a^*, and b^* by 15.90 %, 135.04 %, and 63.30 % respectively (Table 6).

3.6 FRUIT BIOCHEMICAL ATTRIBUTES

Spraying Rice and CalfalB fertilizers improved significantly ($p = 0.0062$) the vitamin C of mandarin fruits. With a spray of Rice and CalfalB, vitamin C in the fruits was respectively 57.86 % and 31.56 % higher than in the unfertilized control. The influence of Rice was noticeably higher than that of CalfalB (Fig. 2).

The content of total phenol and antioxidant activity in the mandarin fruits increased respectively by 60.56 % and 9.87 % with the application of Rice fertilizer. The effect of Rice was superior to that of CalfalB (Figs. 3 and 4).

Fruits of trees sprayed with Rice and CalfalB fertilizers had significantly the higher content of carotenoid than in unsprayed plants. With the application of Rice and CalfalB, the content of carotenoid was respectively 130.76 % and 92.30 % higher than the control (Fig. 5).

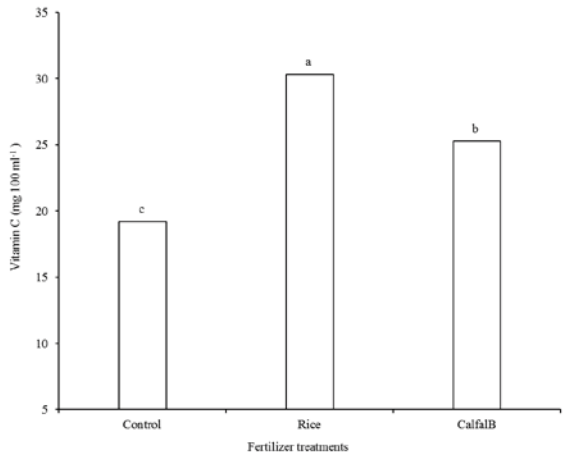

Fig. 2: Effect of spraying fertilizers on the content of vitamin C in the fruits of 'Page' mandarin in Mazandaran province area, Iran. Different letters at the top of columns indicate significant differences ($p \leq 0.05$) among treatments. CalfalB: Ca 8 %, B 0.5 %; Rice: N 15 %, P_2O_5 15 %, K_2O 30 %, MgO 1 %, Mn 1.1 %, Fe 0.1 %, Zn 0.52 %, B 0.25 %, Cu 0.21 %

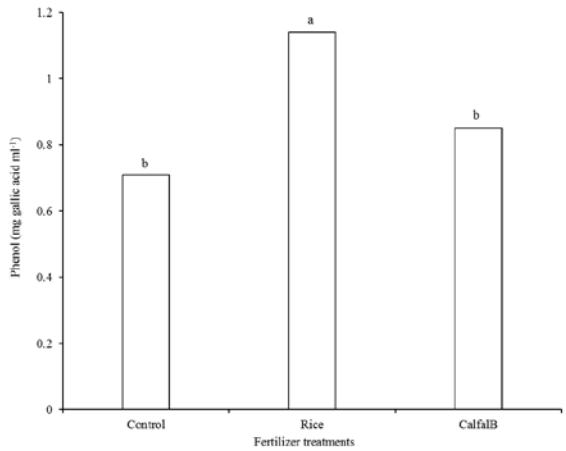

Fig. 3: Effect of spraying fertilizers on the content of total phenol in the fruits of 'Page' mandarin in Mazandaran province area, Iran. Different letters at the top of columns indicate significant differences ($p \leq 0.05$) among treatments. CalfalB: Ca 8 %, B 0.5 %; Rice: N 15 %, P_2O_5 15 %, K_2O 30 %, MgO 1 %, Mn 1.1 %, Fe 0.1 %, Zn 0.52 %, B 0.25 %, Cu 0.21 %

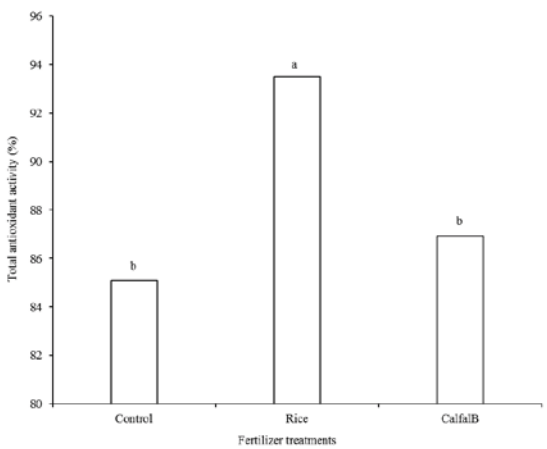

Fig. 4: Effect of spraying fertilizers on the content of antioxidant activity in the fruits of 'Page' mandarin in Mazandaran province area, Iran. Different letters at the top of columns indicate significant differences ($p \leq 0.05$) among treatments. CalfalB: Ca 8 %, B 0.5 %; Rice: N 15 %, P_2O_5 15 %, K_2O 30 %, MgO 1 %, Mn 1.1 %, Fe 0.1 %, Zn 0.52 %, B 0.25 %, Cu 0.21 %

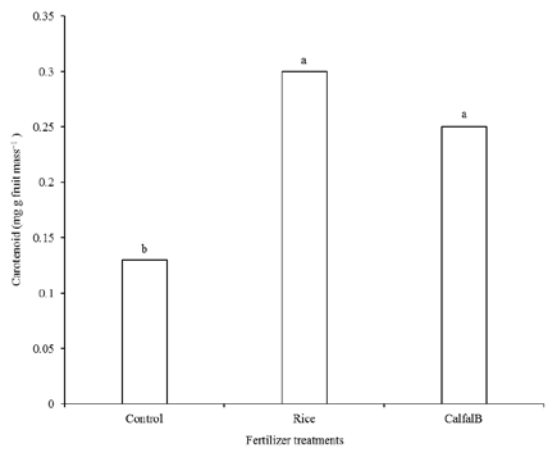

Fig. 5: Effect of spraying fertilizers on the content of carotenoid in the fruits of 'Page' mandarin in Mazandaran province area, Iran. Different letters at the top of columns indicate significant differences ($p \leq 0.05$) among treatments. CalfalB: Ca 8 %, B 0.5 %; Rice: N 15 %, P_2O_5 15 %, K_2O 30 %, MgO 1 %, Mn 1.1 %, Fe 0.1 %, Zn 0.52 %, B 0.25 %, Cu 0.21 %

4 DISCUSSION

Deficiency of essential elements has been one of the main problems in citrus-producing regions of Iran. Compared to the recommended desirable ranges (Menino, 2012; Table 7), the levels of N, K, Ca, Mg, Zn, and Fe in the leaves of unfertilized mandarin trees were lower (Table 7), what shows the deficiency of these minerals. On the other hand, the level of Mn was somewhat similar to the recommended desirable range, and the P concentration was higher than the optimal range (Menino, 2012). However, application of Rice and CalfalB fertilizers by spraying markedly improved leaf levels of N (15.56–26.41 %), P (40–196 %), K (6.38–79.78 %), Ca (12.42–37.85 %), Mg (15.78–94.73 %), Zn (180.19–212.57 %), Mn (59.88–159.20 %), and Fe (67.40–142.68 %) (Tables 4 and 7). Our findings agree with the results published by Hosseini (2018) on lime and Van Dang et al. (2022) on pomelo, who reported that foliar application of fertilizers containing macro- and micronutrients, enhanced the content of minerals in the leaf. Foliar application of fertilizers improves the absorption, movement, and accumulation of mineral elements in the plants (Norozi et al., 2019).

In the current work, due to treatment with fertilizers, especially Rice, chlorophyll a and total chlorophyll increased significantly (Fig. 1). The increase in the content of chlorophyll with foliar application of micronutrients have been described for sweet orange (Nandita et al., 2020), and acid lime (Bastakoti et al., 2022). The increase in the content of chlorophyll because of the application of micronutrients is due to the known roles of micronutrients in the activation of enzymes involved in chlorophyll biosynthesis (Ilyas et al., 2015; Mohammed et al., 2018; Bastakoti et al., 2022). Furthermore, similar findings on the positive effects of macronutrients on the chlorophyll content have been achieved (Oivukkamäki et al., 2023). The positive impact of N and Mg on the content of chlorophyll can be because these elements, as components of a chlorophyll molecule, are necessary for the formation of chlorophyll (Menino, 2012). Moreover, K increases the biosynthesis of chlorophyll and inhibits the decomposition of chlorophyll (Alipour, 2018). The leading cause of reduced chlorophyll content is K deficiency (Ali et al., 2021).

Based on this research, foliar application of fertilizers, especially Rice, improved significantly most traits related to fruit yield and quality of mandarin (Table 5). Using fertilizers containing macro- and micronutrients to improve yield and fruit quality is consistent with Hosseini (2018) for lime, Reetika et al. (2018) for 'Kinnow' mandarin, Bastakoti et al. (2022) for acid lime, and Van Dang et al. (2022) for pomelo. The positive influence of macro- and micronutrients on yield and fruit quality can be ascribed to the effects of these elements on balancing the nutritional status, photosynthetic efficiency, and the transfer of photoassimilate from the source to the sink (Reetika et al., 2018; Cavender et al., 2019; Bastakoti et al., 2022).

Our findings showed that spraying fertilizers, especially Rice, improved TSS and TSS/TA in mandarin fruits (Table 6). These results are in line with those of Van Dang et al. (2022), who indicated that the use of fertilizers containing macro- and micronutrients increases the TSS in pomelo fruits. The rise in the content of TSS in the fruits by spraying P, K, Mg, and Zn can be due to the increase in enzyme activity involved in carbohydrate synthesis (Gerendás & Führs, 2013; Jiang et al., 2014; Davarpanah et al., 2016; Zhang et al., 2018). Conversely, Van Dang et al. (2022) observed that applying fertilizers containing macro- and micronutrients reduced acidity in pomelo fruits, which is dissimilar to our results (Table 6).

Rind color is a vital fruit characteristic for a fresh market. The rind color of citrus fruits is related to many factors, including maturity, environmental conditions, genotype, and plant nutrition (Menino, 2012). According to our study, the fertilizers in particular Rice improved significantly the rind color parameters of mandarin fruits

Table 7: The content of minerals in the leaves of the unfed and fed 'Page' mandarin trees in Mazandaran province area, Iran, and comparison with suggested optimal ranges

Minerals	Fertilized plants	Unfertilized plants	Optimal ranges (Menino, 2012)
N (%)	2.12	2.44–2.59	2.5–2.7
P (%)	0.25	0.30–0.55	0.12–0.16
K (%)	0.94	1.10–1.38	1.2–1.7
Ca (%)	1.77	1.80–2.35	3.0–4.9
Mg (%)	0.19	0.24–0.35	0.30–0.49
Zn (ppm)	16.06	48.16–58.10	25–100
Mn (ppm)	25.10	45.16–60.00	25–100
Fe (ppm)	49.08	78.10–122.04	60–120

(Table 6). Similarly to these findings, increases in fruit color parameters using chemical fertilizers have been described in pomegranate (Almutairi et al., 2021), and strawberry (Kilic et al., 2021). Reported results on the relationship between mineral nutrients and citrus color parameters are scarce and inconsistent.

Fertilizers play a significant role in the content of vitamin C in citrus fruits (Menino, 2012). The fertilizer treatments, especially Rice, increased the content of vitamin C in mandarin fruits (Fig. 2). Our results agreed with those achieved by Maity et al. (2022), Almutairi et al. (2021) on pomegranate, and Kilic et al. (2021) on strawberry, who stated that the application of fertilizers containing different elements enhanced the content of vitamin C in the fruits. Increases in the content of vitamin C in the fruits have been ascribed to the roles of P, K, Mg, and Zn in the accumulation of greater sugars and phytohormones in the fruits (Menino, 2012; Tanari et al., 2019; Maity et al., 2022).

Phenolic compounds in citrus fruits play an important role in human health (Menino, 2012). In our research work, the content of total phenol in the mandarin fruits substantially improved with the application of Rice fertilizer (Fig. 3). Similarly, Cavender et al. (2019) detected that using fertilizers promoted the content of total phenol in blackberry fruits. Many minerals act as cofactors of many enzymes of the phenolic compound pathway (Treutter, 2010).

Carotenoids protect plants from oxidative damage (Menino, 2012). The results revealed that both fertilizers considerably improved the carotenoid content in mandarin fruits (Fig. 5). Similar to our findings, Balázs et al. (2023) observed that applying fertilizers containing different elements promoted carotenoid content in sweet potatoes. The increases in the carotenoid content in the fruits can be ascribed to the point that mineral elements have roles in the activities of carotenoid biosynthesis enzyme (Bruulsema et al., 2012).

An important parameter in evaluating the quality of fruits is the level of antioxidant activity. Our research study indicated that spraying Rice fertilizer increased considerably the antioxidant activity of mandarin fruits (Fig. 4). Antioxidant activity is affected by fertilizer use (Riahi & Hdider, 2013). Using nutrients and fertilizers to increase the antioxidant activity in the fruits is in line with previous studies (Fanasca et al., 2006; Cavender et al., 2019). Stress management is the most effective strategy for increasing antioxidants (Mukherjee et al., 2020), and improving the plant's nutritional status using fertilizers can reduce plant stress and increase antioxidants. Vitamin C, phenols, and carotenoids contribute to the antioxidant activity of citrus fruits (Zou et al., 2016). Consequently, variations in the concentration of these compounds (vitamin C, phenols, and carotenoids) showed a similar trend with the antioxidant activity of mandarin fruits (Figs. 2, 3, 4, and 5).

5 CONCLUSION

Foliar application of CalfalB and Rice fertilizers, especially Rice, improved fruit yield and quality of mandarin 'Page' due to enhanced leaf minerals, leaf chlorophyll, fruit yield, the percentages of juice , pulp , and rind , TSS, TSS/TA, rind color parameters, vitamin C, phenol compounds, carotenoid, and antioxidant activity in the fruits. Accordingly, applying fertilizers containing macro- and microelements can lead to enhanced quality and quantity of mandarin fruits, especially in regions with poor soils.

6 CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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doi:10.14720/aas.2024.120.2.19117 Original research article / izvirni znanstveni članek

Mitochondrial DNA analysis of the the Yugoslavian Shepherd Dog – Sharplanina and its phylogenetic relationship within and between breeds

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Received April 19, 2022; accepted June 03, 2024.
Delo je prispelo 19. aprila 2022, sprejeto 03. junija 2024

Mitochondrial DNA analysis of the the Yugoslavian Shepherd Dog – Sharplanina and its phylogenetic relationship within and between breeds

Abstract: The Yugoslavian Shepherd Dog – Sharplanina belongs to the group of Molosser type dog breeds and is an autochthonous dog breed in southwestern Kosovo and northwestern North Macedonia. This breed is characterised by its genetic diversity in the mitochondrial DNA. In our research we found nine haplotypes grouped into three main clades A, B and C, with distribution rates of 43 %, 43 % and 14 %, respectively. Our analyses show that the "Sharplanina dog" exhibits a remarkable genetic heterogeneity, which makes it very difficult to determine its origin and to correlate the haplotypes with the geographical location of the collected samples. The geographical proximity of the breed's origin to the habitat of the extinct ancient Molossian hound and the similarities of its haplotypes with certain dog breeds in Europe and East Asia make it a very interesting breed for further research.

Key words: livestock guardian dogs, breeds, Sharplanina shepherd dog, genetics, phylogenetics, haplotype, mitochondrial DNA

Analiza mitohondrijske DNA pri psih pasme šarplaninec in njena uporaba za ugotavljanje filogenetskih povezav med pasmami in znotraj pasme

Izvleček: Šarplaninec spada v skupino moloških psov in je avtohtona pasma na področju jugozahoda Kosova in v severozahodni Severni Makedoniji. Za to pasmo psov je značilna genetska pestrost mitohondrijske DNA. V naši raziskavi smo odkrili devet različnih haplotipov, razvrščenih v tri glavne veje, A, B in C, z deleži 43 %, 43 % in 14 %. Naše analize kažejo, da je šarplaninec genetsko izjemno heterogen, zaradi česar je zelo težko določiti njegov izvor in povezati haplotipe z geografsko lego izvora zbranih vzorcev. Zaradi geografske bližine področja razširjenosti pasme področju habitata izumrlih starodavnih moloških psov in podobnosti haplotipov šarplanincev z določenimi pasmami psov v Evropi in vzhodni Aziji, je pasma zelo zanimiva za nadaljnje raziskave.

Ključne besede: pastirski psi, pasme, šarplaninec, genetika, filogenetika, haplotip, mitohondrijska DNA

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1 INTRODUCTION

The "Sharplanina dog" is an autochthonous breed of the Sharri Mountains, a region located southeast of Kosovo and northwest of North Macedonia. For the first time, the breed was registered with the Federation Cynologique Internationale (FCI) in 1931 under the name "Sharplanina dog". In 1957 the FCI accepted the name change into "Yugoslavian shepherd dog - Sarplaninac". It is one of the representatives of Molossoid dog breeds that are grouped into section 2 of group 2 according to the FCI breed nomenclature, as a Yugoslavian shepherd dog - Sharplanina (http://www.fci.be) In Kosovo it is known as the "Deltari Ilir" and it represents one of the most popular dogs in the country. The "Sharplanina dog" was traditionally used for herding sheep and guarding, especially against wolves, as it has natural guarding abilities and an independent mind. Today, it is also used as a pet by people living in the urban areas and therefore spread all over the world. The phylogeny of molossoid dog breeds is highly controversial. It is debated that they were either domesticated in Mesopotamia or that they originally came from Tibet. Some researches go further and postulate that the Tibetan mastiff originated from the Molosser dog and not vice-versa (Savolainen et al., 2002). So far, no molecular genetic studies have been conducted to determine the phylogeny or to evaluate the existing diversity within the breed and/or between the "Sharplanina dog" and other breeds. In Western Balkan it is hard and complex to follow the genetic lineage of dog breeds (Ceh et al. 2014). Here we provide and insight in the genetic diversity of the "Sharplanina dog" by evaluation of its D-loop region on the mtDNA. To determine the diversity within the species and the genetic relationships between species, dogs belonging to the Molossoid breeds were taken for the comparison: the Portugal Serra da Estrela mountain dog, the Caucasian Shepherd Dog and the Anatolian Shepherd Dog. Although recognized as separate breeds by Turkey and several western kennel clubs, the FCI has combined Kangal and Akbash into one breed called the Anatolian Shepherd Dog with the breed number 331. Consequently, this breed reveals, in fact, a broad range of different characteristics and a high genetic variety (Vila et al; 1997).

Sequencing and analysis of the canine mitochondrial DNA has been used to determine origin of domestic dogs (Savolainen & Lundeberg, 1999; Angleby & Savolainen, 2005) and in forensic analyses (Angleby & Savolainen, 2005; Pereira et al., 2004). The phylogenetic relationship is determined by comparing the mitochondrial D-loop region and more specifically the hypervariable control region I, which are 262 and 582 base pairs (bp) long, respectively (Savolainen & Lundeberg, 1999; Angleby & Savolainen, 2005).

Six haplogroups or clades (A-F) have been reported so far (Vila et al; 1997; Angleby & Savolainen, 2005; Wayne & Ostrander, 1999). More than 71 % of all DNA samples analysed to date had haplotypes assigned to clade A (Angleby & Savolainen, 2005). More than 95 % of all haplotypes belong to the three main phylogenetic clades A, B, and C, which suggests that almost all dog populations worldwide originate from a common gene pool (Savolainen & Lundeberg, 1999). The genetic diversity of dog breeds is explained by the fact that they have a common ancestor that originated from a diverse and well-mixed gene pool. Most of them originated from east Asia and then spread througout the world (Savolainen et al., 2002; Wayne & Ostrander, 1999; Leonard et al., 2002). The high number of different mitochondrial haplotypes suggests that females were more involved in the development of a given breed than males (Sundqvist et al., 2006). Moreover, genetically diverse founders from occasional crossbreeding between different breeds and between dogs and wolves (Vila et al; 1999, Vila et al; 1997), contributed significantly to the increase of genetic heterogeneity. Most of the dog breeds show remarkable heterogeneity, which sometimes is higher between the individuals of the same breed than it is for individuals of different dog breeds (Angleby & Savolainen, 2005). Similarly, the Molosser group of dogs has a high number of haplotypes per breed.

2 MATERIALS AND METHODS

2.1 RESEARCH POPULATION

Swabs of 72 "Sharplanina dogs" (40 male and 32 female) were collected in the Sharri Mountain region in Kosovo for sequence analyses of the mtDNA. All animals belonged to private owners and were selected based on breed-specific morphological characteristics and with respect to the pedigree information (in cooperation with the Kennel Kosova Federation). The swabs were deep-frozen immediately after collection until DNA extraction. Mitochondrial DNA (mtDNA) was isolated with the Qiagen Cell Kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany). We amplified the fragment of 721bp. For comparing the mtDNA data within samples we analysed only 582 bp in the first hypervariable segment of the mtDNA control region (HV1) of D-loop. Primers were designed according to the sequence deposited in GenBank (Acc. Nr. U96639). Primers were designated as H15404 (5′-CTCTTGCTCCACCATCAGC-3′) and L16125 (5′-AAACTATAT-

GTCCTGAAACC-´3). PCR and sequencing reactions were performed on a Biometra PCR thermocycler (Biometra, Goettingen). PCR was performed using 20 ng DNA, 0.2 µM of each primer, 5 µl Q-solution, 200 mM of each dNTP and 0.2 µl Taq polymerase (1U) in 1x PCR buffer as recommended by the manufacturer (Qiagen, Hilden, Germany) in a final volume of 25 µl. Reactions were performed for 30 cycles (denaturation at 93 °C for 30s, annealing at 58 °C for 30 s, extension at 72 °C for 30s) following pre-denaturation at 95 °C for 2 min and ended with a final extension at 72 °C for 2 min. Sequencing was performed bi-directionally using the Big Dye Terminator (v 3.1) cycle sequencing kit (ABI, Weiterstadt, Germany). All sequencing reactions were performed on an ABI PRISM® 3100 DNA analyzer (ABI, Weiterstadt, Germany). DNA sequencing was performed using 10 µM of the respective oligonucleotide, 3 µl Big Dye premix and 20 ng of purified PCR product as a template in a total volume of 10 µl. he sequencing conditions were 95 °C for 10 s, followed by 29 cycles of 95 °C for 10 s, annealing at 58 °C for 5 s and an extension at 60 °C for 4 min. After sequencing, a BLAST comparison was performed (http://www.ncbi.nlm.nih.gov). Analyses of the sequenced raw data were performed using Sequencing Analysis Software 3.7 (ABI, Weiterstadt, Germany). The processed data was assembled into a contig using the DNAstar SeqMan software (DNASTAR Inc. Madison, USA).

2.2 PHYLOGENETIC ANALYSES

The sequences obtained were compared by alignment with all available D-loop sequences from Molosser breed dogs (ftp://ftp.ebi.ac.uk/pub/software/clustalw2) and haplotypes were generated using Collapse1.2 (http://darwin.uvigo.es/). Medium–spanning networks were calculated using TCS software (Clement et al., 2000) and the median-joining network algorithm (Bandelt et al., 1999) using Network version 4.1 at http://www.fluxus-engineering.com. Analysis of molecular variance (AMOVA), diversity measures and FST distances were determined using Arlequin 2.0 software (Schneider et al., 2000). For Kangal and Akbash dogs, samples were collected from NCBI GenBank with accession numbers from EF660078 to EF660191. For the Caucasian Shepherd Dog, GeneBank accession numbers AF531664 and AF531731) were used. The haplotype data for the Serra da Estrela mountain dog was taken from Van Asch et al. (2005). The sequence with accession number U96639 was used as a reference for the mitochondrial DNA of the dog.

3 RESULTS AND DISCUSSION

After mitochondrial DNA analysis, we found a total of 10 haplotypes, of which one was newly described and 9 previously reported, in the 72 DNA samples analysed, as shown in Table 1.

Table 1: Total of 10 haplotypes founded on "Sharplanina dog" based on mtDNA analyses

Base pair position	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	16	16
	55	55	55	56	56	56	56	56	56	56	56	56	56	56	58	58	58	59	59	59	60	60	
	08	26	95	12	12	20	27	26	36	93	43	05	25	50	04	15	52	18	35	52	03	25	
Haplotype																							
U96639	C	C	C	T	T	T	A	C	T	T	A	T	G	T	T	C	T	C	G	C	A	T	
A11	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	.	C	
A17	.	.	.	.	.	C	G	.	.	A	.	.	.	.	.	T	.	.	.	T	.	.	
A20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	
A24	.	.	.	.	.	.	G	.	.	G	.	-	-	-	-	T	.	.	.	T	.	.	
A71	.	.	.	.	.	C	G	.	.	A	.	.	.	C	.	T	.	.	.	.	.	C	
B1	.	T	T	.	C	.	-	T	.	G	G	.	A	.	C	T	C	T	.	T	G	.	
B10	.	T	,	.	C	.	.	T	.	G	G	.	A	.	C	T	C	T	.	T	G	.	
nB1	.	T	T	.	C	.	.	T	C	G	G	.	A	.	C	T	C	T	.	T	G	.	
C5	T	T	.	C	.	.	.	.	.	A	.	C	.	.	C	T	.	T	_	T	G	.	

The new haplotype is very similar to the haplotype B1and differs only in one nucleotide base pair (T15636C). For understanding and standardization purposes the names of clades and haplotypes were kept the same as in the previous nomenclature used in several studies (Pereira et al., 2004). We found that the nucleotide frequencies for the entire D-loop region of the mtDNA in the "Sharplanina dog" were A = 0.268, C = 0.2730, T = 0.299, and G = 0.159. Almost the same frequencies were found in the Kangal and Akbash dog breeds, which are common in the mtDNA of all vertebrates (Tamura & Nei, 1993). Our analyses revealed the presence of 21 polymorphic sites, 20 transitions, one transversion and one insertion-deletion (indel).

The haplotypes belong to three main clades A, B and C with a frequency of 43 %, 43 % and 14 % respectively. B1 is the main haplotype found in 29.3 % of the samples, followed by A17 with a frequency of 26.3 %, while the new haplotype was found in 4.1 % of the samples analysed (Table 2).

The median joining network algorithm was used with the sequencing data generated from the samples, and the results were confirmed by the medium spanning network performing calculations using a statistical parsimony algorithm with the TCS software, as shown in the following figure.

Both methods revealed that there are 17 different single nucleotide polymorphisms (SNPs) between the haplotypes. The samples analysed were collected in 5 different regions of Kosovo and we observed that they did not always have the same haplotypes, both within breed and within samples collected in the same region. Although Kosovo is a relatively small region, we did anticipate some level of heterogeneity. However, the extent of genetic diversity revealed by the mtDNA analysis of the "Sarplanina dog" exceeded our expectations. According to Ceh (2014), a total of 15 haplotypes of this breed have been identified thus far. The pair-wise genetic distance is significant for some of the inter-region correlation, indicating no geographical correlation (data not shown). This could be explained by the high number of haplotypes within the breed and the random sampling.

Table 2: Haplotype distribution - frequencies of haplotypes for the total number of "Sharplanina dog"samples, showing nine haplotypes. The haplotype nomenclature is similar as in Angelby and Savolainen (2005). Designation nB1 represents the newly discovered haplotype.

Haplotype ID	No of samples	Frequency (%)
A11	1	1.4
A17	19	26.3
A20	3	4.2
A24	2	2.7
A71	6	8.3
B1	21	29.2
B10	7	9.7
C5	10	14.0
nB1	3	4.2
Total	72	100

3.1 COMPARISON ANALYSIS WITH THE OTHER BREEDS

Four breeds were chosen for comparative sequence analyses of the D-loop region with "Sharplanina dog". The chosen breeds were Kangal (113 dogs), Akbash (20 dogs), for which the data was collected from the NCBI repository and the literature (Savolainen et al., 2002), Caucasian Shepherd Dog (3 dogs) (Savolainen et al., 2002), and Cao de Serra da Estrela (34 dogs) (Van Asch et al., 2005). We decided to make a comparative analysis with these breeds because of their desired specifics, belonging to the same group and geography. There were

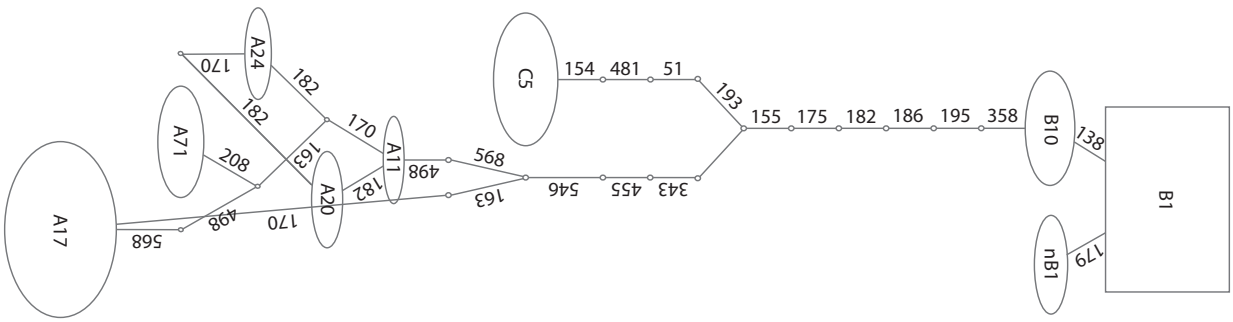

Figure 1: Sequencing data generated from the "Sharplanina dog" samples and the results were confirmed by the Medium-spanning network that performs calculations by using statistical parsimony algorithm with TCS software

6 haplotypes shared between the "Sharplanina dog" and Kangal breed, 2 haplotypes with Akbash dog, 2 haplotypes with Caucasian Ovcharka that are the two known haplotypes for this breed so far, and three haplotypes with Serra da Estrela dog, as shown in the (Fig. 2).

Only the haplotype A17 and the new haplotype B1 are not found within the breeds used for comparison. Based on the total number of dogs reported so far, the common haplotypes in percentages are as follows: 50 % of the Kangal breed dogs share the same haplotypes with the "Sharplanina dog", 52 % of the Serra da Estrela, 40 % of the Akbash and 100 % of the Caucasian Ovcharka breed populations share the same haplotypes with the Sharplanina Dog. It is noteworthy that only four haplotypes have been reported so far for the Caucasian Shepherd Dog, but the number of samples analyzed was very small (Ceh et al., 2014). The frequency of haplotype A17 was high in the "Sharplanina dog" (approx. 26 %). The haplotype is mainly found in dogs living in the northern part of Europe and in East Asia, but not in Southern Europe, except for the Azorean Mountain Dog in the Azores. This observation suggests that this breed may be maternally descended from the northern parts of Europe rather than the Portuguese mainland dog, which is consistent with the historical report (Van Asch et al., 2005). Among the ten haplotypes, haplotype A11 is the least frequent. This haplotype together with B1 and A17 represent the most dispersed haplotypes in the world, including in the mountain/molosser dogs, such as Kangal, Akbash and Caucasian Shepherd breeds. The frequency of haplotype A20 was found to be 4 % in the "Sharplanina dog". This haplotype is also rare in the Kangal and Akbash dogs. A20 haplotype is characteristic of northern Europe, and not found in Asia. The haplotype A24 was found in only two samples of the "Sharplanina dog" and was also found in Pyrenean Mountain dog, Serra da Estrela Mountain dog, and in one sample of Kangal dog. A24 haplotype seems to be characteristic for the south of Europe, except for the Irish wolfhound which is located in the British Isles in the northern part of Europe. A71 haplotype, represented in Sharplanina breed with a frequency of 6 %, was previously found in Kisha dog and reported only in Japan (east Asia) (Savolainen et al., 2002), but latter it has been found at a very high frequency in the Serra da Estrela Mountain Dog (around 26 %) (Van Asch et al., 2005). B1 haplotype is one of the haplotypes found in all regions of Europe and Asia, and we found that it is the most prevalent in the "Sharplanina dog" (29 %). A frequency is also high in the Kangal (44 %) and Serra da Estrela (17 %) breeds. B10 is one of the two haplotypes found in Caucasian Ovcharka (Savolainen et al., 2002), and this haplotype is represented with a frequency of approximatelly 10 % in our samples. Also, it was found in one sample of the Kangal breed and based on the published data it was not found in other breeds selected for our comparative analysis. C5 haplotype, represented in 14 % of the studied population has been reported in one China dog of the unknown breed (Savolainen et al., 2002), one Mongolian dog (Tsuda et al.,

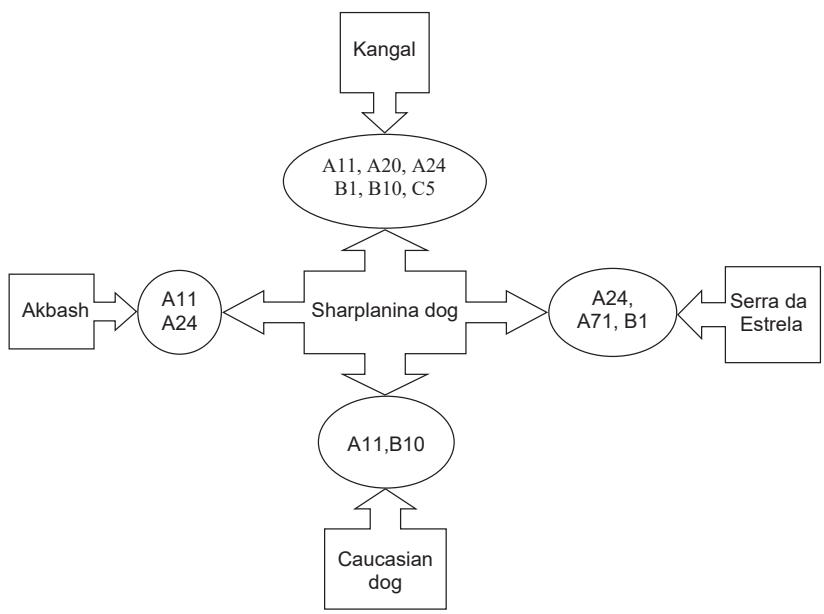

Figure 2: Schematic connection of sharing the haplotype, between the "Sharplanina dog" and the other breeds. In the square are the names of breed and in the circle the shared haplotype.

Table 3: Diversity statistics of the mtDNA D-loop region across selected dog breeds

Breed	N	Number of Haplotypes	Haplogroups	Haplotype diversity	Mean n° of pairwise differences	Nucleotide diversity
Sharplanina dog	72	9	3	0.816 ± 0.024	8.212 ± 3.852	0.014 ± 0.007
Kangal	104	21	4	0.796 ± 0.036	8.369 ± 3.906	0.014 ± 0.007
Akbash	20	10	3	0.836 ± 0.064	4.241 ± 2.195	0.007 ± 0.004
Serra da Estrela	24	8	4	0.852 ± 0.030	9.802 ± 4.601	0.017 ± 0.009
Tibetan Masttif	5	2	1	0.600 ± 0.175	3.673 ± 2.228	0.006 ± 0.004
Kaukazskaia Outcharka	3	2	2	0.666 ± 0.314	8.000 ± 5.126	0.014 ± 0.011

1997), in Anatolian Shepherd Dog (Gundry et al., 2007), and in three dogs of Kangal breed. Also, this haplotype has been reported in three samples of the "Sharplanina dog" from two different sources (Gundry et al., 2007), one originating from North Macedonia and two other samples of unknown origin. A very common clade for breeds of Europe and Asia is clade A with frequencies of about 71 %, and is also common for the Molossian dogs (Savolainen et al., 2002). In the "Sharplanina dog" clade A, was represented in 43 % of the studied dogs and is equally represented with clade B (43 %), the latter not being present in other European breeds. Also, clade A is present in 35 % of the population of Kangal breed with 12 different haplotypes. Clade A is represented in 55.9 % of the Serra da Estrela breed population, and in 90 % of the Akbash breed. In Caucasian shepherd dog a fixation index (F_{ST}) was used as a measure of genetic distance between the dog breeds and showed no geographical correlation among breeds, which might be explained by the high genetic heterogeneity of the mtDNA (Savolainen et al., 2002) . The "Sharplanina dog" has different haplotypes and this is typical of other breeds as well. Also the Tibetian Mastif showed similar distribution of clades as "Sharplanina dog", with 51.4 % of haplotypes belonging to clade A, 45.9 % to clade B and only 1 % on clade C (Li et al., 2017). It represents a breed that has a large number of haplotypes, compared with for example Serra de Estrela Mountain dog (Van Asch et al., 2005) that has 8 haplotypes classified in 4 clades or German Shepherd dog that has 7 haplotypes classified in 2 clades (Volkel, personal communication) or Shiba, the Japanese dog that has 8 haplotypes in three clades (Okumura et al., 1996). A large number of haplotypes was also observed in the Kangal breed (21 haplotypes in four clades) and in the Akbash breed (10 haplotypes in two clades), as shown in Table 3.

The reason for such a high number of haplotypes found in the Kangal dogs is explained (Altunok et al., 2005). The diversity observed in Kangal dogs may be due to repeated mating between Kangal and wild dogs, a crossbreeding known to produce offspring that resemble the Kangal type (Altunok et al., 2005). In general, the haplotype diversity found in the "Sharplanina dog" is most likely due to the origin of their ancient ancestors, that may have come from a very diverse gene pool. To this argument, we would add the fact that for thousands of generations, before the advent of modern breeding methods, dogs around the world were mating randomly (Savolainen et al., 2002). The "Sharplanina dog" itself may not originate directly from the wolf lines, but most likely descended from the oldest dogs in Europe (Molosser dogs) and was subsequently mixed with other dogs in the region.

Our results are not in accordance with the theory that the Molosser dogs are descendants of the Tibetan mastiff. We did not find any sample containing the A44 or A45 haplotype (Savolainen et al., 2002), which are typical of the mastiff breed. Not only the breeds we studied but also all other Molosser type breeds studied previously share no haplotypes with the new Tibetan mastiff which is the descendant of the old Tibetan mastiff. (Savolainenet al., 2002). However, because deep molecular analyses were not performed yet for the mastiff breeds and the Tibetan mastiff specifically, we cannot claim the above with certainty.

The second theory is that the old ancestors of the "Sharplanina dog" were from the Caucasus. Our haplotype analyses suggest that the "Sharplanina dog" might have common ancestor with the Caucasian shepherd breed. The B10 and A11 haplotypes are represented in 11 % of all our samples, the frequency that is very similar to the Caucasian Shepherd Dog. However, it cannot be considered as a fact since the haplotypes are represented with a moderate frequency value (11 %), and second because it is well-known that breeds from different regions can also have more similar haplotypes than breeds that co-exist in the same habitat (van Asch et al., 2005). The breed exists many years in the region, and is considered autochthonous, and unique for Europe. However, sampling strategy is very important for evolutionary studies (Webb et. al, 2010). The "Sharplanina dog" has three haplotypes, C5, A17, and nB1, with a cu-

mulative frequency of 37 %, which is not characteristic for any other breed in Europe. What makes this breed very specific is that clades A and B are represented in an equal frequency of 43 % and 43 %, respectively. This could be expected, as was suggested that clade A (the oldest clade) has its origin from east Asia and clade B from Europe and South Asia (Savolainen et al., 2002). We suggest that the dog ancestors of this breed came from old breed of south Asia and new breed of Europe.

Similar to other breeds, the "Sharplanina dog" has a large number of haplotypes in three main clades. Based on this discovery, it is assumed that at least ten female lines from the breed's population probably played a role in the development of the breed. Nevertheless, Ceh and Dovc (2014) identified 15 haplotype lines, but this discrepancy could be due to the sampling distribution, suggesting that the lower number of haplotypes could be a result of the sampling procedure, which was only conducted in the Kosovo region. Genetic diversity within breeds of the Molossian dog group is often high, reflecting the origin of a genetically diverse founder population, followed by occasional interbreeding between breeds and between dogs and wolves (Vila et al., 1999). Given that the Molossian group is heterogeneous, we should consider both possibilities. First, the grey wolf is very heterogeneous, and as a descendant, so is the dog.

Secondly, in the past, owners neglected to maintain the purity of the dog breed because they were not interested in genetics. Females were often copulated with males of other breeds and could also randomly copulate with wolfs (Sundqvist et al., 2006). Anyway, the breeds of the Molloser group survived through history, because of the practical utility - guarding the flock and house against predators or foreigners. The "Sharplanina dog" has high genetic diversity and very unique distribution of haplotypes, representing a mix between breeds of Europe and Asia and between northern and southern Europe. Three haplotypes are very specific for this breed especially the haplotype C5, found only in this breed in Europe, haplotype A71 only in this breed and in Serra da Estrela (Portugal), and a newly discovered haplotype, nB1 which we suggest to name haplotype B20, if we consider the haplotype designations from other studies (Angleby & Savolainen, 2005), there is no haplotype with the initials B20.

4 CONCLUSION

Looking at the origin of this breed and the breeds studied, it appears that they are descended from the ancient dog, which originated in Mesopotamia, for example, or, as some assume, in Tibet. The archaeological data, the historical data and the phylogenetic data make it seem more likely that the Molosser group breed originated somewhere more nearby. The fact that the breed's ancestors were well known at the time of Aristotle and Alexander the Great also confirms that the breed could have originated in this region. This may be explained with better genetic characterisation of other breeds in the region and the other breeds in Asia, and also wolfs in this region. The data does not give a perfect view of the phylogenetic relationship between this breed and the similar breeds, also taking into consideration that the phylogenetic correlation between breeds is/was done with different methods, for example using the chromosome Y or SNP analyses or combining maternal and paternal DNA analyses. Our study characterises the breed in a way of maternal factors and represents a step forward in phylogenetic studies of "Sharplanina Dog", which could also be used as tools for forensic purposes, especially in Kosovo and North Macedonia, representing regions with a high number of the "Sharplanina dog".

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doi:10.14720/aas.2024.120.2.17019 Review article / pregledni znanstveni članek

Biotehnološki procesi kot sredstvo za povečanje dostopnosti in antioksidativne aktivnosti fenolnih spojin iz zrn krušne pšenice in pire

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Received December 11, 2023; accepted May 01, 2024.
Delo je prispelo 11. decembra 2023, sprejeto 1. maja 2024.

Biotechnological processes as means to increase the accessibility and antioxidant activity of phenolic compounds from bread wheat and spelt grains

Abstract: Cereal grains, especially bran, are a rich source of phenolic compounds with antioxidant activity. The potential positive effects of phenolics from whole grains of spelt and bread wheat on human health are limited by the poor bioaccessibility and bioavailability of their bound phenolics. Studies have shown that biotechnological processes (germination/fermentation/enzymatic treatment) are an effective strategy for improving the release of bound phenolics from the cell wall matrix of cereal grains. In this review article, the effects of biotechnological processes on the composition, antioxidant activity and bioaccessibility of phenolics from spelt and bread wheat grains are discussed in detail. Existing research indicates the presence of a different phenolics in spelt and bread wheat grains, making whole grains excellent for improving nutritional value of products. It has been shown that biotechnological processes can effectively increase the content of bioaccessible and bioavailable phenolics in cereal grains, which enables improved *in vitro* antioxidant activity. Currently, there is a lack of *in vivo* studies to confirm the findings obtained *in vitro*, so *in vivo* studies to determine the biological activity of phenolic compounds from pre-treated grains will be crucial in the future.

Key words: phenolic compounds, antioxidant activity, accessibility, germination, fermentation, enzymatic treatment, LC-MS/MS

Biotehnološki procesi kot sredstvo za povečanje dostopnosti in antioksidativne aktivnosti fenolnih spojin iz zrn krušne pšenice in pire

Izvleček: Žitna zrna, zlasti otrobi, so bogat vir fenolnih spojin z antioksidativnim delovanjem. Potencialni pozitivni učinki fenolnih spojin iz polnozrnatih zrn krušne pšenice (*Triticum aestivum* L.) in pire (*Triticum spelta* L.) na človeško zdravje so zaradi slabe biološke dostopnosti in razpoložljivosti vezanih fenolnih spojin omejeni. Študije so pokazale, da so biotehnološki procesi (kaljenje/fermentacija/encimsko tretiranje) učinkovita strategija za izboljšanje sproščanja vezanih fenolnih spojin iz matriksa celičnih sten žitnih zrn. V preglednem članku temeljito obravnavamo vplive biotehnoloških procesov na sestavo, antioksidativno aktivnost in biološko dostopnost fenolnih spojin iz zrn krušne pšenice in pire. Obstoječe raziskave kažejo na prisotnost raznovrstnih fenolnih spojin v zrnih krušne pšenice in pire, zaradi česar so polnozrnata žitna zrna odlična za uporabo v izdelkih, z namenom izboljšanja njihove hranilne vrednosti. Dokazano je, da biotehnološki procesi učinkovito povečajo vsebnost biološko dostopnih fenolnih spojin v žitnih zrnih, kar omogoča izboljšano *in vitro* antioksidativno delovanje. Trenutno primanjkuje *in vivo* študij za potrditev ugotovitev dobljenih *in vitro*, zato bodo v prihodnosti ključne *in vivo* študije določanja biološke aktivnosti fenolnih spojin iz predhodno obdelanih zrn.

Ključne besede: fenolne spojine, antioksidativna aktivnost, dostopnost, kaljenje, fermentacija, encimsko tretiranje, LC-MS/MS

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1 UVOD

Krušno pšenico (*Triticum aestivum* L.*)* in piro (*Triticum spelta* L.) taksonomsko uvrščamo v družino trav (Poaceae), rod pšenice (*Triticum*). Pšenica predstavlja eno najpomembnejših rastlin namenjenih prehrani. Poznamo več vrst pšenice, vendar pa je v svetovnem merilu najbolj razširjena navadna ali krušna pšenica. To potrjujejo podatki FAOSTAT, ki navajajo, da so leta 2021 pridelali več kot 770 milijonov ton krušne pšenice na več kot 200 milijonih hektarjev (FAOSTAT, 2023). Krušna pšenica in izdelki iz nje predstavljajo dober vir energije, esencialnih aminokislin, mineralov, vitaminov in prehranskih vlaknin (Escarnot in sod., 2012). Pira predstavlja najstarejšo obliko heksaploidne pšenice. V začetku 20. stoletja se je pridelava pire močno zmanjšala, v zadnjih letih pa postaja pomembna alternativa navadni pšenici, zaradi večjega povpraševanja po ekološko pridelani hrani in pozitivnih učinkov na zdravje ljudi (Bojňanská in Frančáková, 2011). Pridelava pire ima številne prednosti, kot so majhni stroški pridelave, primerna je za gojenje brez pesticidov, saj ima dobro odpornost proti boleznim in škodljivcem, raste tudi v težkih pridelovalnih razmerah, vendar pa je hektarski pridelek manjši kot pri pšenici. Iz prehranskega stališča pa pira v primerjavi s krušno pšenico postaja bolj zaželena zaradi večje vsebnosti beljakovin (Pruska-Kedzior in sod., 2008), prostih sladkorjev (Zörb in sod., 2007), maščobnih kislin in lipidov (Ruibal-Mendieta in sod., 2005). Tako pri krušni pšenici kot piri predstavljajo ogljikovi hidrati glavne sestavine (59-71 %) zrna (Escarnot in sod., 2012), ki so bogata tudi s prehranskimi vlakninami (2 %). Poleg tega, da ima pira večjo vsebnost proteinov kot krušna pšenica, pa se razlikuje tudi razmerje med gliadinom in gluteninom, ki se pri piri giblje med 2,8 in 4,0, pri krušni pšenici pa med 1,5 in 3,1, kar nakazuje na večjo razteznost testa in manjši volumen pirinega kruha (Geisslitz in sod., 2019).

Pri vsebnosti bioaktivnih spojin nekateri avtorji navajajo, da večjih razlik med piro in krušno pšenico ni mogoče opaziti (Bonafaccia in sod., 2000), medtem ko drugi avtorji pripisujejo beli pirini moki večjo vsebnost antioksidantov kot moki iz krušne pšenice (Wang in sod., 2020). Bioaktivne spojine se v žitnih zrnih večinoma nahajajo v zunanjih plasteh zrna (perikarp, testa, alevronska plast), zato med bolj zdrave živilske izdelke sodijo polnozrnati izdelki. V žitnih zrnih so najpogostejše bioaktivne spojine: prehranske vlaknine (β-glukani, arabinoksilani, rezistentni škrob), fenolne spojine (fenolne kisline, flavonoidi), karotenoidi, steroli, fitati, itd. (Gani in sod., 2012).

Fenolne spojine se v rastlinski celici nahajajo v topni obliki, kamor spadajo proste in konjugirane fenolne spojine, ter v netopni vezani obliki. Proste fenolne spojine so v nevezani obliki, konjugirane pa so vezane na topne molekule z majhno molekulsko maso (sladkorji, maščobne kisline), običajno pride med njimi do tvorbe kovalentnih vezi, lahko pa tudi do nekovalentnih vezi, vodikovih vezi ali hidrofobnih interakcij med različnimi molekulami. Večina topnih fenolnih spojin se nahaja v vakuolah rastlinskih celic (Shahidi in Yeo, 2016; Xu in sod., 2020). Proste in konjugirane fenolne spojine tako uvrščamo med ekstraktibilne fenolne spojine, netopne vezane fenolne spojine pa med neekstraktibilne (Xu in sod., 2020). Količinsko najbolj zastopana oblika so netopne vezane fenolne spojine, kar so potrdile tudi številne raziskave (Chen in sod., 2017; Pang in sod., 2018). Vezane fenolne spojine tvorijo različne vezi z molekulami v celičnih stenah zrna, tako lahko karboksilna skupina fenolnih kislin (benzojske in cimetne kisline) tvori estrske vezi s hidroksilno skupino komponent celične stene (strukturni ogljikovi hidrati, proteini). Med hidroksilnimi skupinami fenolnih spojin in komponentami celične stene (celuloza, lignin) se lahko tvorijo etrske vezi (Acosta-Estrada in sod., 2014). Nadalje se lahko med ogljikovim atomom fenolnih spojin in ogljikovim atomom komponent celičnih sten tvorijo tudi C-C vezi (C-glikozidi) ali pa fenolne spojine sodelujejo pri tvorbi vodikovih in elektrostatskih vezi (Shahidi in Yeo, 2016). Vezane fenolne spojine predstavljajo glede na skupno količino fenolnih spojin: 92 % v piri (Mencin in sod., 2022a), in 75 % v krušni pšenici (Adom in Liu, 2002). Nahajajo se v otrobih žitnih zrn, zato je pomembno poudariti, da uživanje žit pripomore k večjemu vnosu fenolnih spojin le pri uživanju polnozrnatih izdelkov.

Fenolne spojine zaradi svoje strukture izražajo antioksidativno aktivnost, tako da prostim radikalom oddajo elektron ali vodikov atom in jih na ta način stabilizirajo (Terpinc in Abramovič, 2010). V raziskavah kjer so z *in vitro* testi določali antioksidativno aktivnost, so ugotovili, da vezane fenolne spojine izkazujejo znatno večjo antioksidativno aktivnost v primerjavi s prosto in konjugirano obliko (Acosta-Estrada in sod., 2014; Chen in sod., 2017; Pang in sod., 2018). V *in vivo* raziskavi, ki je bila narejena na 32 zdravih prostovoljcih, so Costabile in sod. (2008) ugotovili, da uživanje polnozrnate krušne pšenice prispeva k povečani koncentraciji ferulne kisline v krvi, hkrati pa pozitivno vpliva na črevesno mikrobioto, saj se je povečala populacija koristnih bifidobakterij in mlečnokislinskih bakterij.

Struktura matriksa celičnih sten v otrobih in način, kako so fenolne spojine vključene v matriks, močno vplivata na njihovo biološko dostopnost. Zato so se razvile različne tehnologije, s katerimi olajšamo cepitev vezi, povečamo količino sproščenih vezanih fenolnih spojin in posledično izboljšamo njihovo biološko dostopnost (Wang in sod., 2014). Biotehnološki procesi, kamor spa-

dajo kaljenje, fermentacija in encimsko tretiranje, trenutno predstavljajo eno izmed najbolj aktualnih tem s področja izboljšanja biološke dostopnosti in razpoložljivosti fenolnih spojin.

Kaljenje predstavlja ekonomično in učinkovito naravno metodo, ki zmehča strukturo in izboljša hranilno vrednost zrna - poveča se vsebnost bioaktivnih spojin, hkrati pa se zmanjša vsebnost antinutritivnih komponent (Singh in Sharma, 2017). Kaljenje poveča aktivnost hidrolitičnih encimov, spodbudi nastanek novih in aktivacijo tistih, ki so v nekaljenem zrnu v neaktivnem stanju. Med procesom kaljenja pride do razgradnje makrohranil (ogljikovih hidratov, proteinov, lipidov), kar poveča količino njihovih presnovnih produktov (enostavnih sladkorjev, prostih aminokislin, organskih kislin), istočasno pa se sproži sinteza sekundarnih metabolitov. Z razgradnjo makrohranil se posledično sproščajo tudi vezane fenolne spojine, kar v začetni fazi kaljenja pomeni upad vezane frakcije in porast proste (Terpinc, 2019). Na omenjene spremembe lahko dodatno vpliva abiotski stres med kaljenjem. Rastline se na stresne razmere odzivajo tako, da inducirajo sintezo različnih zaščitnih spojin, tudi številnih fenolnih spojin, ki omogočajo preživetje in nadaljnjo rast v takšnih razmerah (Falcinelli in sod., 2017; Chen in sod., 2019; Ma in sod., 2019). Različne raziskave so pokazale, da je količina fenolnih spojin in razmerje med ekstraktibilnimi in vezanimi fenolnimi spojinami odvisno od obdelave zrn pred kaljenjem, razmer namakanja (Yang, 2001; Xu in sod., 2009) in od razmer, v katerih zrno kali: temperature (Paucar-Menacho in sod., 2017; Chavarín-Martínez in sod., 2019; Mencin in sod., 2021a), časa kaljenja (Terpinc in sod., 2016; Paucar-Menacho in sod., 2017; Kim in sod., 2018), načina osvetljevanja (Xiang in sod., 2017) in relativne vlage (Yang, 2001).

Druge postopke, s katerim lahko povečamo biološko dostopnost bioaktivnih spojin, predstavljajo različni tipi fermentacije (alkoholna, mlečnokislinska, spontana, itd.) (Angelino in sod., 2017). Prednost tega procesa je, da poleg encimov iz žitnih zrn sodelujejo tudi encimi eksternih mikroorganizmov, ki pripomorejo k razgradnji celične stene. Prav tako pa fermentacija spodbuja sintezo novih spojin kot tudi encimsko transformacijo že prisotnih bioaktivnih spojin. Dosedanje študije o vplivu fermentacije na vezane fenolne spojine v različnih žitnih zrnih so potrdile povečanje njihove biološke dostopnosti in razpoložljivosti (Wang in sod., 2014). Vpliv fermentacije na fenolne spojine je v glavnem odvisen od vrste zrn (Đorđević in sod., 2010), vrste mikroorganizmov (Katina in sod., 2007; Đorđević in sod., 2010; Mencin in sod., 2022a) in razmer fermentacije (temperatura, pH, čas) (Boskov Hansen in sod., 2002; Katina in sod., 2007).

Prav tako učinkovit postopek za sproščanje vezanih fenolnih spojin iz komponent celične stene žitnih zrn je encimsko tretiranje, za katerega velja, da je okolju prijazno, energetsko učinkovito in sprošča le specifične spojine, ne da bi pri tem poškodoval ostale spojine (Ferri in sod., 2020). Pri encimskem tretiranju zrna neposredno obdelamo s hidrolitičnimi encimi, s čimer izboljšamo biološko dostopnost in razpoložljivost fenolnih spojin. Encimi, ki hidrolizirajo celične stene (celulaze, ksilanaze, esteraze itd.), so bili že večkrat uporabljeni za razgradnjo matriksa celičnih sten v žitnih zrnih (Moore in sod., 2006; Acosta-Estrada in sod., 2014; Bei in sod., 2018; Mencin in sod., 2022b). Feruloil esteraze spadajo v skupino esteraz, ki hidrolizirajo estrsko vez med hidroksicimetnimi kislinami in hemicelulozo, prisotno v celičnih stenah žitnih zrn. Prav tako je feruloil esteraza sposobna katalizirati cepitev kovalentne vezi med dvema ferulnima kislinama, ki sta pritrjeni na sosednja arabinoksilana. Feruloil esteraza postane aktivna tudi med kaljenjem, našli pa so jo tudi pri laktobacilih, prisotnih v črevesju človeka (Faulds in sod., 2004; Gänzle, 2014). Številni znanstveni članki (Sancho in sod., 2001; Mathew in Abraham, 2004; Moore in sod., 2006) opisujejo sinergistične interakcije med ksilanazami, ki naključno cepijo β-1,4 ksilansko strukturo, in med feruloil esterazami, ki olajšajo ksilanazam dostopnost do komponent celičnih sten. V povezavi z razgradnjo celičnh sten se omenjajo tudi α-amilaze in proteaze (Singh in sod., 2016).

Nekaj študij (Katina in sod., 2007; Anson in sod., 2009) je bilo narejenih tudi na področju uporabe različnih kombinacij biotehnoloških procesov (kaljenja/fermentacije/encimskega tretiranja) na različnih žitnih zrnih. Raziskave kažejo na sinergističen učinek pri sočasni uporabi različnih biotehnoloških procesov, saj pripomorejo k večji koncentraciji in aktivnosti hidrolitičnih encimov.

Uživanje bioaktivnih spojin je ključno predvsem zaradi njihovih ugodnih učinkov na naše zdravje. Preden lahko posamezni spojini pripišemo pozitivno delovanje, je potrebno poznati njeno biološko dostopnost in razpoložljivost v človeškem organizmu. Biološka dostopnost (ang. bioaccessibility) je definirana kot količina snovi, ki se lahko sprosti iz matriksa hrane v prebavni trakt in je na voljo za absorpcijo v tankem črevesu. Razgradnja hrane, ki je ključna za biološko dostopnost in razpoložljivost, se prične v ustih, nadaljuje se v želodcu in v črevesu. Vezane fenolne spojine niso dostopne encimom gastrointestinalnega (GI) trakta, tako ima več kot 90 % fenolnih spojin v žitnih zrnih majhno biološko dostopnost. Neabsorbirane fenolne spojine potujejo v debelo črevo, kjer njihovo razgradnjo deloma katalizirajo encimi črevesne mikrobiote (Vitaglione in sod., 2008).

Prispevek povzema rezultate raziskav, ki so bile opravljene na zrnih krušne pšenice in pire z namenom

izboljšanja dostopnosti in antioksidativne aktivnosti fenolnih spojin z različnimi biotehnološkimi procesi.

2 BIOTEHNOLOŠKI PROCESI IN NJIHOVA APLIKACIJA NA PODLAGI DOSEDANJIH RAZISKAV

V okviru svoje študije so Mencin in sod. (2021) raziskovali vpliv kaljenja pirinih zrn v različnih stresnih razmerah na antioksidativne lastnosti fenolnih spojin. Zanimala jih je vsebnost ekstraktibilnih in vezanih skupnih fenolnih spojin, določena s Folin-Ciocalteu metodo, hkrati pa tudi identifikacija in kvantifikacija posameznih fenolnih spojin, določena s HPLC-MS/MS metodo. Ekstraktibilne fenolne spojine so ekstrahirali z absolutnim metanolom, iz trdnega preostanka po metanolni ekstrakciji pa so netopne vezane fenolne spojine sprostili iz vezane oblike s pomočjo alkalne hidrolize. V raziskavi so zrna pire kalili 144 h v temi, stresne razmere so zagotovili z manj ugodno temperaturo kaljenja, manjšim dodatkom vode, povečano slanostjo in osmolarnostjo, z mehansko poškodbo kalčkov ter z različnimi kombinacijami naštetega. Prav kombiniranje različnih stresnih razmer je rezultiralo v večjih vsebnostih ekstraktibilnih in vezanih fenolnih spojin, hkrati pa se je povečala tudi sposobnost lovljenja DPPH$^{\bullet}$ radikalov v primerjavi s kontrolo, tj. zrna kaljena 144 h pri 20 °C. Ne glede na vrsto abiotskega stresa, so opazili znatne razlike v vsebnosti ekstraktibilnih in vezanih fenolnih spojin pri kaljenih zrnih, slednje so predstavljale kar dve tretjini vseh fenolnih spojin. Kaljenje pirinih zrn ob dodatku 25 mM NaCl in 50 mM sorbitola brez aplicirane mehanske poškodbe je med vsemi preizkušenimi kombinacijami najbolj prispevala k povečanju vsebnosti fenolnih spojin in njihove antioksidativne aktivnosti. Na podlagi preliminarnih poskusov so avtorji ugotovili, da sta ustrezna temperatura kaljenja in dodatek vode predpogoj za povečano tvorbo fenolnih spojin med kaljenjem, saj so optimalno kombinacijo stresa aplicirali pri 25 °C in razmerju med maso zrn in dodatkom vode: 1:2 (15 g:30 ml). Prav tako so opazili, da so ekstraktibilne frakcije fenolnih spojin izkazovale relativno veliko antioksidativno aktivnost, glede na znatno večjo vsebnost fenolnih spojin v vezanih frakcijah. Avtorji so predvidevali, da večja heterogenost ekstraktibilne frakcije prispeva k antioksidativni aktivnosti.

Hübner in Arendt (2013) sta poročala, da je vsebnost fenolnih spojin, ki jih rastlina sintetizira in kopiči med kaljenjem, odvisna predvsem od odziva rastline na stresne dejavnike. Nadalje so različne raziskave pokazale, da kaljenje zrn poveča vsebnost topnih fenolnih spojin, kar pripisujejo sintezi *de novo* in različnim biološkim transformacijam (Gan in sod., 2017; Kim in sod., 2018). Transformacije, ki se dogajajo med kaljenjem, so odvisne tudi od dolžine kaljenja, saj se v zgodnji fazi kaljenja zaradi razgradnje gradnikov celičih sten poveča vsebnost enostavnih sladkorjev in aminokislin, hkrati pa se začnejo sproščati tudi fenolne spojine vezane na komponente celičnh sten. Z daljšim časom kaljenja se začnejo sintetizirati rastlinske celice z novimi celičnimi stenami in nekatere *de novo* sintetizirane topne fenolne spojine se vežejo z novo nastalimi komponentami celičnih sten. Tako se pri daljšem času kaljenja začne povečevati vsebnost vezanih fenolnih spojin (Wang in sod., 2014).

Večina raziskav poroča, da kaljenje znatno izboljša tudi antioksidativno aktivnost ekstraktibilnih fenolnih spojin v primerjavi z nekaljenimi žitnimi zrni (krušna pšenica, riž, koruza, ječmen), kar pripisujejo povečani vsebnosti antioksidativnih spojin v kaljenih zrnih (Donkor in sod., 2012; Ti in sod., 2014; Žilić in sod., 2015).

Živković in sod. (2023) so poročali, da je kaljenje (96 h) pirinih zrn znatno povečalo vsebnost prostih in vezanih metabolitov. Večina fenolnih spojin pa je bila prisotna v vezani frakciji, kjer je prevladovala *trans*-ferulna kislina. V prosti frakciji so našli veliko vsebnost apigenin di-*C*-glikozidov, kaljenje pa je najbolj vplivalo na vsebnost šaftozida, saj se je vsebnost le-tega povečala za trikrat. Opazili so tudi povečanje antioksidativne aktivnosti kaljenih zrn pire, predvsem na račun kopičenja sekundarnih metabolitov.

Prav tako so Mencin in sod. (2021) poročali, da sta *trans*-ferulna in *p*-kumarna kislina glavni predstavnici identificiranih vezanih fenolnih spojin v 144 h kaljenih zrnih pire, njuna vezana oblika pa predstavlja kar 99 % od skupne vsebnosti (ekstraktibilne + vezane) posamezne kisline. Zanimivo je, da so stresne razmere vplivale na zmanjšanje vsebnost posameznih ekstraktibilnih fenolnih kislin, po drugi strani pa se je vsebnost vezanih povečala v vzorcih, ki so bili izpostavljeni povečani slanosti in osmolarnosti.

Fermentacija je še ena koristna tehnika predhodne obdelave zrn, ki učinkovito sprošča fenolne spojine iz žitnih otrobov (Angelino in sod., 2017). Mencin in sod. (2022a) so pirina zrna izpostavili različnim tipom fermentacije (mlečnokislinska, alkoholna, kombinirana (mlečnokislinska + alkoholna), spontana), ki so jih kombinirali s kaljenjem in z encimskim tretiranjem. Ugotovili so, da se je ne glede na prisotno mikrofloro, po fermentaciji neobdelanih, kaljenih in encimsko tretiranih zrn povečala vsebnost ekstraktibilnih in vezanih fenolnih spojin. Hkrati se je znatno zmanjšalo razmerje med vezanimi in ekstraktibilnimi fenolnimi spojinami, kar je posledično pozitivno vplivalo na dostopnost pirinih antioksidantov. Rezultati so pokazali, da tako obdelava pirinih zrn s kaljenjem in encimskim tretiranjem

pred fermentacijo, kot tudi vrsta fermentacije vplivata na spremembe vsebnosti skupnih fenolnih spojin.

Wang in sod. (2014) so poročali, da kombinacija kaljenja in fermentacije vodi do sinergističnih učinkov, saj kaljena zrna predstavljajo bogat vir fermentabilnih virov (sladkor, dušikove spojine), hkrati pa tako kaljenje kot tudi fermentacija prispevata k večji koncentraciji in aktivnosti hidrolitičnih encimov, kar vodi k boljši biološki dostopnosti fenolnih spojin. Med fermentacijo zrn se vsebnost bioaktivnih komponent spreminja zaradi metabolne aktivnosti prisotnih mikroorganizmov, ki razgrajujejo estrske vezi in hidrolizirajo β-glukozidne vezi, pri čemer se sproščajo vezane fenolne spojine (Adebo in Medina-Meza, 2020). Po drugi strani pa na vsebnost fenolnih spojin med fermentacijo lahko vpliva tudi endogena sinteza fenolnih spojin v mikroorganizmih (Chrzanowski, 2020).

Mencin in sod. (2022a) so poročali, da so največjo vsebnost ekstraktibilnih in vezanih fenolnih spojin ter njihovo antioksidativno aktivnost določili pri kaljenih zrnih fermentiranih s kvasovko *Saccharomyces cerevisiae* Meyen ex E.C. Hansen. Zanimivo, pri encimsko tretiranih pirinih zrnih je najučinkoviteje povečala vsebnost ekstraktibilnih fenolnih spojin fermentacija z bakterijo *Lactobacillus plantarum* (Orla-Jensen 1919) Bergey et al. 1923 (Approved Lists 1980) . medtem ko je imela ista fermentacija z *L. plantarum* negativen vpliv na vsebnost vezanih fenolnih spojin. O zmanjšanju vsebnosti fenolnih spojin so poročali tudi Spaggiari in sod. (2020), ki so predvidevali, da je razlog v metabolnih lastnostih mikroorganizmov, ki lahko transformirajo fenolne spojine v različne metabolite. Vsebnost ekstraktibilne *trans*-ferulne kisline se je najbolj povečala pri kaljeni piri fermentirani s kvasovko *S. cerevisiae* in to za kar 2922 %. Podobno so poročali tudi Anson in sod. (2009), fermentacija krušne pšenice s kvasovko *S. cerevisiae* je povečala biološko dostopnost ferulne kisline. Prav tako so Konopka in sod. (2014) poročali, da alkoholna fermentacija poveča vsebnost ekstraktibilne ferulne kisline v pšenici za kar 10-krat v primerjavi z neobdelanimi zrni.

Mencin in sod. (2022a) so ugotovili tudi, da so fermentirana neobdelana, kaljena in encimsko tretirana pirina zrna, ki so imela večjo vsebnost skupnih fenolnih spojin, izkazovala tudi boljšo antioksidativno aktivnost, določeno z DPPH in ABTS testom. Avtorji so poudarili, da je kombiniranje biotehnoloških procesov najučinkovitejši način za znatno povečanje vsebnosti fenolnih spojin in njihove antioksidativne aktivnosti.

Moore in sod. (2006) so potrdili, da obdelava s kvasovkami (*S. cerevisiae*) znatno poveča skupno vsebnost prostih fenolnih spojin in antioksidativno aktivnost pšeničnih otrobov. Avtorji študije so izpostavili različno zmožnost kvasovk za presnavljanje posameznih fenolnih spojin. Spaggiari in sod. (2020) so v fermentiranih pšeničnih otrobih ugotovili relativno veliko vsebnost kavne kisline, kar nakazuje na metabolno aktivnost mikroorganizmov. Tudi Žilić in sod. (2015) so v svoji raziskavi na krušni pšenici izpostavili, da lahko bakterije *Lactobacillus* spp. proizvajajo kavno kislino iz klorogenske kisline. Raziskava, ki so jo naredili Montemurro in sod. (2019), je pokazala, da fermentacija kaljenih zrn krušne pšenice s kislim testom ne vpliva zgolj na povečanje fenolnih spojin, ampak tudi na povečanje vsebnosti peptidov, prostih aminokislin, γ-aminomaslene kisline ter zmanjša koncentracijo fitinske kisline, kondenziranih taninov in inhibitorjev tripsina.

Tretji biotehnološki proces, s katerim lahko izboljšamo biološko dostopnost fenolnih spojin v zrnih, je encimsko tretiranje. Mencin in sod. (2022b) so pirina zrna tretirali s celulazami, ksilanazami, feruloil esterazami, α-amilazami in proteazami, posamezno in v različnih kombinacijah. Ugotovili so, da je encimsko tretiranje zrn, ne glede na vrsto uporabljenih encimov, izboljšalo vsebnost ekstraktibilnih skupnih fenolnih spojin do 5-krat v primerjavi z netretiranimi zrni. Po drugi strani se je vsebnost vezanih fenolnih spojin zmanjšala po encimskem tretiranju. Tretiranje pirinih zrn z vsemi petimi encimi hkrati je vplivalo na največje povečanje vsebnosti ekstraktibilnih fenolnih spojin. Nadalje so avtorji poročali, da tretiranje zrn samo s feruloil esterazami ni bistveno povečalo vsebnost ekstraktibilnih skupnih fenolnih spojin. Kot razlog so navedli, da feruloil esteraze niso sposobne samostojno hidrolizirati estrskih vezi med hidroksicimetnimi kislinami in hemicelulozo v zrnih pšenice in pire zaradi morebitnih steričnih ovir, ki jih povzroča struktura polisaharidov, s čimer je okrnjena tudi migracija encimov. Po drugi strani pa so feruloil esteraze v kombinaciji s proteazami pokazale veliko sposobnost sproščanja vezanih fenolnih spojin, kar nakazuje na to, da lahko feruloil esteraze hidrolizirajo estrsko in etrsko vez med fenolnimi spojinami in komponentami celičnih sten zrn, po tem, ko proteaze hidrolizirajo strukturne proteine (Mencin in sod. 2022b). Čeprav so avtorji uporabili kar pet različnih hidrolitičnih encimov, pa encimsko tretiranje vseeno ni v celoti sprostilo vezanih fenolnih spojin. Slednje je v skladu z navedbo Moore in sod. (2006), da matriks celičnih sten zrn vsebuje tudi strukturne elemente, ki jih encimi ne morejo hidrolizirati.

Kombiniranje encimskega tretiranja z ostalima dvema biotehnološkima procesoma (kaljenjem in fermentacijo) je dodatno povečala vsebnost ekstraktibilnih skupnih fenolnih spojin v primerjavi s samo kaljenimi oz. fermentiranimi zrni. Poleg tega je encimsko tretiranje kaljenih oz. fermentiranih zrn rezultiralo v povečanju deleža ekstraktibilnih fenolnih spojin glede na skupne (ekstraktibilne + vezane) in to za 10 % oz. 38 % (Mencin

in sod., 2022b). Bei in sod. (2018) so na podlagi zaporedne uporabe encimskega tretiranja in fermentacije na zrnih ovsa ugotovili, da poleg transformacije netopnih fenolnih spojin v topne, omogoča oslabitev kovalentnih vezi med netopnimi fenolnimi spojinami in komponentami celičnih sten, zato netopne fenolne spojine lažje ekstrahiramo in tako določimo njihovo večjo vsebnost.

Mencin in sod. (2022b) so poročali, da se je vsebnost prevladujočih fenolnih kislin (*p*-kumarne, *trans*-ferulne, kavne, *p*-hidroksibenzojske kisline) v ekstraktibilni frakciji pirinih zrn po encimskem tretiranju znatno povečala v primerjavi z netretiranimi zrni. Največje povečanje ekstraktibilne *trans*-ferulne kisline so zasledili pri zrnih tretiranih hkrati s ksilanazami in feruloil esterazami. Svoje rezultate so potrdili s teorijo, da ksilanaze naključno cepijo β-1,4 ksilansko strukturo, medtem ko so feruloil esteraze sposobne sproščati ferulno kislino (Sancho in sod., 2001). Podobne raziskave so delali tudi Rakariyatham in sod. (2020), ki so ugotovili, da tretiranje zrn s celulazami poveča sproščanje *o*-kumarne kisline, nadalje so Peixoto Araujo in sod. (2019) ugotovili, da tretiranje s proteazami in celulazami poveča vsebnost ekstraktibilne ferulne in *p*-hidroksibenzojske kisline. Mencin in sod. (2022b) so poročali tudi o znatnem povečanju vsebnosti ekstraktibilne *trans*-ferulne kisline za kar 5899 % oz. 8263 % pri kombiniranju encimskega tretiranja s kaljenjem in fermentacijo pirinih zrn. Rezultati nakazujejo na to, da kaljena in fermentirana zrna, v primerjavi z neobdelanimi zrni, predstavljajo znatno boljši substrat za tretiranje z eksternimi encimi. Avtorji so zaključili, da predhodna obdelava zrn omogoča eksternim encimom lažji dostop do njihovih substratov, v primerjavi z neobdelanimi zrni.

Prav tako so Mencin in sod. (2022b) potrdili, da je kombinacija kaljenja oz. fermentacije z encimskim tretiranjem dobra strategija za izboljšanje antioksidativne aktivnosti ekstraktibilnih fenolnih spojin. Tudi Azmir in sod. (2013) so v preglednem članku poročali, da encimsko tretiranje poveča antioksidativno aktivnost žitnih zrn, razlog pa vidijo v sproščanju polarnih antioksidantov, vezanih na komponente celičnih sten, in/ali s hidrolizo biopolimerov, kot so polipeptidi in polisaharidi. Wang in sod. (2018) pa navajajo še dve možni razlagi za povečanje antioksidativne aktivnosti encimsko tretiranih zrn: prva je, da z encimskim tretiranjem povečamo topnost fenolnih spojin iz zrn, druga pa je, da po encimskem tretiranju pridobimo fenolne spojine z večjo antioksidativno aktivnostjo.

Zanimive so tudi ugotovitve Mencin in sod. (2021, 2022a, 2022b), kjer so poleg *in vitro* antioksidativne aktivnosti pirinih zrn obdelanih z biotehnološkimi procesi, določali tudi antioksidativno aktivnost v živi celici – kvasovki *S. cerevisiae*, ki omogoča vpogled v živo okolje. Fenolne spojine kaljenih zrn niso pokazale antioksidativne aktivnosti v celici, ker identificirane spojine niso uspele vstopiti v celico in jo zaščititi pred oksidacijo. Po drugi strani pa so ekstraktibilne frakcije fermentiranih in encimsko tretiranih pirinih zrn izkazale antioksidativno aktivnost v celici, medtem, ko vezane frakcije po večini niso izkazovale antioksidativne aktivnosti v celici. Avtorji so poudarili, da je za antioksidativno delovanje fenolnega ekstrakta v živi celici, ključno tudi razmerje med različnimi fenolnimi spojinami, ki vstopajo v celico. V njihovem primeru so določili manjšo znotrajcelično oksidacijo pri prehajanju večje količine flavonoidov in manjše količine hidroksicimetnih kislin v celico.

Biotehnološke procese raziskovalci pogosto uporabljajo z namenom izboljšanja biološke dostopnosti različnih bioaktivnih komponent v matriksu celičnih sten rastlin. Tako so Mencin in sod. (2022c) po obdelavi pirinih zrn z različnimi biotehnološkimi procesi določali tudi biološko dostopnost prisotnih fenolnih spojin. Uporabili so *in vitro* statični prebavni model INFOGEST (Brodkorb in sod., 2019), s katerim so posnemali prebavo v ustih, želodcu in tankem črevesu. Avtorji so poročali, da so biotehnološki procesi statistično značilno povečali vsebnost biološko dostopnih skupnih in posameznih fenolnih spojin iz pirinih zrn v primerjavi z neobdelanimi zrni. Nadalje so poudarili, da kombiniranje biotehnoloških procesov, še posebej kaljenja in alkoholne fermentacije, najučinkoviteje izboljša biološko dostopnost fenolnih spojin. Biotehnološko obdelana zrna imajo znatno večjo začetno vsebnost fenolnih spojin kot neobdelana, posledično jih lahko več vstopa v debelo črevo. Po prebavi je bila vsebnost biološko dostopnih skupnih fenolnih spojin v kaljenih pirinih zrnih fermentiranih s kvasovko *S. cerevisiae* kar 7-krat večja kot pri neobdelanih zrnih. Podobno so opazili tudi Anson in sod. (2009), ki so poročali, da je kombinacija uporabe eksternih encimov in fermentacije pri otrobih krušne pšenice učinkovito povečala biološko dostopnost ferulne kisline za kar 5-krat v primerjavi z neobdelanimi otrobi. Večina raziskav omenja velike izgube fenolnih spojin med procesom prebave (Ortega in sod., 2011; Ydjedd in sod., 2017; Chait in sod., 2020). Drastične izgube fenolnih spojin po GI prebavi so lahko posledica spremembe v molekulski strukturi fenolnih spojin zaradi različnih kemijskih reakcij, predvsem oksidacije in polimerizacije, in zaradi encimskega delovanja, ki lahko povzroči spremembe v njihovi topnosti (Ortega in sod., 2011). Mencin in sod. (2022c) so poročali, da so se v primeru *trans*-ferulne kisline, ki prevladuje v pirinih zrnih, še posebej izkazale kombinacije dveh biotehnoloških procesov, ki so njeno biološko dostopnost povečale za od 24-krat (kaljena + encimsko tretirana zrna) do 63-krat (encimsko tretirana + fermentirana zrna) v primerjavi z neobdelanimi zrni. Zanimive so tudi ugotovitve Zeng in sod. (2016), ki so poročali, da je bila

vsebnost biološko dostopnih fenolnih spojin v krušni pšenici manjša kot pri ovsu kljub večji vsebnosti skupnih fenolnih spojin in močneje izraženi antioksidativni aktivnosti. To nakazuje, da žitna zrna z večjo vsebnostjo fenolnih spojin niso nujno tista z večjo biološko dostopnostjo. Očitno igra matriks celičnih sten žitnih zrn ključno vlogo pri prebavljivosti fenolnih spojin. Lima in sod. (2019) so izpostavili, da na stabilnost fenolnih spojin med postopkom GI prebave znatno vpliva njihova kemijska struktura, saj imajo fenolne spojine različno občutljivost na spremembo vrednosti pH in aktivnost prebavnih encimov. Mencin in sod. (2022c) so ugotovili, da je kljub uporabi različnih biotehnoloških postopkov, ki so znatno povečali biološko dostopnost fenolnih spojin, večji del fenolnih spojin ostal v biološko nedostopni obliki, ki nadalje vstopa v debelo črevo. V debelem črevesu pa poteka fermentacija, kjer bakterijski encimi olajšajo sproščanje fenolnih spojin, ki v tankem črevesu niso bile dostopne (Li in sod., 2022).

3 ZAKLJUČKI

Žitna zrna predstavljajo pomemben vir vlaknin in nanje vezanih bioaktivnih spojin, ki imajo majhno biološko dostopnost. Da bi izkazale svoje pozitivne učinke na zdravje, se morajo fenolne spojine sprostiti iz matriksa celičnih sten v hrani in biti dostopne v prebavnem traktu. Znatno večja izhodiščna vsebnost fenolnih spojin v biotehnološko obdelanih zrnih sovpada z večjo količino fenolnih spojin, ki bo uspešno prešla proces prebave. Dosedanji izsledki raziskav kažejo, da imajo biotehnološki procesi pozitiven učinek na povečanje vsebnosti biološko dostopnih fenolih spojin in njihove antioksidativne aktivnosti v žitnih zrnih. Rezultati predstavljenih raziskav odpirajo možnosti za razvoj različnih funkcionalnih živil, saj prav s hranili osiromašena živila znatno pripomorejo k večji pojavnosti kroničnih bolezni. Zaradi številnih pozitivnih lastnosti biotehnoloških procesov, pa bi bili potencialni živilski izdelki z izboljšano hranilno vrednostjo dobro sprejeti med potrošniki. V prihodnje bi bilo potrebno dati poudarek na raziskavah, ki se bodo dotikale kombinacij biotehnoloških procesov, zlasti kaljenja in fermentacije, saj se je prav ta kombinacija izkazala za najučinkovitejšo metodo povečanja vsebnosti biološko dostopnih fenolnih spojin. Nadaljnje študije so potrebne tudi na področju biološke transformacije fenolnih spojin v njihove metabolite, saj lahko ti lažje vstopajo v živo celico, hkrati pa bi bilo potrebno prenesti *in vitro* študije določanja biološke dostopnosti in razpoložljivosti fenolnih spojin na *in vivo* nivo.

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