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Ovitek: Entomopatogeni glivi *Isaria fumosorosea* Wize in *Beauveria bassiana* (Bals.-Criv.) Vuill., ki sta okužili gosenico vrste *Chaetoprocta odata* (Hewitson, 1865); (A in B) Razgradnja telesa gosenice zaradi rasti micelija po okužbi; (C) Okužba z glivo *Beauveria bassiana*; (D) Okužba z glivo *Isaria fumosorosea* (Foto: Shaziya GULL in Tariq AHMAD, 1–8)
Cover: Entomopathogenic fungi Isaria fumosorosea *Wize and* Beauveria bassiana *(Bals.-Criv.) Vuill. infesting larvae of* Chaetoprocta odata *(Hewitson, 1865); (A and B) Disruption of insect body tissue due to mycelial growth after infestation; (C) Infestation by* Beauveria bassiana*; (D) Infestation by* Isaria fumosorosea *(Photo: Shaziya GULL and Tariq AHMAD, 1–8)*

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Soilless greenhouse cultivation: growth and yield of ginger in response to the pot size and culture media

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Soilless greenhouse cultivation: growth and yield of ginger in response to the pot size and culture media

Abstract: The increasing shortage of arable land and adverse weather conditions have caused a significant part of crops, especially medicinal plants, to be cultivated through soilless cultivation methods. In this research, the soilless greenhouse cultivation of ginger was studied. The experiment was laid out in a 2 × 3 factorial scheme based on a completely randomized design with 3 replications. Treatments consisted of two different sizes of pots (10 l and 20 l), and three types of culture media, including sand, perlite, and an equal (1:1) mixture of sand and perlite. Based on the results, except for leaf area and rhizome sodium content, the interaction effect was significant for most of the traits. The highest rhizome dry mass (58.13 ± 2.05), rhizome dry material content (43.68 ± 1.19), were recorded in larger pots containing the mixture medium. There were positive and significant correlations between leaf area and some of important traits such as rhizome dry mass (0.48) and rhizome dry matter content (0.58). The results of this research confirmed that by taking into account the most suitable pot size and cultivation medium (i.e. 20 l pot and mixed culture medium), the maximum yield of rhizome fresh mass can be obtained up to 133.5 ± 7.94 g.

Key words: culture medium, perlite, sand

Odziv rasti in pridelka na velikost lonca in substrat pri gojenju ingverja v rastlinjaku

Izvleček: Naraščajoče pomanjkanje orne zemlje in neugodne vremenske razmere so povzročile, da se številne gojene rastline, še posebej zdravilne zdravilne rastline, gojijo v različnih načinih brez tal. V tej raziskavi je bilo preučevano gojenje ingverja v rastlinjaku v loncih. Poskus je bil zasnovan kot popolni naključni 2 × 3 faktorski poskus s tremi ponovitvami. Obravnavanja so obsegala dve velikosti loncev (10 l in 20 l) in tri vrste substrata in sicer pesek, perlit in mešanico peska in perlite v razmerju 1:1. Rezultati so pokazali, da je bila interakcija med obravnavanji z izjemo listne površine in vsebnosti natrija v koreniki značilna za večino lastnosti. Največja suha masa korenike (58,13 ± 2,05) in vsebnost suhe snovi v njej (43,68 ± 1,19) sta bili ugotovljeni v večjih loncih, ki so vsebovali mešanico substratov. Ugotovljena je bila tudi značilna pozitivna povezava med listno površino in nekaterimi pomembnimi lastnostmi kot sta suha masa korenike (0,48) in vsebnostjo suhe snovi v njej (0,58). Rezultati raziskave so potrdili, da je potrebno upoštevati najprimernejšo velikost loncev in sestavo substrata (20 l-lonci in mešanica substrata), če hočemo doseči največji pridelek sveže mase korenik, to je do 133,5 ± 7,94 g.

Ključne besede: gojitveni medij, perlit, pesek

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1 INTRODUCTION

Ginger, (*Zingiber officinale* Roscoe), belongs to the Zingiberaceae family with numerous medicinal, nutritional, and ethnomedical values. This plant is widely used as a spice, flavoring, and herbal medicine all over the world (Zhang et al., 2021). Traditionally, ginger has been used in many medicinal systems to treat a variety of ailments including nausea and vomiting, indigestion and constipation, asthma and cough, heart palpitations, inflammation, loss of appetite, etc. (Shahrajabian et al., 2019). Ginger is cultivated mainly in tropical regions up to 1500 m above sea level, both in dry and irrigated conditions (Guo et al., 2023).

The expansion of urbanization, industrialization of societies, and rise of the sea level have caused an increasing shortage of arable land. In addition, conventional soil cultivation faces increasing problems such as successive droughts, the unpredictability of climatic patterns, inefficient management of water resources, etc (Nerlich & Dannehl, 2021). As a result, today, a considerable part of crops are cultivated using soilless methods. Besides, such methods have a unique ability to optimally use inputs as well as dense agriculture (Ragaveena et al., 2021). In comparison with field cultivation, the volume of the culture medium used for each plant in soilless cultivation is considerably reduced and thus plant growth is significantly affected by the physical and chemical characteristics of the growing medium. A suitable substrate directly affects the growth, development, and maintenance of a functional root system. Moreover, the substrate acts as a reservoir of nutrients and water and is directly responsible for the diffusion of oxygen into the roots and other gas exchanges, so choosing a suitable culture medium is of particular importance. Various inorganic compounds are used as a cultivation medium in soilless agriculture, for example, pumice (Uzun et al., 2021), light expanded clay aggregates (LECA) (Mlih et al., 2020), prockwool (Nerlich et al., 2022), pearlite (Asaduzzaman et al., 2018), and sand (Robinson et al., 2013). Expanded perlite is produced by roasting perlite ore at temperatures up to 1000 °C. Due to its unique properties, such as low mass, and the ability to retain water, perlite is used as a substrate (Son, 2000). A suitable culture medium should be readily available, relatively inexpensive, stable, and easy to make it easy to handle and inexpensive to transport (Nemati et al., 2015).

Cultivation and processing of plants in cultivation containers have become popular as a prominent strategy in greenhouse industries. Studies show that plants grown in containers have generally different characteristics than field crops (Balliu et al., 2021). A meta-analysis revealed that by halving the pot size, biomass production decreased by an average of 43 % (Poorter et al., 2012). Logically, an ideal pot size depends on the size of the plant to be grown in it. Also, an incorrect choice of pot size may lead to changes in the results of the experiment. Therefore, this study focuses on the effect of the type of cultivation media and pot size on the growth and rhizome yield of ginger under greenhouse conditions.

2 MATERIALS AND METHODS

2.1 SITE SPECIFICATIONS

This study was carried out in the research greenhouses of the Faculty of Agriculture of Shahed University (35° 33’ 15” N/ 51° 20’ 24” E), Tehran, Iran. The average temperature and humidity of the greenhouse were 28 °C and 70 %, respectively.

2.2 STATISTICAL DESIGN AND STUDIED TREATMENTS

The research was conducted in the form of a 2 × 3 factorial experiment based on a completely randomized statistical design with 3 replications. The treatments were: two different sizes of the pot (including 35 × 30 cm (10 l) and 45 × 35 cm (20 l)) and three different types of media, including sand, perlite, and an equal mixture (1:1) of sand and perlite.

2.3 PREPARATION OF SEEDS, GROWING MEDIA, POTS, AND PLANTING

Chinese ginger rhizomes were cut into pieces with an approximate weight of 30 ± 10 g and immediately disinfected using fungicide-bactericide (2 % Brodofix, www.baghbantak.com). To prepare the sand substrate, some beach sand was prepared and washed to remove its salinity and then dried in the open air. Perlite was also purchased from Madankavan Co. (https://madankavan.com). The mixed medium was also prepared by mixing equal parts of sand and perlite (1:1). Subsequently, pots and media were disinfected using a 2 % Brodofix solution. The pots were then filled with the appropriate amount of growing media. Inside each pot, 3 to 4 seeds were planted at a depth of 8 cm and were watered immediately. All the seeds were planted on February 4, 2022.

After planting, during the first two months, the pots were irrigated daily with 100 ml of distilled water. After these two months, and for 30 days, 100 ml of 50 % of Hoagland’s nutrient solution (Table 1) was added to each pot

Table 1: Components of Hoagland solution

Macronutrients		Micronutrients	
Component	Quantities in solution (g l^{-1})	Component	Quantities in solution (g l^{-1})
2M KNO_3	202	$MnCl_2$•$4H_2O$	1.81
2M $Ca(NO_3)2$•4H2O	472	$ZnSO_4$•$_7H_2O$	0.22
2M $MgSO_4$•7H2O	493	$CuSO_4$•$5H_2O$	0.08
1M KH_2PO_4	136	Na_2MoO_4•$_2H_2O$	0.12
Iron Chelate	15		

(Hoagland & Arnon, 1950). Subsequently, for the next 30 days, 100 ml of 70 % Hoagland's nutrient solution was added to each pot. Finally, rhizomes were harvested in July 2022.

2.4 STUDIED TRAITS

Before emptying the pots, leaf number (LN) was counted and the leaf area (LA) in cm^2 was determined using a leaf area meter (Delta-T, Cambridge, UK). Additionally, leaf fresh mass (LFM) and leaf dry mass (LDM) were also measured.

The yield was determined as rhizome fresh mass (RFM) at the end of the experiment. Also, root number (RN), root length (RL), and root fresh mass (RFM) were measured. Root dry mass (RDM) and rhizome dry mass (RDM) were determined after air-drying. The rhizome dry matter content (RDMC) was calculated by the ratio of the rhizome dry mass to the rhizome fresh mass and expressed in percent.

2.5 RHIZOME SODIUM AND POTASSIUM CONTENT

The sodium and potassium content of the rhizomes were measured using the standard method for determining Na and K (Vdlufa, 2012). 2 g of powdered rhizome was ashed at 700 °C for 15 hours. The obtained ash was then dissolved in hydrochloric acid (25 %), heated in a water bath, and filtered to obtain the extract. Then, Na and K content was measured by a flame photometer device (Model 410, Sherwood Scientific Ltd, Cambridge, UK).

2.6 SUBSTRATE SOLUTION CHARACTERISTICS

The properties of the substrate solution that were studied included EC, and pH values. To obtain the substrate solution, the substrates were completely dried in the oven for 14 hours at 110 °C. Then, 5 g of each of the dried substrates was weighed and dissolved in 50 ml of distilled water for 2 hours in a shaker.

2.7 STATISTICAL ANALYSIS

Data were statistically analysed using SAS software (SAS Institute Inc., Cary, NC, USA). The data were subjected to the Kolmogorov-Smirnov normality test before the analysis of variance (ANOVA). A *post hoc* analysis was performed using Duncan's Multiple Range Test. Pearson correlations between traits were calculated using IBM SPSS software (version 22). Principal components analysis was performed using Minitab software (version 19).

3 RESULTS AND DISCUSSION

3.1 ANALYSIS OF VARIANCE

The results obtained from the analysis of the variance of the effects due to pot size and type of culture medium on some growth indices of ginger in soilless greenhouse cultivation conditions have been shown in Table 2. As can be seen in this table, the interaction effect was significant for most of the traits, except for leaf area and rhizome sodium content. Generally, main effects are not interpreted when the interaction term is significant

Table 2: Analysis of variance of the effect of pot size and type of culture medium on the medicinal properties of ginger in soilless greenhouse cultivation conditions

	Mean squares			
Traits	Culture medium (DF = 2)	Pot size (DF = 1)	Interaction (DF = 2)	Error (DF = 12)
Leaf number	7.17n.s	88.89**	17.39**	2.5
Leaf area pot^{-1}	17116.67**	5477.56**	748.22n.s	436.39
Leaf area	57.07**	1.03n.s	0.11n.s	1.45
Leaf fresh mass	18.92*	71.16**	28.49*	4.11
Leaf dry mass	8.41*	27.38**	14.86**	1.72
Root number	0.5n.s	4.5**	3.5**	0.33
Root length pot^{-1}	188.39**	1250**	225.17**	17.39
Root length	20.11*	18.97n.s	17.22*	4.11
Root fresh mass	13.99**	55.3**	5.58**	0.62
Root dry mass	3.84**	16.65**	1.42*	0.33
Rhizome fresh mass	3889.35**	5742.35**	1405.01**	119.75
Rhizome dry mass	1082.67**	1907.56**	491.76**	27.6
Rhizome dry matter	252.49**	375.65**	85.76*	12.44
Rhizome sodium content	665.26*	196.68n.s	266.68n.s	121.68
Rhizome potassium content	41.76*	0.13n.s	46.54*	7.54
Substrate pH	0.44**	0.01n.s	0.07*	0.01
Substrate EC	310722.67n.s	732050*	606980.67*	128280.28

*, and ** mean significance at the probability level of 0.05 and 0.01 respectively, and n.s means non-significant.

in the ANOVA table. Hence, in most traits, the focus is mainly on the interpretation of the interaction effect and the main effects were interpreted only for leaf area and rhizome sodium content.

3.2 CORRELATION COEFFICIENTS BETWEEN TRAITS

Pearson's correlation coefficients between the traits studied have been shown in Table 3. In this Table, only significant correlations ($p \leq 0.05$) are shown. As can be seen, the negative correlations were not significant and therefore have been removed from the Table. Since investigating the ginger rhizome yield was one of the important goals of this research, the correlation between rhizome mass and other studied traits was of high importance. Rhizome mass showed a positive and significant correlation with root number, root length, root fresh mass, root dry mass, rhizome dry mass, rhizome dry matter percent, leaf dry mass, leaf number, leaf area, and substrate electrical conductivity (Table 3). Also, leaf area pot^{-1} was positively and significantly correlated with, root fresh mass, root dry mass, rhizome fresh mass, rhizome dry mass, rhizome dry matter percent, leaf dry mass, root length pot^{-1}, and substrate electrical conductivity (Table 2). Similar correlations were reported by Islam et al. (2008), and Jatoi and Watanabe (2013).

3.3 LENGTH, NUMBER, AND MASS OF ROOTS

Figure 1 demonstrates the comparisons of mean traits related to ginger root in soilless greenhouse cultivation as affected by substrate type and pot size. The highest root fresh mass of 9.03 g was observed in 20 l pots and mixed culture medium, which was not statistically different from that recorded in 20 l pots and perlite culture medium while, the minimum root fresh mass (2.65 g) was observed in 10 l pots and sand substrate. A similar trend was observed for root dry mass. Likewise, the maximum root length (12 cm) was found in the mixed substrate and larger pots (Fig. 1A). As shown in figure 1B, the highest number of roots (4.33) belonged to larger pots containing mixed culture medium, which showed more than 85 % growth compared to that recorded in smaller pots.

Figure 1 shows that in each of the three substrates, root number, root mass, as well as root length increased with pot size. This result suggests that root physical ap-

Table 3: Pearson's correlation coefficients between studied traits in ginger medicinal plant under the effect of pot size and substrate type in greenhouse conditions.

	LN	LAP	LA	LFM	LDM	RN	RLP	RL	RFM	RDM	RFM	RDM	RDMC	RSC	RPC	SpH	SEC
LN	1	0.53		0.85	0.86	0.61	0.85		0.79	0.87	0.72	0.71	0.63				
LAP		1	0.79		0.57		0.63		0.70	0.76	0.77	0.78	0.84				0.52
LA			1									0.48	0.58				
LFM				1	0.94	0.54	0.69		0.59	0.67			0.48				0.47
LDM					1	0.57	0.77		0.69	0.77	0.57	0.62	0.66				0.51
RN						1	0.74		0.62	0.55	0.69	0.74	0.66				0.72
RLP							1		0.95	0.90	0.84	0.87	0.84				0.60
RL								1	0.55	0.54				0.53	0.51		
RFM									1	0.94	0.81	0.85	0.86				0.57
RDM										1	0.84	0.86	0.82				0.56
RFM											1	0.98	0.85				0.58
RDM												1	0.93				0.64
RDMC													1				0.67
RSC														1	0.80		
RPC															1		
SpH																1	
SEC																	1

1.0, 0.8, 0.6, 0.4, 0.2, 0.0, -0.2, -0.4, -0.6, -0.8, -1.0

LN: leaf number, LAP: leaf area pot^{-1}, LA: leaf area, LFM: leaf fresh mass, LDM: leaf dry mass, RN: root number, RLP: root length pot^{-1}, RL: root length, RFM: root fresh mass, RDM: root dry mass, RFM: rhizome fresh mass, RDM: rhizome dry mass, RDMC: rhizome dry material content, RSC: rhizome sodium content, RPC: rhizome potassium content, SpH: substrate acidity, SEC: substrate electrical conductivity

pearance was a function of pot size independent of growing media. In line with this result, Hess and De Kroon (2007) reported that regardless of the type of culture medium or the concentration of nutrients, increasing the pot volume will lead to an increase in the size of the roots (i.e. root mass is a function of available rooting volume independent of nutrients). Unlike the Hess and De kroon hypothesis, Murphy et al. (2013) reported that root mass increased with pot size only in the treatment with high water-soluble fertilizer where plant size increased greatly with pot size. In this research, Murphy's report could not be confirmed or rejected since only a Hoagland solution was used to feed the plants. Figure 1 also shows that in the larger pot size, the root growth of plants in mixed media or perlite was significantly higher than that found in sand. This result suggests that a suitable pot size along with a suitable substrate led to the expansion of the root system of the ginger plant.

3.4 MASS, NUMBER, AND AREA OF LEAVES

The effects due to pot size and the type of culture medium on ginger plant leaf attributes have been demonstrated in Figures 2 and 3. The results showed that in similar substrates, leaf attributes (mass, number, area) were significantly higher in larger pots, which seems to be due to the improvement in root growth. As previously shown, larger pots were found to have greater root length and mass, which would logically lead to improved water and nutrient uptake and thus increased plant growth. The significant positive correlation between leaf growth indices (i.e. number, area, and mass), and root attributes (i.e. length and mass) listed in Table 1 confirms this hypothesis. Also, the significant positive correlation between the leaf area and root growth indices suggests that decreased root growth indices observed in smaller pots were mainly due to the decrease in the photosynthetic area of the plant (Table 1). In line with this suggestion, it has been reported that the growth of plants in the pot is a function of photosynthesis per unit of leaf area (Poorter et al., 2012).

According to Figure 3A, the leaf area in the pots containing perlite as well as mixed substrates was significantly higher than in the pots containing sand. In line with this result, Vanaei et al. (2008) by studying the effects of pot size and the type of culture medium on potato seedlings observed that the highest number and mass of small tubers found in the pearlite culture medium.

The physical characteristics of the cultivation substrate have an effective role in root growth and water absorption. By definition, the water holding capacity (WHC) of a substrate is the volume of water retained by a saturated growing medium after it is allowed to drain,

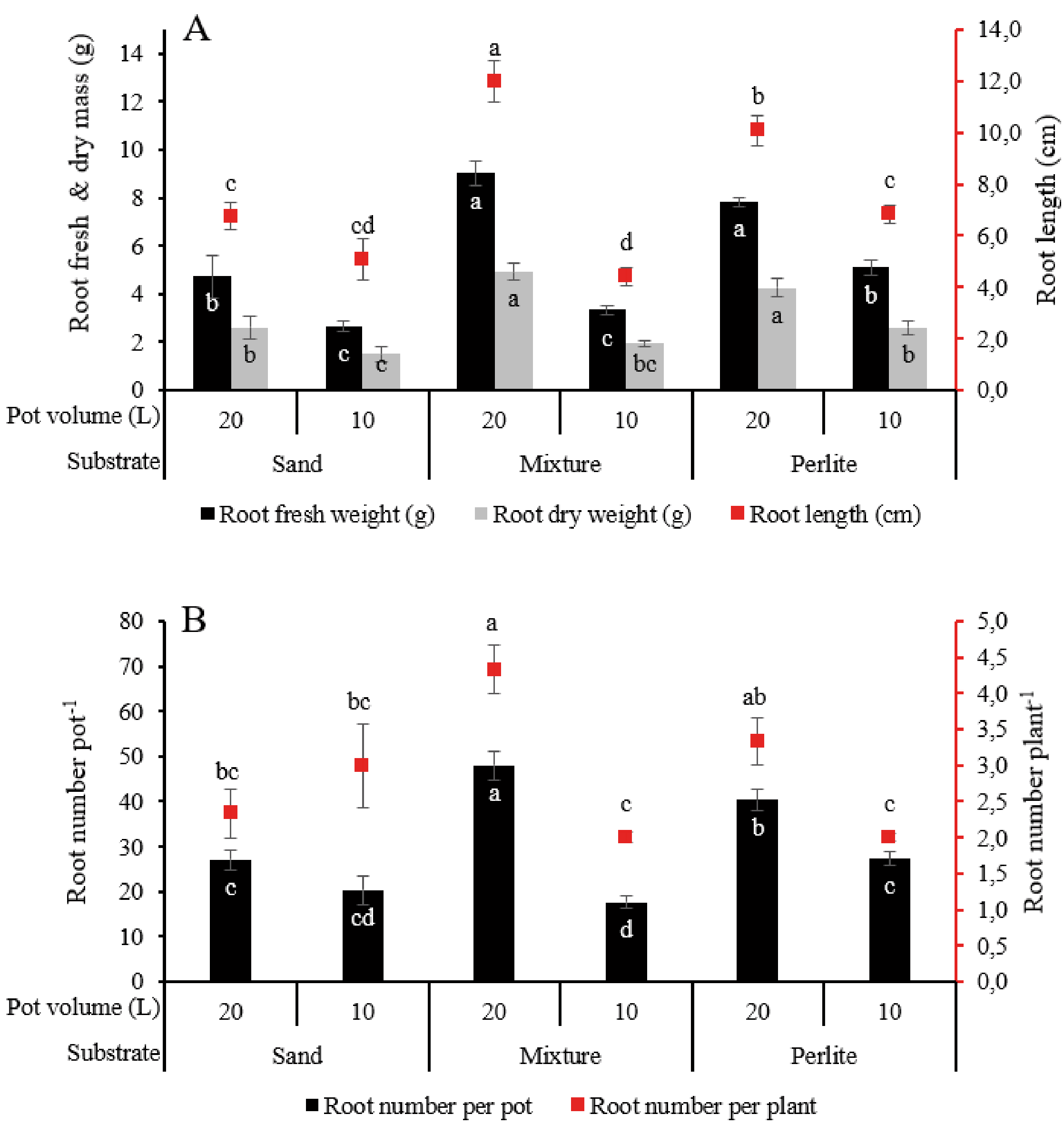

Figure 1: Comparisons of mean traits related to the ginger root (*Zingiber officinale*) in soilless greenhouse cultivation as affected by substrate type (A) and pot size (B). Within each trait, the difference between the means that have common letters is not statistically significant according to Duncan's Multiple Range Test (α = 0.05)

which is greatly influenced by the size of the particles. Therefore, in this research, it seems that compared to sand mediums, perlite, as well as the mixture substrates, had a higher WHC, which resulted in more appropriate growth indices of ginger plants. This conclusion is supported by Kakoei and Salehi (2013) who reported that the WHC of perlite is much higher than that of sand.

3.5 YIELD AND DRY MATTER CONTENT OF RHIZOMES

Figure 4 shows the comparison of the average rhizome yield of the ginger plant under the influence of pot size and culture medium. The maximum rhizome yield (fresh and dry mass) was obtained in larger pots and

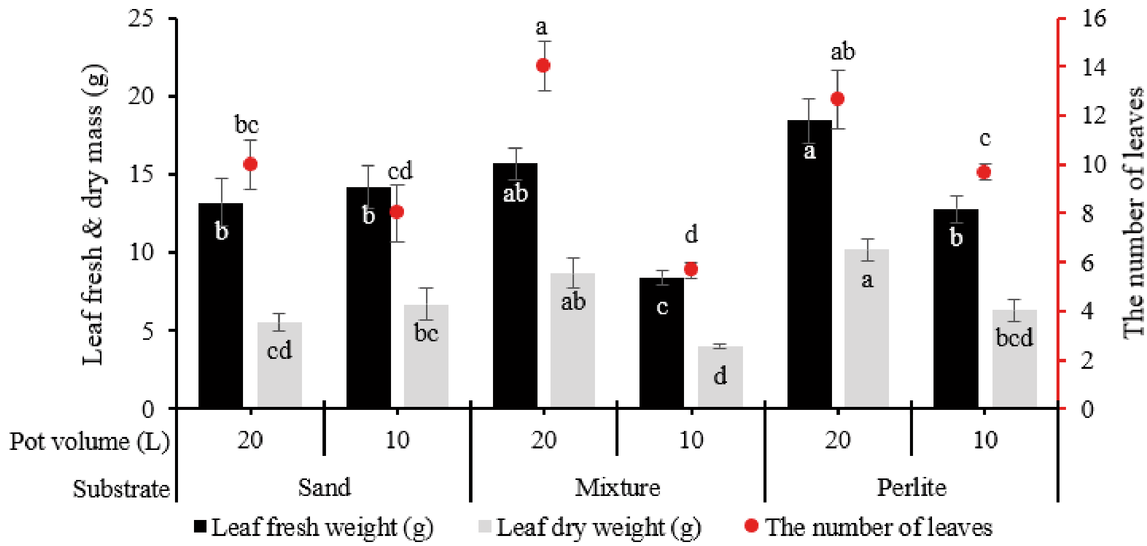

Figure 2: Comparisons of mean traits related to the ginger leaves (*Zingiber officinale*) in soilless greenhouse cultivation as affected by substrate type and pot size. Within each trait, the difference between the means that have common letters is not statistically significant according to Duncan's Multiple Range Test (α = 0.05)

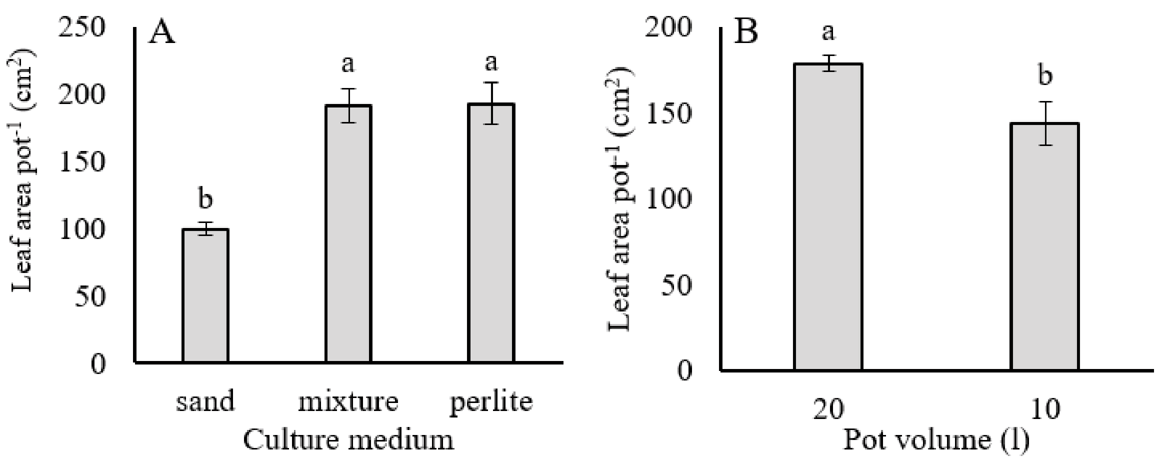

Figure 3: Comparisons of the mean area of ginger leaves (*Zingiber officinale*) under the effect of culture medium (A) and pot size (B) in soilless greenhouse cultivation. The difference between the means that have common letters is not statistically significant according to Duncan's Multiple Range Test (α = 0.05)

mixed culture medium, while the lowest rhizome yield belonged to smaller pots and sand culture medium (Figure 4). Similarly, the larger pots containing either perlite or mixture substrates were found to have the highest percentage of rhizome dry matter content (Figure 4), while the lowest percentage was found for sand substrates in both pot sizes. These results show that the better allocation of photosynthetic products within the rhizomes has occurred in the large pots containing mixed media or perlite, which can be caused by the improvement of the leaf area and as a result, the increase in photosynthetic products. In agreement with the results of this study, Hemmat et al. (2014) reported that larger pots (3 l) containing an equal mixture of peat moss and sand significantly improved the number and dry matter of minitubers.

3.6 SODIUM, AND POTASSIUM CONTENT OF RHIZOMES

The average rhizome sodium content fluctuated from 44.50 mg g^{-1} for the sand medium to 65.33 mg g^{-1} for the perlite medium (Figure 5).

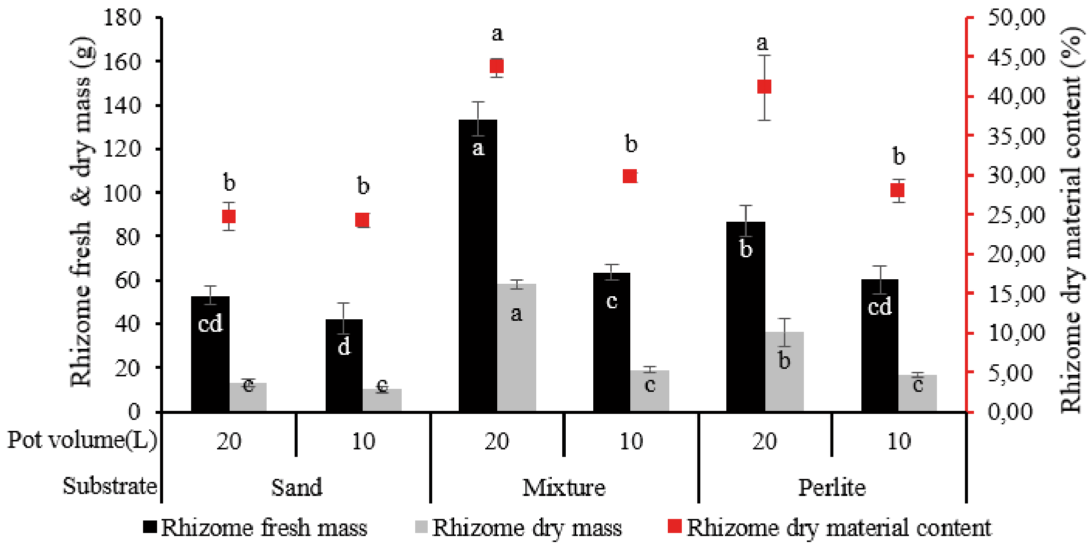

Figure 4: Comparisons of mean traits related to ginger rhizome (*Zingiber officinale*) under the effects of substrate type and pot size in soilless greenhouse cultivation. Within each trait, the difference between the means that have common letters is not statistically significant according to Duncan's Multiple Range Test (α = 0.05)

The absorption of metal ions by the plant root system is a function of the concentration of these ions in the culture medium. In this study, compared to the perlite, the lower water-holding capacity of pots containing sand caused the nutrients to be removed from the pots during the leaching process, as a result, the plant's access to nutrients decreased. Consequently, the sodium content of the rhizome showed a significant decrease in the sand culture medium. On the contrary, in perlite culture medium (or mixture), due to the higher capacity of holding water (i.e. reduction in the leaching rate of nutrition solution), the absorbable sodium ions increased and as a result, the sodium content of the rhizome was observed at the highest level (Figure 5).

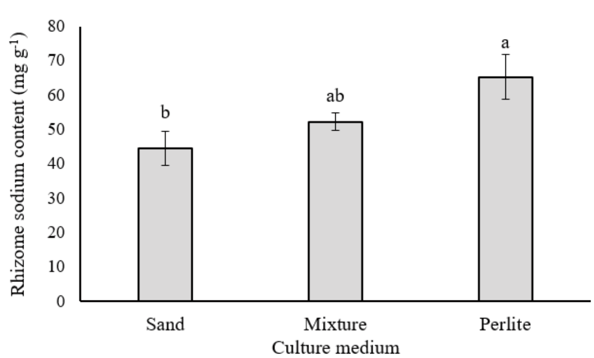

Figure 5: Comparisons of mean sodium content in ginger rhizome tissue (*Zingiber officinale*) under the effects of substrate type and pot size in soilless greenhouse cultivation. The difference between the means that have common letters is not statistically significant according to Duncan's Multiple Range Test (α = 0.05)

The effects of substrate type and pot size on potassium content of ginger rhizome tissue in soilless greenhouse cultivation have been shown in Fig. 6. On average, the potassium content measured in rhizome tissue ranged from 3.00 mg g^{-1} in smaller pots and sand medium to 13.67 mg g^{-1} in smaller pots and perlite medium (Fig. 6).

As discussed the sodium content of the rhizome, due to the lower WHC of the pot, the concentration of potassium in the pots containing sand was lower compared to other culture media, which led to a decrease in potassium absorption and therefore, in the sand substrate, the potassium content of the rhizome showed a significant decrease. On the contrary, in the perlite culture medium, after increasing the WHC and reducing nutrient leaching, the absorbable potassium ions increased and as a result, the rhizome potassium content was at the highest level (Figure 6). The pattern governing the changes of this trait among the experimental treatments showed that in any culture medium, the larger pots had more rhizome potassium content.

3.7 ACIDITY AND ELECTRICAL CONDUCTIVITY OF SUBSTRATES

The comparison of the mean acidity of culture medium in pots of different sizes has been presented in Figure 7. In general, the acidity fluctuated from 6.62

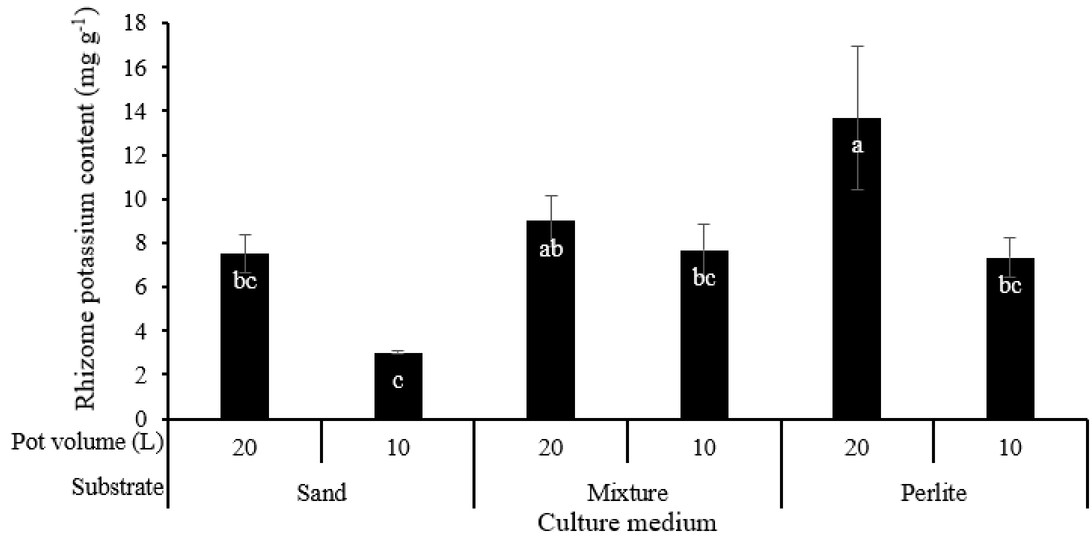

Figure 6: Comparisons of mean potassium content in ginger rhizome tissue (*Zingiber officinale*) under the effects of substrate type and pot size in soilless greenhouse cultivation. The difference between the means that have common letters is not statistically significant according to Duncan's Multiple Range Test (α = 0.05)

(for the treatment combination of mixed substrate-10 l pots) to 7.34 (for the treatment combination of perlite substrate-10 l pots). It has been reported that perlite is neutral with an acidity between 7 and 7.5, which is consistent with the results of this research (Bar-Tal et al., 2019). Also, it has been reported that the acidity of sand is around 7 (Chen et al., 2022). In this research, the acidity of each sand and perlite culture medium was around 7, but when perlite was mixed with sand, the pH value decreased significantly. The unusually high pH when treating perlite 10 l pots was probably more related to the use of the nutrient solution itself than to the substrate. Figure 7 also shows the comparison of the mean electrical conductivity (EC) of the substrates. The pattern governing the changes of EC among the experimental treatments showed that, in general, the pots containing sand substrate had lower EC. Also, for the sand substrate, smaller pots had more EC, while for the other two substrates (i.e. perlite and mixture), larger pots showed more EC. The highest EC (1.43 dS m^{-1}) was observed for larger

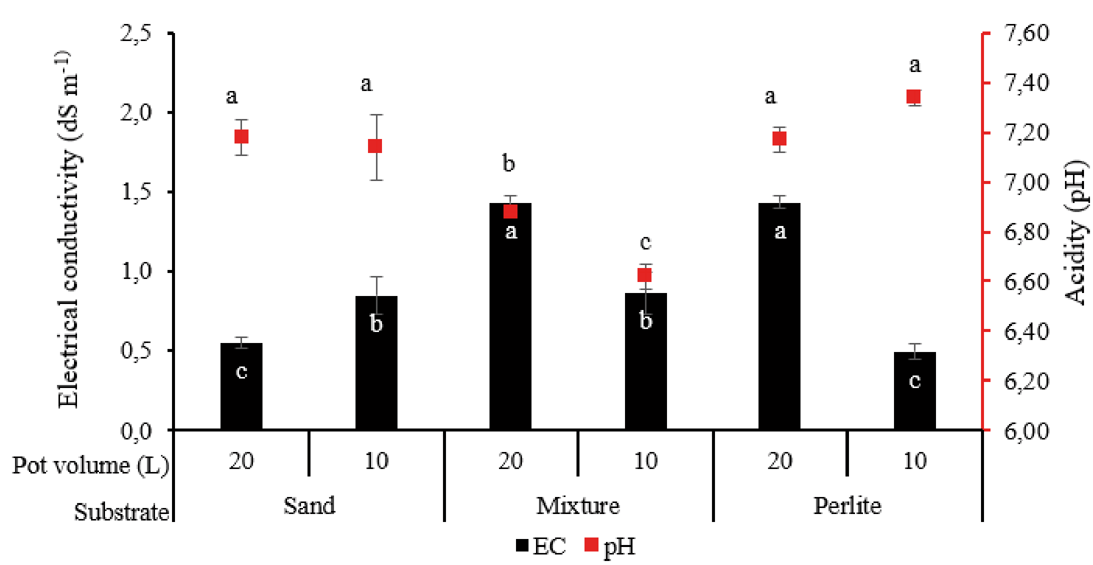

Figure 7: Comparison of mean electrical conductivity and acidity of culture media used in the soilless greenhouse cultivation of ginger (*Zingiber officinale*). Within each trait, the difference between the means that have common letters is not statistically significant according to Duncan's Multiple Range Test (α = 0.05)

pots containing perlite as well as mixed substrates while the lowest EC (0.49 dS m^{-1}) belonged to the smaller pots containing the perlite substrate, which was not statistically different from that recorded for sand substrate and larger pot (0.55 dS m^{-1}).

In this study, based on the Wilcox diagram, the EC of the culture media was in class c1, which was not salty at all. However, there were significant differences between the substrates EC. Since perlite and sand are neutral (Bush, 2001; Chen et al., 2022), the observed increase in the EC of the substrates seems to be due to the elements in the nutrient solution (Hoogland's solution) added to the pots. Considering that the volume and concentration of the nutrient solution added to all the pots were the same, therefore, the change in EC should logically be caused by the difference in leaching rate. The WHC of perlite is higher than that of sand, which means that compared to sand, the leaching rate in perlite (as well as the mixed substrate) is lower which naturally leads to more accumulation of nutrient solution and subsequently, increased concentration of ions in the substrate. Consequently, the increase of ions will rise the EC of the substrate. Moreover, by increasing the size of the pot, the leaching rate will decrease and as a result, more nutrients will accumulate in the substrate. Consequently, the larger the size of the pot, the greater the EC.

4 CONCLUSIONS

The results obtained from this research confirmed the possibility of economically growing ginger in a greenhouse. In general, 20 l pots significantly increased rhizome yield and were therefore more suitable for greenhouse cultivation. Since most rhizome-related traits were recorded in the sand-perlite mixture medium, this type of substrate was recognized as the most suitable substrate for potted ginger plant cultivation. The results of this research confirmed that by taking into account the most suitable pot size and cultivation medium (20 l pot and a mixture medium) the maximum yield of rhizome fresh mass is 133.5 g. Examining Pearson's correlations between traits showed that the leaf area was positively significantly correlated with important traits such as rhizome dry mass and rhizome dry matter content.

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Investigation of diflovidazin and fenpropathrin on two-spotted spider mite, *Tetranychus urticae* Koch, 1836 (Acari: Tetranychidae): population and interaction study

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Investigation of diflovidazin and fenpropathrin on two-spotted spider mite, *Tetranychus urticae* Koch, 1836 (Acari: Tetranychidae): population and interaction study

Abstract: *Tetranychus urticae* is one of the most important pests of agricultural crops around the world. This research investigated the lethal effects of diflovidazin and fenpropathrin on different life stages of two-spotted spider mites, the interaction of binary mixture of these two compounds, and sublethal effects of diflovidazin on the deutonymphs under laboratory conditions. The Potter spray tower was used for the bioassay of acaricides on different life stages of *T. urticae*. The results showed that diflovidazin was effective on different developmental stages excluding female's adults. Also, fenpropathrin showed toxicity on all life stages except eggs. LC_{50} value and combination index (CI) of their mixture against deutonymph were 4.85 mg l^{-1} and 0.5 mg 1^{-1}, respectively, which revealed a synergistic effect on *T. urticae*. Sublethal effects of LC_{30} concentration of diflovidazin were evaluated on life table parameters of *T. urticae*. The value of the intrinsic rate of increase (r), the finite rate of increase (λ), and the net reproductive rate (R_0) significantly decreased in treated mites in comparison to control. These results suggested that diflovidazin could have significant roles in the control of *T. urticae* due to negative effect on population parameters as well as synergistic effect of binary mixtures of this acaricide with fenpropathrin.

Key words: two-spotted spider mite, life table, mixture of pesticides, interaction

Preučevanje učinkov diflovidazina in fenpropatrina na navadno pršico (*Tetranychus urticae* Koch, 1836) (Acari: Tetranychidae): populacijska in interaktivna raziskava

Izvleček: Navadna pršica (*Tetranychus urticae*) je eden izmed najpomembnejših škodljivcev gojenih rastlin širom po svetu. V raziskavi so bili preučevani letalni učinki diflovidazina in fenpropatrina na različne razvojne stopnje navadne pršice, učinek mešanic teh dveh snovi in subletalni učinki diflovidazina na deutonimfe v laboratorijskih razmerah. Za preučevanje učinkov teh akaricidov na različne razvojne stopnje navadne pršice je bil uporabljen Potterjev pršilni stolp. Rezultati so pokazali, da je bil diflovidazin učinkovit v vseh razvojnih stopnjah, z izjemo odraslih samic. Tudi fenpropatrin je bil strupen za vse razvojne stopnje pršice, z izjemo jajčec. LC_{50} vrednost in kombinacijski indeks (CI) mešanic akaricidov na deutonimfe sta bila 4,85 mg l^{-1} in 0,5 mg 1^{-1}, kar kaže na njun sinergijski učinek pri zatiranju navadne pršice. Vrednosti maksimalne rasti populacije (r), končne rasti populacije (λ) in neto reprodukcije (R_0) so se pri obravnavanih pršicah značilno zmanjšale v primerjavi s kontrolo. Rezultati nakazujejo, da bi diflovidazin lahko imel pomebno vlogo pri nadzoru navadne pršice zaradi njegovih negativnih učinkov na populacijske parametre kot tudi zaradi sinergističnih učinkov mešanic tega akaricida s fenpropatrinom.

Ključne besede: navadna pršica, preživetvena sposobnost, mešanica pesticidov, interakcija

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1 INTRODUCTION

The two-spotted spider mite, *Tetranychus urticae* Koch, 1836 (Acari: Tetranychidae) is one of the most destructive polyphagous mites in the greenhouses and fields and causes significant damage to the yield of many horticultural, agricultural, and ornamental crops (Jeppson et al., 1975). Nearly 1,200 plant species have been reported as hosts of this mite, of which more than 150 species of these hosts such as cotton, corn, tomatoes, and ornamental trees have economic importance. This mite can produce more than 37 generations per year under favorable conditions (Riahi et al., 2013). The management of *T. urticae* populations has relied on application of different chemical acaricides (Cooper & Dobson, 2007) that have increased the public health concern (Owusu & Yeboah, 2007) and adverse effects on beneficial organisms (Bajc et al., 2017; Eziah et al., 2016). Due to high reproductive ability and short life cycle, two-spotted spider mite could develop resistance to many acaricides (Devine et al., 2001; Stumpf & Nauen, 2001). Despite the discovery and commercialization of acaricides belonging to different chemical groups, the number of acaricides on the market is much lower than insecticides that were estimated 7 % of the total insecticides market (Sparks & Nauen, 2015). Therefore, it is important to prevent the resistance of acari mites to acaricides and using a mixture of pesticides is one of the ways to prevent pest resistance to pesticides.

One of the resistance management programs is to use a mixture of pesticides for more effective control (Ahn et al., 1993). A mixture of two or more pesticides may improve pest control, enhance their pesticidal properties, decrease the costs and pesticide consumption and prevent pesticide resistance (Yuya et al., 2009). However, the influence of this way depends on the amount of application and the formulation of the pesticides. Widespread use of pesticide mixtures is usually common in greenhouses and seedling production centers where a collection of pests are present (Hosseininaveh & Ghadamyari 2013). In addition, multi-pesticide mixtures usually alter the uptake, transport, metabolism, and toxicity at the target site of pesticides, thus improving performance in many cases (Flint, 2002). On the other hand, sublethal insecticide exposure is expected to increase the competitiveness of resistant phenotypes (Wang et al., 2018), acting as a selection pressure for the evolution of insecticide resistance (Hamedi et al., 2010). The effects of sublethal concentrations of acaricides have been studied on spider mites (Landeros et al., 2002; Marčić, 2005, 2007). To better estimation of pesticides, the investigation on life table and population fitness could clarify the impacts of pesticide on pests and non-target species. Thus eco toxicological improvement could associate with altering the way of the evaluation of pesticide effects (Kammenga et al., 1996; Stark & Banks, 2003).

Different groups of pesticides are used for control of the tetranychid family in Iran (Saeidi & Arbabi, 2007). Fenpropathrin (Danitol®) is a pyrethroid insecticide used to control this pest (IRAC group 3A, sodium channel modulators) which was documented for reducing the rate of reproduction by feeding prevention and disruptive vital functions of adult mites (Nourbakhsh, 2019). Recently, diflovidazin (Flumite®) is a compound in the tetrazine chemical (IRAC group 10A, mite growth inhibitors affecting CHS1) (Merzendorfer, 2013) has been registered for management of *T. urticae* and *Panonychus ulmi* (Koch, 1836) (Acari: Tetranychidae) in Iran (Nourbakhsh, 2019).

Therefore, in this study, the lethal effects of fenpropathrin and diflovidazin were assayed on different stages of two-spotted spider mite. After determining the LC_{50}, the mixture of these compounds was examined for detection of synergistic, antagonistic, and potentiation effects on deutonymphs. Finally, the influence of LC_{30} concentration of diflovidazin was evaluated on life-table parameters as population growth parameters, female longevity, and life expectancy of *T. uticae*.

2 MATERIALS AND METHODS

2.1 MITE POPULATION GROWTH AND CHEMICAL COMPOUNDS

The mite population was collected from Siahkal (Gilan province, Iran) considered as susceptible to acaricides, in the summer of 2016 without a history of applying synthetic pesticides. The population was established on black-eyed pea (*Vigna unguiculata* (L.) Walp) under the laboratory conditions (25 ± 2 °C, 60 ± 10% relative humidity (RH), and a 16:8 h (L:D) photoperiod).

Commercial formulations of fenpropatrin 10 % EC (Danitol®) and difluvidazin 20 % SC (Flumite®) were obtained from Kimia Gohar Khak and Agro-Chemie Ltd, respectively.

2.2 BIOASSAYS ON DIFFERENT DEVELOPMENTAL STAGES

The toxicity status of pesticides was determined on each developmental stage of *T. urticae* (Vassiliou & Kitsis, 2013). Briefly, 10-15 same age larvae (0-24 hour old), deutonymph, and female adults were transferred on the upside of each leaf discs of black-eyed pea, *Vigna unguiculata* (L.) Walp. (4 cm^2). After mite's settlement, different concentrations of fenpropathrin were sprayed on leaf discs containing deutonymph (4, 8, 16, 32 and 65 mg l^{-1}), larvae (2, 4, 6, 10 and 16 mg l^{-1}) and adult (10, 20, 40, 80 and 100 mg l^{-1}). Also, different concentrations of difluvidazin were applied on egg (1, 2, 3, 5, 7 and 10 mg l^{-1}), larvae (1, 2, 3, 5, 10 and 15 mg l^{-1}) and deutonymph (1, 2.5, 5, 10 and 20 mg l^{-1}) by a Potter spray tower (1 bar pressure, 1.5 ± 0.05 mg spray fluid deposit/cm^2). The distilled water was used as control. Mortality was assessed 48 h after treatment. Mites that did not walk normally after touching with a camel's hair brush were considered as dead.

For the egg developmental stage, 24 hours before bioassay, 10 female adult mites were put on a not-treated leaf disc for oviposition. Then, 20 eggs were used for each replication. For each treatment, 5 replications were prepared. All sample for each bioassay were transferred to the controlled conditions in an incubator at 25 ± 2 °C, 60 ± 10 % RH, and a 16:8 h (L:D) photoperiod.

2.3 THE MIXTURE BINARY ASSAY OF FENPROPATHRIN AND DIFLOVIDAZIN

Toxicity interaction studies were based on LC_{50} values for deutonymph stage of *T. urticae*. The mixture ratio was prepared based on LC_{50} values of fenpropathrin and difluvidazine using the potter tower bioassay method as discussed above. So, the binary mixture effects of these two pesticides were evaluated in 1 (diflovidazin): 2 (fenpropathrin) ratios. As above, the bean leaf discs containing deutonymph were sprayed with different doses (1, 2, 7, 14 and 20 mg l^{-1}) of a mixture of two pesticides, and the value of LC_{50} in the mixture was determined as described above.

2.4 DIFLUVIDAZIN SUBLETHAL EFFECT AND LIFE-TABLE ASSAY

For evaluation of the sublethal effects of diflovidazin, the LC_{30} concentration was investigated on deutonymph of two-spotted spider mite. In order for this assay, 100 deutonymphs were transferred on leaf discs and treated with LC_{30} concentration. Distilled water was used as control. After being adults, 15 pairs of *T. urticae* were transferred on new leaf discs, separately, and their oviposition as well as mortality were documented until the death of all individuals. Moreover, same aged-eggs laid on leaf discs (within a 24-h for both treatments) were used for life history experiments. During this test, developmental times and mortality of different immature stages were checked daily until adult emergence. Therefore, the raw life history data of treated and untreated *T. urticae* were obtained.

2.5 STATISTICAL ANALYSIS

LC_{30} and LC_{50} values, slopes, toxicity ratios between developmental stages in 95 % confidence intervals were determined by Probit analysis (POLO-PC, LeOra Software, Berkeley, USA). Furthermore, LC_{50} of two pesticides and LC_{50} of each pesticide on different life stages of two-spotted mite were compared by POLO-PC software and toxicity ratios were calculated to find the higher effects (Robertson et al., 2017).

Binary mixture of two acaricides were analyzed based on the method of Chou & Talalay, 1984. For this purpose, CompuSyn software was used which by certain ratios of each compound, the combination index, CI was calculated and the type of interaction effect was detected as synergistic, additive, and antagonistic. The combination index (CI) has been used for the quantitative determination of synergism (CI < 1), antagonism (CI > 1), and additive effect (CI = 1), and CI is calculated by Compusyn software.

The figures were designed by Microsoft Excel 16. The recorded raw data for life history and population growth parameters of *T. urticae* were analyzed based on the life table theory (Chi & Liu, 1985) and computer program TWOSEX-MS Chart (Chi, 2015). The age-specific survival rate (l_x), the age-stage-specific survival rate (s_{xj}), the age-specific fecundity (*mx*), the age-stage-specific reproductive values (v_{xj}), and population growth parameters such as intrinsic rate of increase (r), finite rate of increase (λ), net reproductive rate (R_0) and mean generation time (T) were calculated, accordingly. Moreover, the bootstrap method (100,000 replications) was used for the means and standard errors (Chi, 2015). The means were compared using the paired bootstrap test at 5 % significant level based on the confidence interval of difference (Efron and Tibshirani, 1993; Chi, 2015).

3 RESULTS AND DISCUSSION

3.1 EVALUATION OF THE LETHAL EFFECT OF DIFLOVIDAZIN AND FENPROPATHRIN

The LC_{50} and LC_{30} values of diflovidazin and fenpropathrin are presented in Table 1 and Table 2, respectively.

Table 1: Diflovidazin bioassay on different developmental stages of *Tetranychus urticae*

Developmental stage	N*	Slope ±SE	Chi-square	df	LC_{30} (mg l^{-1}) (95 % CI^{+})	LC_{50} (mg l^{-1}) (95 % CI^{+})
Egg	280	3.34 ± 0.57	2.29	4	2.21 (1.42-2.83)	3.18 (2.38-3.83)
Larvae	280	2.23 ± 0.44	4.50	4	3.29 (0.80-5.31)	5.65 (2.60-8.63)
Deutonymph	240	2.34 ± 0.49	1.01	3	3.93 (2.00-5.51)	6.59 (4.42-8.60)
Adult	240	-	-	-	NC	10000**

*The number of mites were used in bioassays
CI^{+}: The upper and lower confidence interval at 95 % level
NC: Not calculated
**: It was not possible to calculate the LC_{50} value due to the phytotoxicity property

Table 2: Fenpropathrin bioassay on different developmental stages of *Tetranychus urticae*

Developmental stage	N*	Slope ± SE	Chi-square	df	LC_{30} (mg l^{-1}) (95 % CI^{+})	LC_{50} (mg l^{-1}) (95 % CI^{+})
Larvae	240	3.38 ± 0.60	1.11	3	3.95 (2.51-5.07)	5.65 (4.20-6.89)
Deutonymph	240	2.15 ± 0.32	1.02	3	7.94 (4.93-10.86)	13.92 (10.06-18.16)
Female adults	240	2.11 ± 0.35	1.78	3	20.04 (11.46-27.75)	35.51 (25.15-46.39)
Egg	-	-	-	-	NC	10000**

*The number of mites were used in bioassays
CI^{+}: The upper and lower confidence interval at 95 % level
NC: Not calculated
**: It was not possible to calculate the LC_{50} value due to the phytotoxicity property

The LC_{50} values on the eggs, larvae, and deutonymphs indicated that the eggs had a higher susceptibility to diflovidazin. The greater slope of the logarithm-probit line in diflovidazin treatments was related to the egg stage that showed the lower increase in concentration caused more mortality in eggs in comparison to other developmental stages (Figure 1). Diflovidazin did not have any toxicity on females up to 10000 ppm (Table 1). Besides, fenpropathrin had no lethal effect on eggs up to 10000 mg l^{-1} (Table 2). The larval stage has the highest sensitivity to fenpropathrin rather than deutonymph and female adult (Table 2 and Figure 2). According to results (Table 3), the larval stage has the same sensitivity to fenpropathrin and difluvidazine (Figure 3). However, larvae showed most sensitivity to these pesticides.

The toxicity ratio comparison of LC_{50} of three developmental stages in diflovidazin treatment is shown in Table 3. Due to overlap of the diflovidazin LC_{50} values of deutonymph and larval

stage (1.16- fold), that involvement of number one between the high and low 95 % confidence limits. The toxicity ratio of fenpropathrin was found larva as the most sensitive stage. The various biological stages were as follows: larvae > deutonymph > female adults. The comparison of toxicity of diflovidazin and fenpropathrin on larval and deutonymph stages showed that there was not a significant difference in the larval stage (1- fold). However, the deutonymph stage had more sensitivity to diflovidazin with 2.11 folds more than fenpropathrin.

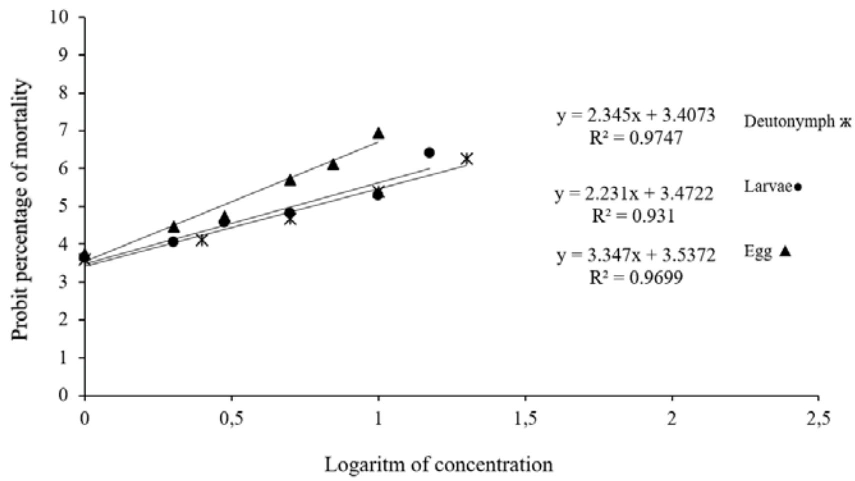

Figure 1: Logarithm of concentration-probit relationship percentage of mortality of different developmental stages of *Tetranychus urticae* in response to diflovidazin

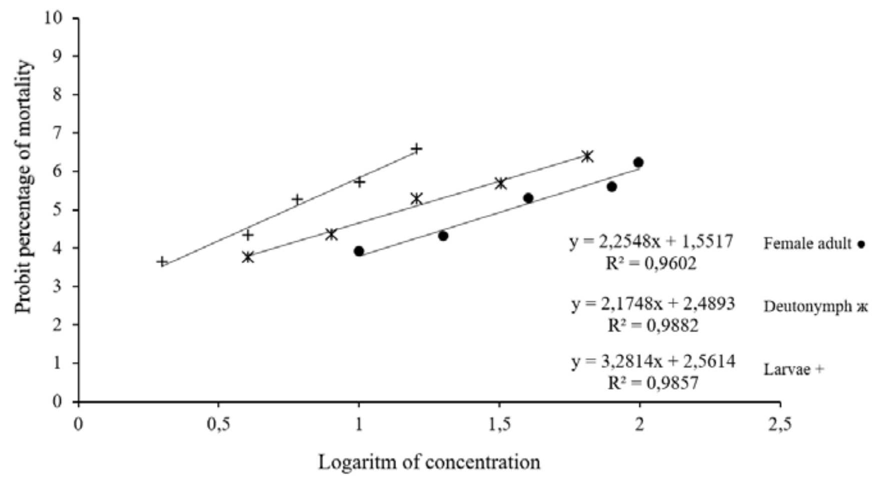

Figure 2: Logarithm of concentration-probit relationship percentage of mortality of different developmental stages of *Tetranychus urticae* in response to fenpropathrin

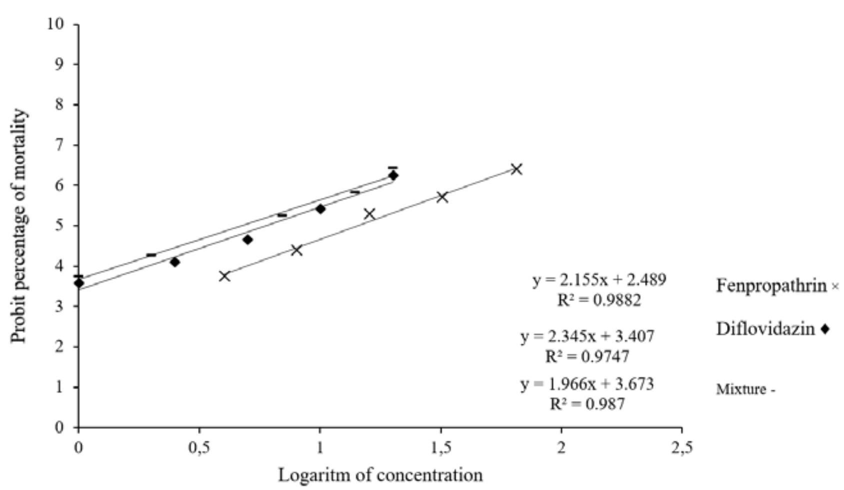

Figure 3: Logarithm of concentration-probit relationship percentage of mortality of different fenpropathrin, diflovidazin and their mixture on *Tetranychus urticae*

Table 3: The comparison of diflovidazin and fenpropathrin toxicity on different developmental stages of *Tetranychus urticae*

Treatment	Developmental stage	Toxicity ratio	(95 % Confidence Interval)*
Diflovidazin	LC_{50} of deutonymph/ LC_{50} of larvae	1.16	0.75-1.80
	LC_{50} of deutonymph/LC_{50} of egg	2.07*	1.42-3.01
	LC_{50} of larvae/LC_{50} of egg	1.77*	1.21-2.61
Fenpropathrin	LC_{50} of deutonymph/ LC_{50} of larvae	2.46*	1.70-3.55
	LC_{50} of adult/LC_{50} of deutonymph	2.55*	1.69-3.84
	LC_{50} of adult/LC_{50} of larvae	6.38*	4.32-9.11
$\frac{LC_{50}\ \text{Fenpropathrin}}{LC_{50}\ \text{Diflovidazine}}$	Larvae	1.00	0.67-1.47
	Deutonymph	2.11*	1.39-3.20

*The significant difference in Toxicity ratio: the upper and lower confidence at 95 % level

In the study of clofentzine (IRAC group 10A), propargite (IRAC group 12C), tetradifon (IRAC group 12D), etoxazole (IRAC group 10B), fenpyroximate (IRAC group 21A), amitraz (IRAC group 19), fenpropathrin, hexythiazox (IRAC group 10A), bromopropylate (unknown Mode of Action (MOA)), and fenazaquin (IRAC group 21A), the most effective compounds were reported hexythiazox and etoxazole, but fenpropathrin had the least effect on deutonymphs of Iranian population of *T. urticae* (Saeidi & Arbabi, 2007). The toxicity of difluvidazine on the larval stage of citrus red mite, *Panonychus citri* (McGregor, 1916) (Acari: Tetranychidae) (Gao et al., 2004) is 173.39- times higher than its adult stage which was consistent with the results obtained in this research. Since diflovidazin inhibits chitin synthesis (IRAC group 10A) less or no effective was observed on females.

Based on the results of Marčić (2000), eggs had more sensitivity to diflovidazin and clofentzin acaricides in comparison to larva and deutonymph of *T. urticae*. The LC_{50} value of diflovidazin on females of *T. urticae* was 2362.2 ppm (Havasi et al., 2018). Aveyard et al. (1986) reported the effectiveness of clofentzine acaricide on the egg, larval, and deutonymph of *T. urticae* without influence on adults. The egg stage had a lower LD_{50} (0.16 ppm) than other stages which were consistent with the results of this study. Fenpropathrin had no effect on the two-spotted mite egg stage. Similar to the results of this research, propargite did not show any toxicity effect on the egg stage of this pest (Ashley et al., 2006).

The acaricide activity of bifenazate and diazene was reported on all developmental stages of *T. urticae* and *P. citri*; while etoxazole and tebufenpyrad (IRAC group 21A) had no effect on adult comparison bifenazate (Ochiai et al., 2007). In the survey of clofentezine, deltamethrin (IRAC group 3A), fenpyroximate, and hexythiazox, hexythiazox had the lower LC_{50} on larvae of three laboratory strains of *T. urticae* (Nauen et al., 2001).

3.2 INTERACTION OF DIFLOVIDAZIN AND FENPROPATHRIN

The mortality of adult female *T. urticae* in response to different concentrations of a combination of diflovidazin and fenpropathrin (in a ratio of 1: 2 of LC_{50} values) were reported in Table 4. The CI of the binary mixture of diflovidazin and fenpropathrin was determined 0.5 which is in the range of $0.3 < CI < 0.7$, according to Table 4, the effect of the mixture was synergistic.

The combination toxicity of diflovidazin LC_{50} with fenpropathrin LC_{50} in a ratio of 1: 2 was tested on *T. urticae* deutonymph. The LC_{50} values obtained from the ef-

Table 4: The toxicity index values of diflovidazin and fenpropathrin mixture on *Tetranychus urticae*

Mortality percentage	Combination Ratio (2:1) diflovidazin + fenpropathrin	Combination Index (CI)
10	0.91	0.514
20	1.62	0.516
30	2.38	0.517
40	3.27	0.518
50	4.37	0.518
60	5.83	0.519
70	8.00	0.520
80	11.75	0.521
90	20.97	0.523
95	35.74	0.525

Table 5: Diflovidazin + fenpropathrin bioassay on deutonymph of *Tetranychus urticae*

Developmental stage	N*	Slope ± SE	Chi-square	df	LC_{50} (mg l^{-1})
Deutonymph	240	1.96 ± 0.26	0.89	3	4.85 (3.71-6.10)

*The number of mites were used in bioassays

fect of the mixture of them had been reported in Table 5. Based on the data in these table, the combined mixture of these two acaricides on the deutonymph stage is more toxic than either acaricide alone.

A comparison of the toxicity ratios of diflovidazin, fenpropathrin, and binary mixture of these compounds on deutonymph with 95 % confidence has been given in Table 6. If the confidence interval of LC_{50} ratios includes one, it indicates no significant difference between two treatments (Wheeler et al., 2006). Therefore, in this study, the LC_{50} of diflovidazin did not show a significant difference with LC_{50} of the pesticide mixture which was related to the overlap of their LC_{50} confidence limits and involvement of number one between the 95 % confidence limits of LC50 ratios (Table 6).

In the mixture of fenvalerate (IRAC group 3A) and some organophosphate pesticide, the most effective combination was fenvalerate and azinphos-methyl (IRAC group 1B) in a 1:1 ratio on *T. urticae* (Bruce Chapman & Penman, 1980). The result of this study showed the combination of diflovidazin and fenpropathrin, with different mode of action as sodium channel modulator and growth inhibitor, respectively, had a synergistic effect on *T. urticae* control. Similar to the results obtained for the mixture of difluvidazine with fenpropatrin (pyrethroid) in this research, a synergistic effect was observed in a mixture of chlordimeform with some pyrethroid compounds (El-Sayed & Knowles, 1984).

Table 6: Comparison of toxicity of fenpropathrin, diflovidazin and their mixture on deutonymph of *Tetranychus urticae*

Treatment	Toxicity ratio	Up - Down*
Fenpropathrin / Diflovidazin	2.11*	1.39 - 3.20
Fenpropathrin/ Mixture	2.86*	1.90 - 4.32
Diflovidazin/ Mixture	1.35	0.89 - 2.07

*The significant difference in confidence at 95 % level

3.3 THE EFFECT OF LC_{30} CONCENTRATION OF DIFLOVIDAZIN ON *T. urticae*

Developmental times (day) and longevity of the *T. urticae* are presented in Table 7. As shown, the length of the egg incubation period, as well as the protonymph duration, were significantly different in diflovidazin treatment and control (Table 7).

Analysis of variance showed that the adult preovipositional period (APOP) and total preovipositional period (TPOP) were not different in treatment and control (Table 8). The sub-lethal dose of diflovidazin significantly reduced the oviposition period of females, which was due to the reduction of female longevity in diflovidazin treatment (3.47 ± 0.25) in the comparison with the control (10.14 ± 3.5). Also, the number of eggs was significantly reduced by the treated females compared to the control.

In the present study, the effect of LC_{30} concentration of diflovidazin on fecundity, adult preoviposition period, total preoviposition period was calculated (Table 8). The intrinsic rate of population increase (*r*), the finite rate

Table 7: The effect of diflovidazin on different stage duration of *Tetranychus urticae* (mean ± SE)

Treatment	Egg	Larvae	Protonymph	Deutonymph	Pre-adult period	Female longevity	Life span
Control	3.75± 0.44	1.76 ± 0.43	2.11 ± 0.39	2.21 ± 0.52	9.83 ± 0.91	10.14 ± 3.5	19.97 ± 6.01
Diflovidazin	4.02 ± 0.02*	1.62 ± 0..05	1.83 ± 0.06*	2.29 ± 0.11	9.76 ± 0.12	3.47 ± 0.25*	13.22 ± 0.4*

*The significant difference in confidence at 95 % level

Table 8 . The sub-lethal concentration effect of diflovidazin on the reproduction capacity of *Tetranychus urticae* (mean ± SE)

Treatment	APOP** (day)	TPOP*** (day)	Oviposition period (day)	Fecundity (egg/female)
Control	0.0 ± 0.00	9.47± 0.16	10.97 ± 0.65	76.19 ± 5.64
Diflovidazin	0.0 ± 0.00	9.71 ± 0.14	3.64 ± 0.31*	16.39 ± 1.66*

*The significant difference at 95 % level
**APOP: Adult pre-oviposition period
***TPOP: Total pre-oviposition period

Table 9: The sub-lethal concentration effect of diflovidazin on the life table parameters of *Tetranychus urticae* (mean ± SE)

Treatment	r (day^{-1})	λ (day^{-1})	R_0 $\left(\frac{offspring}{individual}\right)$	GRR $\left(\frac{offspring}{individual}\right)$	T (day)
Control	0.266 ± 0.009	1.30 ± 0.012	47.24 ± 6.247	93.87 ± 5.764	14.455 ± 0.214
Diflovidazin	0.162 ± 0.012*	1.176 ± 0.014*	7.21 ± 1.090*	3.035 ± 3.72*	12.177 ± 0.158*

*The significant difference at 95 % level
r: Intrinsic Rate of Increase, λ: Finite Rate of Increase, R_0: Net Reproduction Rate, GRR: Gross Reproduction Rate, T: Mean Generation Time

of increase (λ), and the mean generation time (T) were significantly lower in diflovidazin. The net reproduction rate (R_0) was 7- folds lower than the control. Decrease in T value in diflovidazin treatment is probably due to shorter female longevity compared to the control treatment (Table 9).

The age-stage specific survival rate (S_{xj}) indicates the probability that a newborn will reach any age and stage of life. The egg incubation period, larval duration, and the preoviposition period in LC_{30} treatment of diflovidazin treatment were shorter than control (Figure 4).

The age-specific fecundity of female adults (m_x), the age-specific maternity ($l_x m_x$), and the age-specific survival rate (l_x) in different diflovidazin treatments and control are shown in Figure 5. In these curves, the age-specific fecundity indicates the rate of reproduction of females at different ages, as the onset time and reproduction termination in treatments. The age-specific survival rate (l_x) indicates a newborn egg will survive to x age. According to the results, m_x and l_x in diflovidazin treatment have a sharp decline and the fertility and survival of female adults in this treatment decreased rapidly (Figure 5).

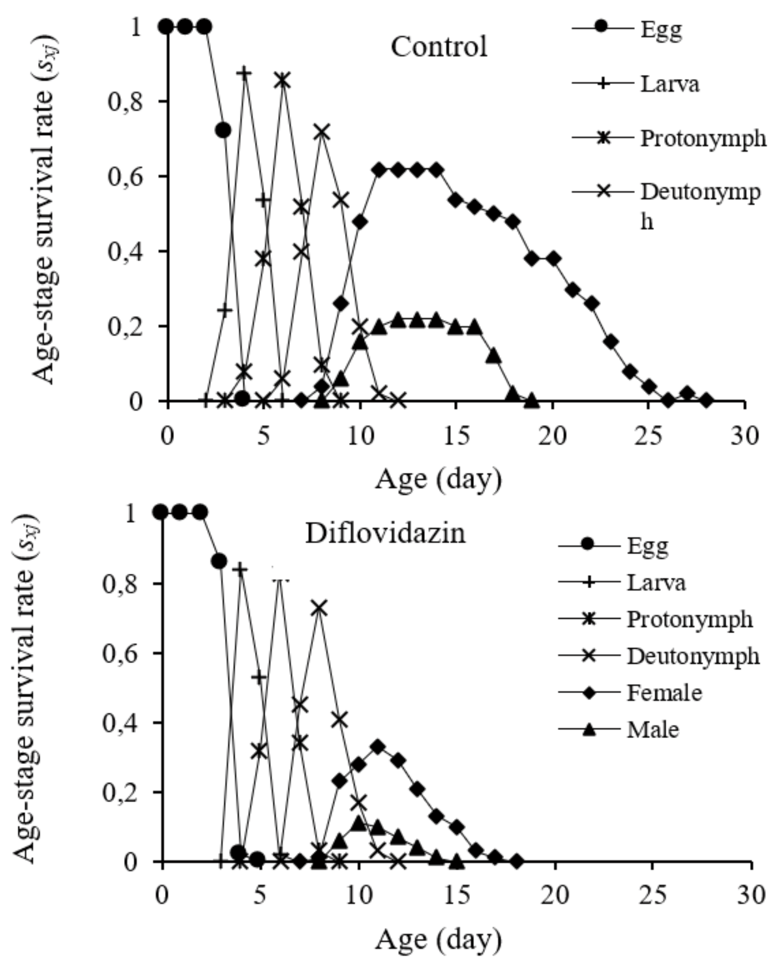

Figure 4: Age-stage survival rate (s_{xj}) of *Tetranychus urticae* at different stages in control and diflovidazin treatments

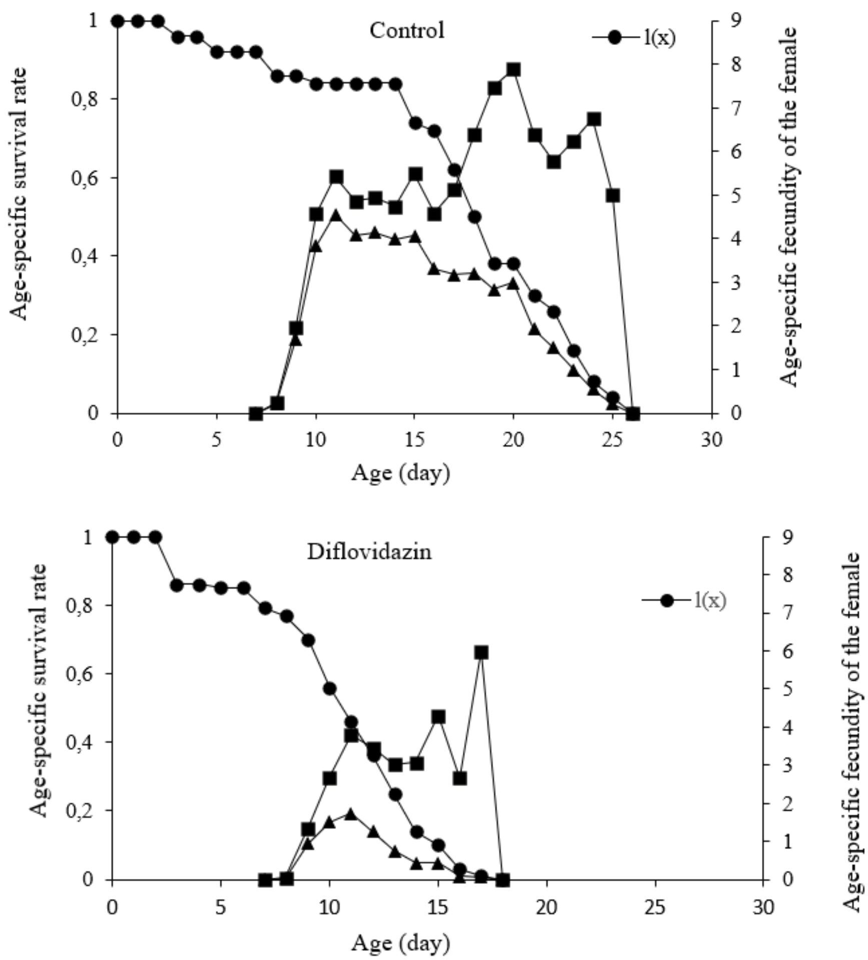

Figure 5: Age-specific survival rate (l_x), age-specific fecundity of the female (m_x) and age-specific maternity ($l_x m_x$) of *Tetranychus urticae* at different stages in control and diflovidazin treatments

Age-stage-specific reproductive value (v_{xj}) represents the number of offspring expected to be generated in the future by any person of x age and j growth stage (Carey, 1993; Fisher, 1958). There was a significant difference in comparison between the control reproductive value curves and the adult stage treatment (Figure 6) and the highest amount of reproductive value occurred in the control treatment on days 9-10. The age-specific life expectancy (e_{xj}) was summarized in Figure 7. Life expectancy changes had an inverse relation to mortality rate (q_x) and it was the lowest in diflovidazin treatment.

The acaricide sublethal effect measurements could clarify the different aspects of acaricides on mites. The sublethal effects of bifenazate (IRAC group 20D) affected the parental generation of *T. urticae* as survival rate, oviposition period, fecundity, and longevity (Li et al., 2017). The study of diflovidazin sublethal effects on *T. urticae* was associated with a significant reduction in biological parameters as female maturation duration, the oviposition period, the net reproductive rate (R_0), intrinsic rate of increase (r), and total fecundity (Havasi et al., 2018). Sublethal concentrations of tebufenpyrad significantly affected the offspring, longevity, fertility, and the intrinsic rate of increase (r) of *T. urticae* (Marčić, 2005).

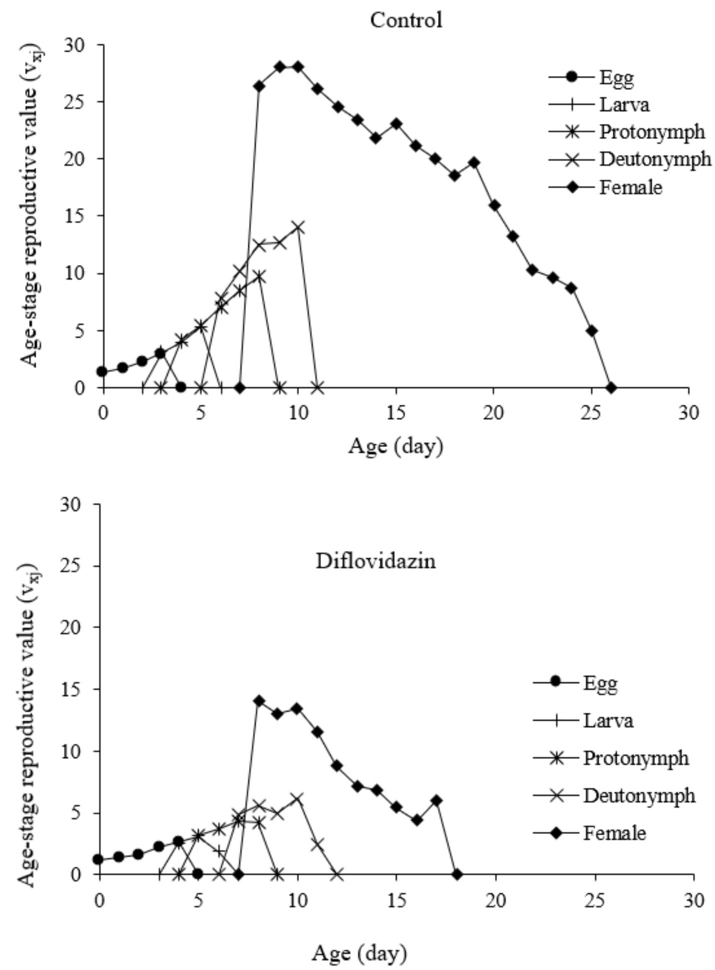

Figure 6: Age-stage reproductive value (*vxj*) of *Tetranychus urticae* at different stages in control and diflovidazin treatments

4 CONCLUSION

Although control of *T. urticae* has proven successful in many protected crops by some beneficial organisms, acaricides play a major role in control of this pest in field and greenhouse's crops (Van Leeuwen et al., 2010). Considering that the cost of chemical control is lower compared to biological control by releasing predatory mites in the field (Vidrih et al., 2020). This justifies the intensive use of acaricides in some areas. In addition to using biological control agents to control this pest, the mixture of pesticides should also be considered as a strategy to delay resistance. The results of diflovidazin studies on *T. urticae* showed that diflovidazin is an effective acaricide on immature stages especially eggs. However, it had no effect on the adult stage. Fenpropathrin had the most influence on the larval stage, without effect on the egg stage. The results of LC_{30} of diflovidazin acaricide were associated with the significant reduction of net reproduction rate (R_0), intrinsic population growth rate (r), and the finite increase in population (λ). Thus, the combination of these pesticides with complication effects on together and a synergistic effect on deutonymph, can be effective recommendation in *T. urticae* control and suitable for delay to acaricide resistance.

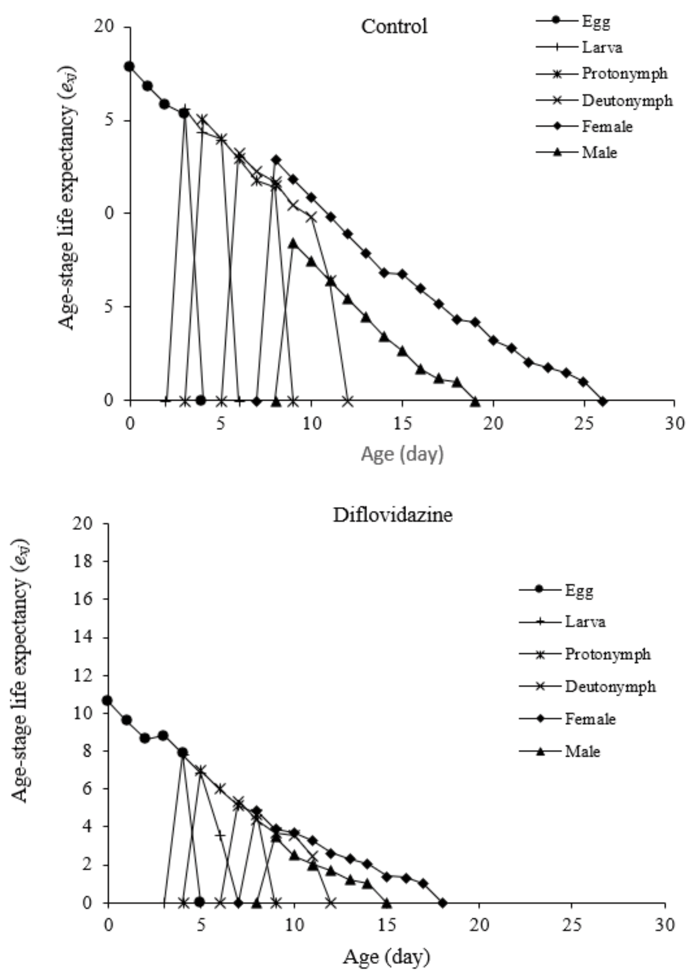

Figure 7: Age-stage life expectancy (e_{xj}) of *Tetranychus urticae* at different stages in control and diflovidazin treatments

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Efficacy of entomopathogenic fungi for control of walnut blue butterfly (*Chaetoprocta odata* [Hewitson, 1865]) in walnut (*Juglans regia* L.) under laboratory conditions

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Efficacy of entomopathogenic fungi for control of walnut blue butterfly (*Chaetoprocta odata* [Hewitson, 1865]) in walnut (*Juglans regia* L.) under laboratory conditions

Abstract: Biological control nowadays is rapidly growing to reduce the incessant use of chemical insecticides for control of various insect pests. In the present study, entomopathogenic fungi are used to determine insecticidal activity against walnut blue butterfly under laboratory conditions. The experimental setup was completely randomized design (CRD) with two treatments along with control with different concentrations of entomopathogenic fungi. The bioassay was carried out by spraying second larval instar of *Chaetoprocta odata* [Hewitson 1865] with 1, 2, 3 & 4 % conidial concentration of *Beauveria bassiana* (Balsamo) Vuill. (1912) and *Isaria fumosorosea* Wize (1904). The results of this study showed that all the concentrations showed remarkable pathogenic activity but *I. fumosorosea* was highly pathogenic and recorded the highest mortality rate of 93.33 % after 144 hours compared to *B. bassiana* where 73.33 % mortality was reported. LC_{50} values for *B. bassiana* (4.15) was higher than that of *I. fumosorosea* (3.34) which indicates that *I. fumosorosea* was more effective against *C. odata* population. Among different concentrations of *I. fumosorosea*, 4 % concentration was the most effective with lowest LC_{50} values.

Key words: *Juglans regia*; biological control; *Chaeotoprocta odata*;, virulence; *Beauveria bassiana*; *Isaria fumosorosea*

Učinkovitost entomopatogenih gliv za zatiranje gosenic orehovega modrina (*Chaetoprocta odata* [Hewitson 1865]) na orehu (*Juglans regia* L.) v laboratorijskih razmerah

Izvleček: Biotično varstvo rastlin danes pridobiva na pomenu, kar vpliva na manjšo rabo kemičnih insekticidov pri zatiranju različnih vrst škodljivih žuželk. V pričujoči raziskavi sta bili uporabljeni dve vrsti entomopatogenih gliv za določitev njunega insekticidnega delovanja na orehovega modrina v laboratorijskih razmerah. Poskus je bil zasnovan kot popolni naključni poskus z dvema obravnavanjema in kontrolo. Poskus z glivama je bil izveden s škropljenjem druge razvojne stopnje gosenic modrina z 1, 2, 3 & 4 % koncentracijo konidijev gliv *Beauveria bassiana* (Bals.-Criv.) Vuill. (1912) in *Isaria fumosorosea* Wize (1904). Rezultati raziskave so pokazali, da so vse koncentracije konidijev imele opazno patogeno aktivnost, a je bila gliva *I. fumosorosea* bolj patogena in je bila v obravnavanjih z njo dosežena največja smrtnost gosenic, 93,33 % po 144 urah, v primerjavi z glivo *B. bassiana*, kjer je bila smrtnost 73,33 %. LC_{50} vrednosti so bile za glivo *B. bassiana* večje (4,15) kot pri glivi *I. fumosorosea* (3,34), kar kaže, da je bila gliva *I. fumosorosea* bolj učinkovita pri zatiranju gosenic orehovega modrina. Med različnimi koncentracijami konidijev glive *I. fumosorosea* je bila 4 % koncentracija najbolj učinkovita z najmanjšimi vrednostmi LC_{50}.

Ključne besede: *Juglans regia,* biotično varstvo rastlin**,** *Chaeotoprocta odata,* učinkovitost, *Beauveria bassiana, Isaria fumosorosea*

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1 INTRODUCTION

The caterpillars of walnut blue butterfly, *Chaetoprocta odata* [Hewitson 1865] Lepidoptera: Lycaenidae are severe leaf defoliators which feed on parenchyma tissue of leaves resulting in scleretonizing of new leaflets. The neonate larvae feed on the succulent leaves and are voracious feeders (Butani, 1979; Masoodi & Trali, 1987; Abbas, 2013). It is one of the pests feeding on walnut trees of Kashmir Valley with devastating potential to defoliate the whole host tree (Mir & Wani, 2005). Chemical insecticides are being used since ages to protect plants due to which insects are developing resistance against them. One of the environmentally friendly techniques is biological control especially, the use of entomopathogenic fungi, forming a key part of integrated pest management (IPM) program (Ren & Chen, 2012). They cause cuticular infection resulting in toxin formation in pest after invasion. Additionally, they can infect any developmental stage of pests (Wang et al., 2010). Around 750 species of fungi are highly effective against various insect pests and offer great potential to manage insects with least hazard effects on environment and human health (Rabindra & Ramanujam, 2007; Laznik et al., 2012). This is the first attempt to use two strains of entomopathogenic fungi, *Beauveria bassiana* and *Isaria fumosorosea* for controlling pests on walnut trees.

2 MATERIALS AND METHODS

2.1 BIOLOGICAL MATERIAL

C. odata proceeding larvae were collected from the walnut orchards of Central Kashmir *Viz.*, Srinagar (34° 04' 54.36" N, 74° 48' 33.00" E, 1587 m), Budgam (34° 01' 2.05" N, 74° 43' 6.71" E, 1610 m) and Ganderbal (34° 13' 39.11" N, 74° 46' 19.78" E, 1619 m) and rearing was done in glass jars placed in an incubator maintained at temperature of 25 (± 1) °C and relative humidity of 65 (± 5) %. Two fungal strains *viz.*, *Beauveria bassiana* and *Isaria fumosorosea* in four different concentrations (1 %, 2 %, 3 % & 4 %) prepared from a stock solution of 1×10^9 conidiaml^{-1}by diluting with double distilled water obtained from Green Life Biotech Laboratory, Somanur, Coimbatore, India were tested against them to know the efficacy.

2.2 BIOASSAYS

In the experiment, freshly cut leaves of walnut trees were placed in 11 cm diameter petri- dishes and leaf petiole was covered with water swabbed cotton. Second instar larvae of *C. odata* were inoculated by immersing them in different conidial suspensions for 30 seconds and in case of the control, larvae were dipped in the distilled water. *C. odata* larvae were relocated to these leaf discs and all the petri-dishes were covered with the white muslin cloth tied with a rubber band for proper ventilation. The experiment design was a randomized complete block where three replicates were taken and each replicate had 10 larvae. Mortality was observed daily for six days at 23 (± 2) °C and 65 (± 5) % RH with a photoperiod of 12:12h (Irigaray et al., 2003). Mycosis test was done on the dead larvae and was transferred to petri-dishes in which moist filter paper was placed for 10 days. Microscopic examination confirmed the cause of mortality by fungal entomopathogens (Cherry et al., 2005).

2.3 MYCOSIS TEST

To know whether insect mortality had occurred due to fungal infection, mycosis test was done on the dead cadavers of insects. In this experiment, three petri plates were taken; two containing distilled water and one having 70 % ethanol. The process started by dipping cadavers of insects one by one in water followed by ethanol and then again in water to kill fungus present on the insect cuticle. Different isolates were dipped in separate Petri dishes. The procedure was repeated for all replicates of different isolates. Further, if fungi showed their growth again, the conidia of different isolates would penetrate the insect cuticle which subsequently enables us to know that death has occurred due to fungal infection (Grund & Hirch, 2010). The collected insect specimens were examined under a Leica M205A stereo zoom trinocular microscope and were photographed with a Leica DFC295 camera having automontage software version 4.10 (Leica Microsystems, Germany).

2.4 STATISTICAL ANALYSES

Experimental data was analysed by using Origin Pro software (Version 15). The data derived on means of percentage mortality and corrected mortality of *M. fotedari* adults in different treatments were analysed by ANOVA at 0.05 % level of significance. Tukey's Honest Square Difference test was used to separate means of treatment. Regression Analysis was done to estimate Lethal Concentration 50 (LC50) values at different concentrations. Abbott's formula (Abbott, 1925) was used for correction mortality data with that in control.

$$CM\ (\%) = T\ (\%) - C\ (\%) / 100 - C\ (\%)$$

Where, *CM* (%) - Corrected mortality
T - Mortality in treatment
C - Mortality in control

3 RESULTS

On treating larvae with *B. bassiana* at 1% concentration, it was found that mortality of larvae occurred on the 4th day after treatment. The corrected mortality after 120 hrs and 144 hrs was 3.33 % and 7.03 % respectively (Fig. 6 and Fig. 1). One way ANOVA depicted that mortalities after 96 hrs, 120 hrs and 144 hrs were statistically similar ($p > 0.05$) with each other. The regression equation for the suspension was calculated as Y=2.09X - 3.999 having a regression coefficient (R^2) value of 0.864 while LC_{50} value of the *B. bassiana* at 1 % concentration was 25.78 whereas on treating larvae with *I. fumosorosea* at 1 % concentration, the average corrected mortality after 120 hrs and 144 hrs was 13.70 % and 17.77 % respectively (Fig. 6 & Fig. 1). The calculated regression equation was Y=5.141X - 9.109 having a regression coefficient (R^2) value of 0.880. On subjecting data to one way ANOVA, insignificant difference was between the mortality rate at 96 hrs and 144 hrs while significant difference was between 144 hrs and 120 hrs at $p \leq 0.05$. LC_{50} value of *I. fumosorosea* at 1 % concentration was 11.49 %

At 2 % concentration, the corrected mortality by Abbott's formula after 120 hrs and 144 hrs was 41.11 % and 52.96 % respectively (Fig. 7 & Fig. 2) for *B. bassiana* with LC_{50} value 5.65 (Fig. 5). On subjecting the data to one way ANOVA, the results showed that the larvicidal activity at 72 hrs, 96 hrs, 120 hrs and 144 hrs was significantly different with $p \leq 0.05$ (Fig. 2). The regression equation was intended as Y=12.38X - 19.997 with an evaluated regression coefficient value (R^2) 0.950 (Fig. 5). On treating with 2 % *I. fumosorosea* the corrected mortality after 120 hrs and 144 hrs was 54.8 % and 71.47 % respectively (Fig. 7& Fig. 2). The evaluated LC_{50} value at 2 % concentration was 4.69. The regression equation at 2 % concentration was Y=15.808X - 24.221 having a regression coefficient (R^2) value of 0.966. From the data analysis, it was revealed that mortalities at 72 hrs, 96 hrs, 120 hrs and 144 hrs were significantly different among themselves as shown by ANOVA at $p \leq 0.05$.

When larvae were inoculated with a spore concentration of 3 %, larvicidal activity of *B. bassiana* against 2nd larval instar depicted that average mortality increased from 72 hrs to 144 hrs. The toxicity of *B. bassiana* was recorded showing the corrected mortality percentages at 120 hrs and 144 hrs were 58.51 and 64.07 respectively (Fig. 8) while LC_{50} value at 3 % was 4.71. Linear regression equation (Y=15.142X - 21.332) showed the value of regression coefficient (R^2) equal to 0.961 (Fig. 5 & Fig. 3). One way ANOVA results of the data showed that mortality rates at 3 % concentration were statistically different at 72 hrs, 96 hrs and 120 hrs although, percent mortalities at 120 hrs and 144 hrs were significantly similar among

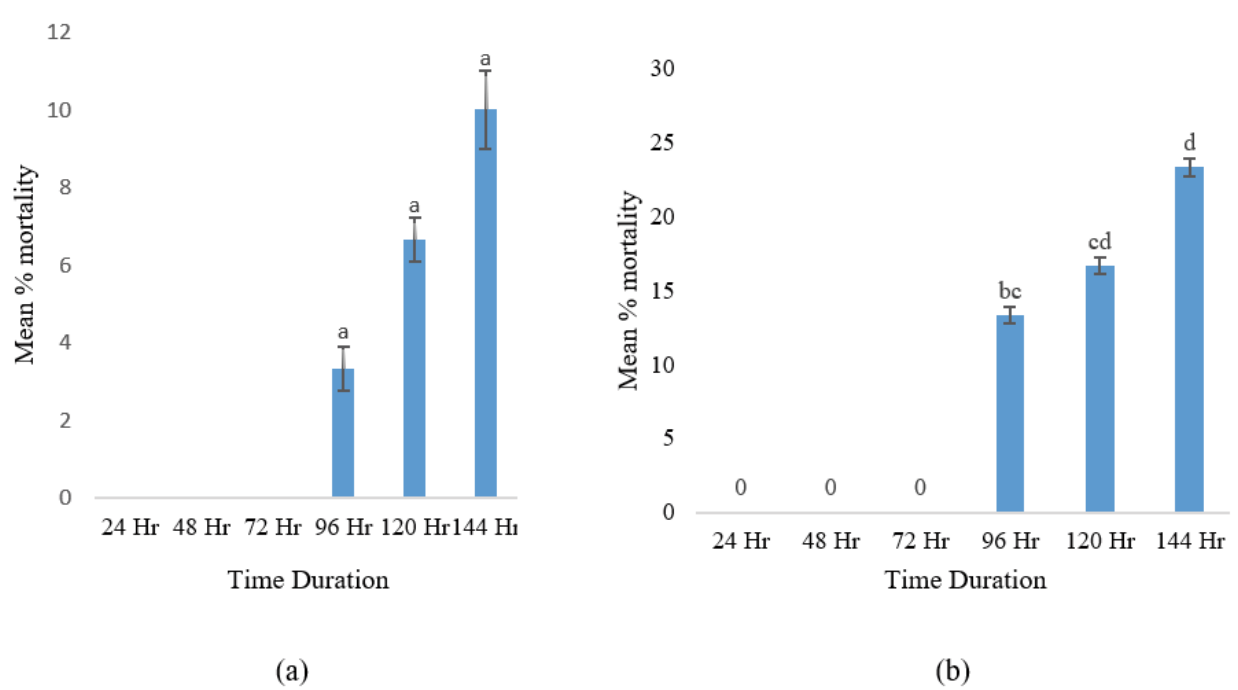
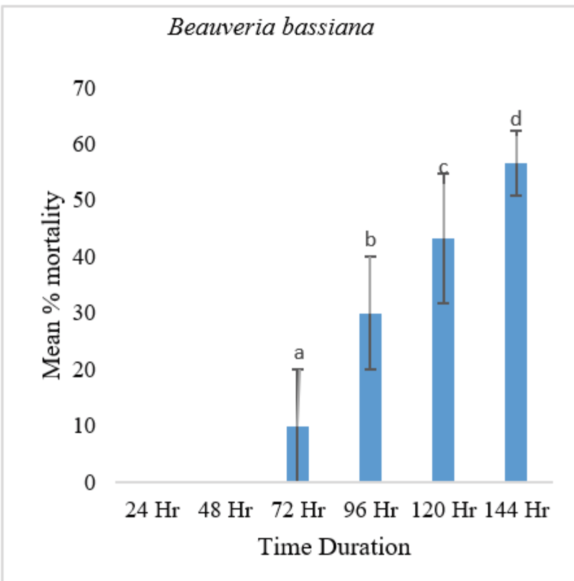


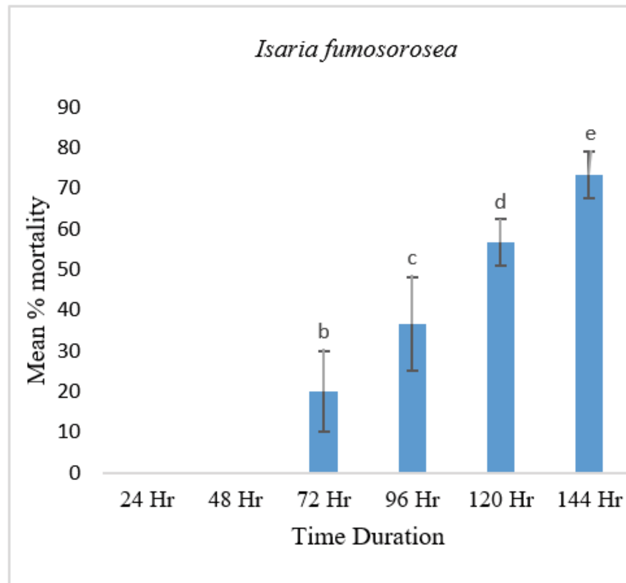
Figure 1: Mean percent mortality (± SD) at 1% concentration of (a) *Beauveria bassiana* (b) *Isaria fumosorosea*

themselves at $p \leq 0.05$. Similarly,when larvae were treated with 3 % concentration of *I. fumosorosea*, the corrected mortalities at 120 hrs and 144 hrs were recorded as 75.92 % and 82.21 % (Fig. 3 & Fig. 8). One way analysis of variance depicted that mortalities at 48 hrs, 72 hrs, 96 hrs and 120 hrs were statistically significant with each other although, mortality at 144 hrs was insignificant to mortality shown at 120 hrs. The obtained regression equation was Y=17.999X - 20.222 with regression coefficient (R^2) value 0.983 while the calculated LC_{50} value was 3.90.

At 4 % concentration, the larvicidal activity of *B. bassiana* was observed soon after 48 hours f treatment.

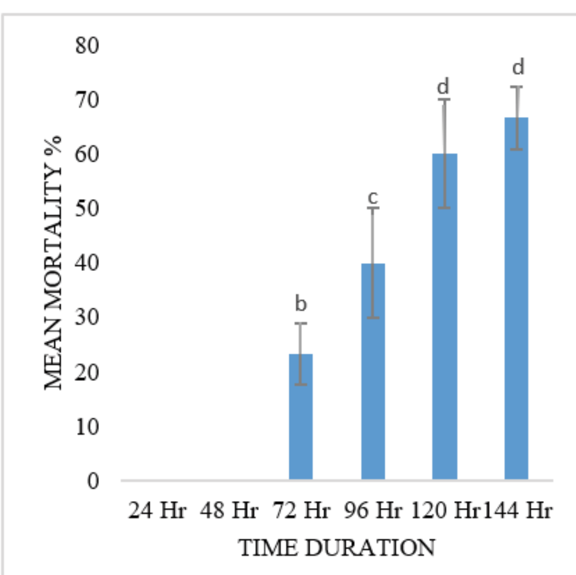


(a)

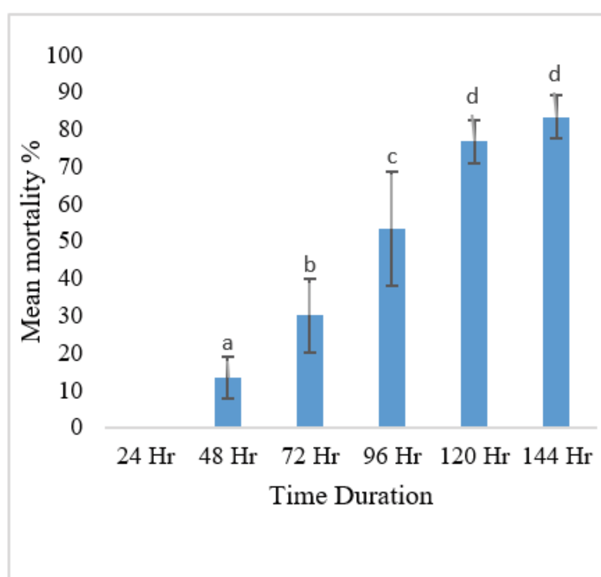


(b)

Figure 2: Mean percent mortality (± SD) at 2 % concentration of (a) *Beauveria bassiana* (b) *Isaria fumosorosea*



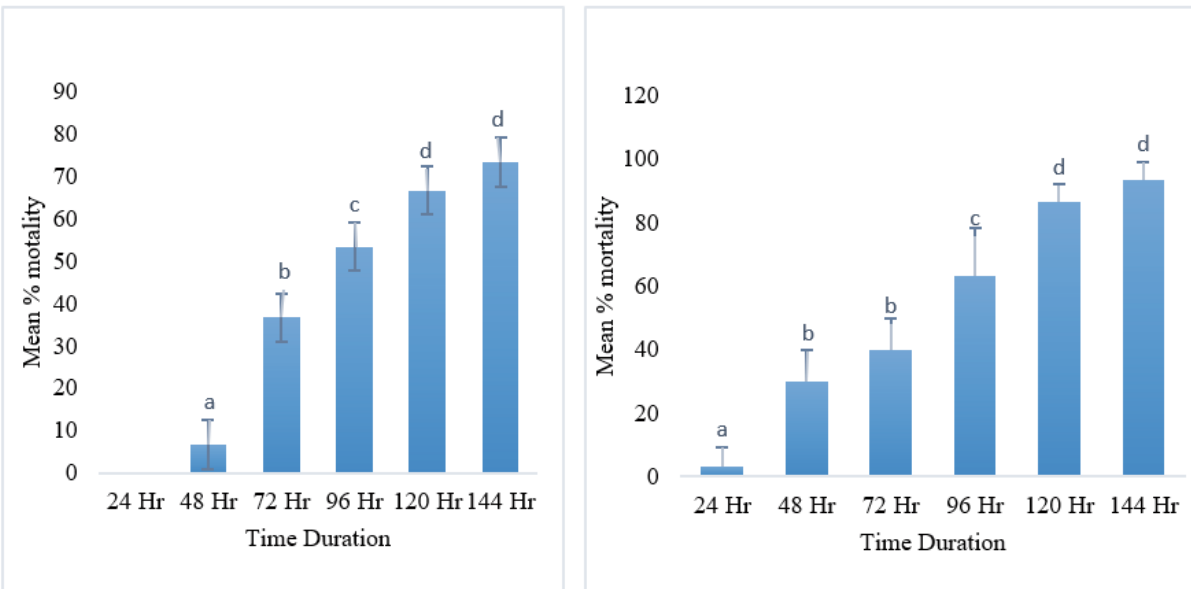
(a)



(b)

Figure 3: Mean percent mortality (± SD) at 3 % concentration of (a) *Beauveria bassiana* (b) *Isaria fumosorosea*

The mortality percentages at 120 hrs & 144 hrs corrected by Abbott's formula were 65.55 and 71.10 correspondingly (Fig. 8 & Fig. 4) whereas the calculated regression equation was Y=16.095X - 16.889 having a regression coefficient (R^2) value of 0.960 (Fig. 5) in contrast to *I. fumosorosea* at 4 % concentration, the corrected mortality percentages acquired through Abbott's formula at 120 hrs and 144 hrs were 86.29 and 92.96 respectively (Fig. 9). One way ANOVA results exhibited that mortality at 24 hrs was significant to the mortalities at 48 hrs, 72 hrs, 96 hrs, 120 hrs and 144 hrs. The mortality at 48 hrs and 72 hrs was insignificant with each other but significant to



(a) (b)

Figure 4: Mean percent mortality (± SD) at 4 % concentration of (a) *Beauveria bassiana* (b) *Isaria fumosorosea*

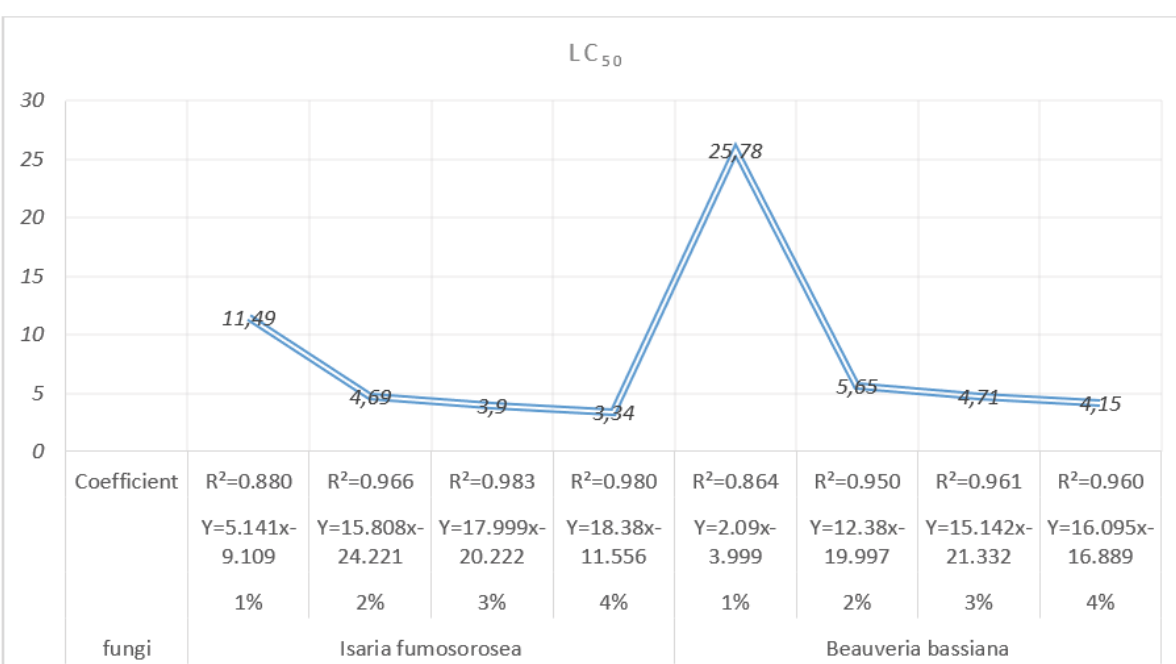


Figure 5: LC_{50} of second larval instar of *Chaetoprocta odata* treated with different suspensions of entomopathogenic fungi

others while the mortalities at 120 hrs and 144 hrs were statistically similar at $p \leq 0.05$. The calculated regression equation was Y=18.38X - 11.556 with a regression coefficient (R^2) value of 0.980. LC_{50} value at 4 % concentration was 3.34 (Fig. 5).

The calculated F values for 1, 2, 3 and 4% concen-

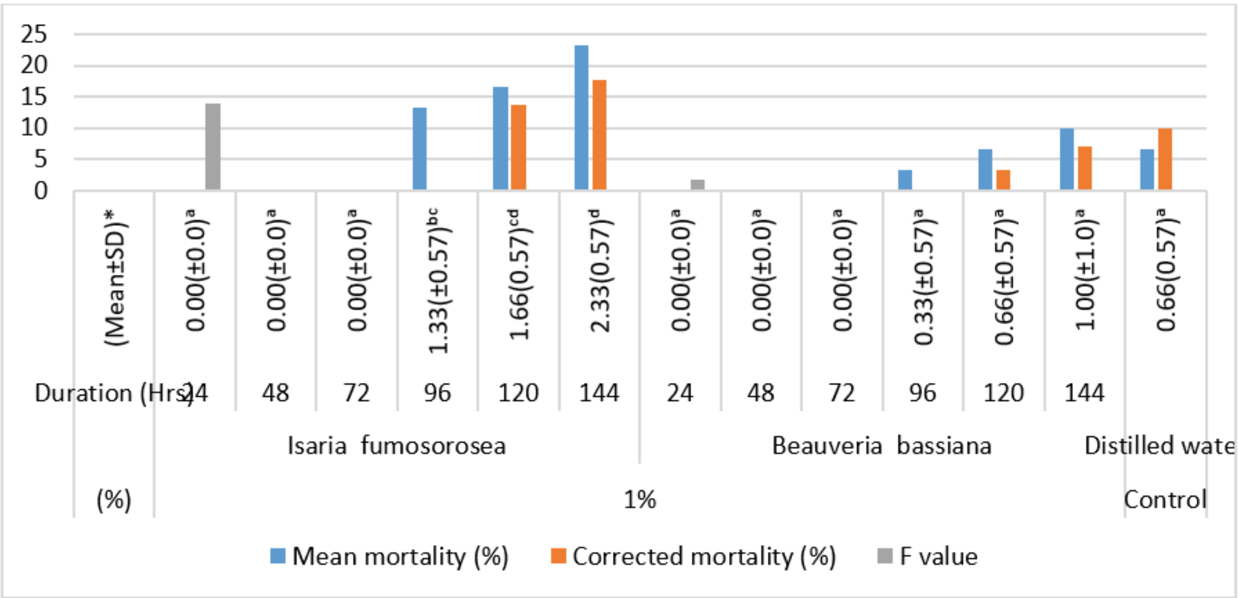


Figure 6: Corrected mortality percent and toxic effects of entomopathogenic fungi (1 % suspension) against second larval instar of *Chaetoprocta odata*\

*Mean of 10 second instar larvae of *Chaetoprocta odata*/ replication/ treatment; means followed by identical letters in lower case each column are not significantly different by Duncan's test at 5 %; Mean mortality % of individuals at the end of experiment corrected for mortality in control using Abbott formula

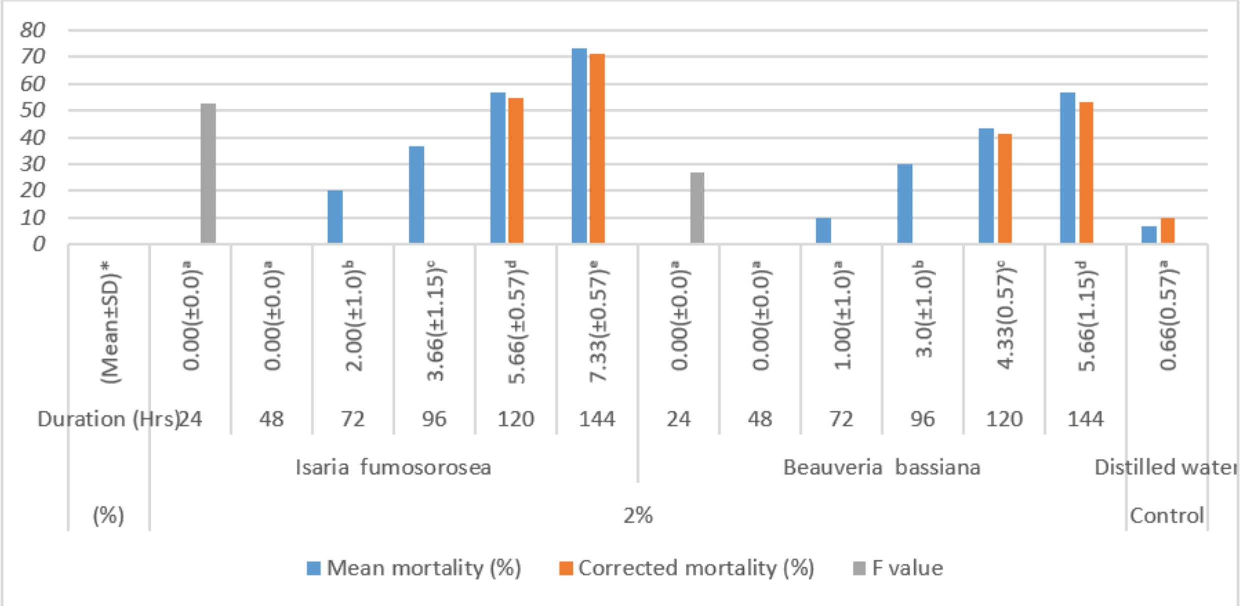


Figure 7: Corrected mortality percent and toxic effects of entomopathogenic fungi (2 % suspension) against second larval instar of *Chaetoprocta odata*

*Mean of 10 second instar larvae of *Chaetoprocta odata*/ replication/ treatment; means followed by identical letters in lower case each column are not significantly different by Duncan's test at 5 %; Mean mortality % of individuals at the end of experiment corrected for mortality in control using Abbott formula tration was high for 4% concentration which means significant mortality had occurred at this concentration predicting that the variance between different mortalities isn't due to the random chance of all the variables used. The calculated regression equation for each concentration of *B. bassiana* demonstrated positive correlation

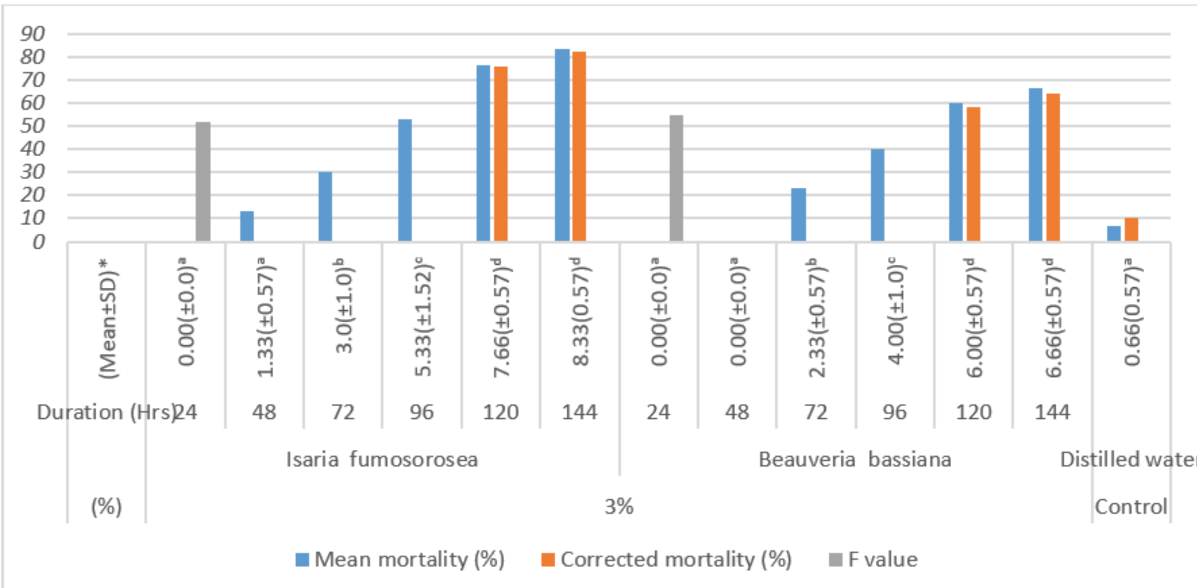


Figure 8: Corrected mortality percent and toxic effects of entomopathogenic fungi (3 % suspension) against second larval instar of *Chaetoprocta odata*

*Mean of 10 second instar larvae of *Chaetoprocta odata* / replication/ treatment; means followed by identical letters in lower case each column are not significantly different by Duncan's test at 5 %; Mean mortality % of individuals at the end of experiment corrected for mortality in control using Abbott formula

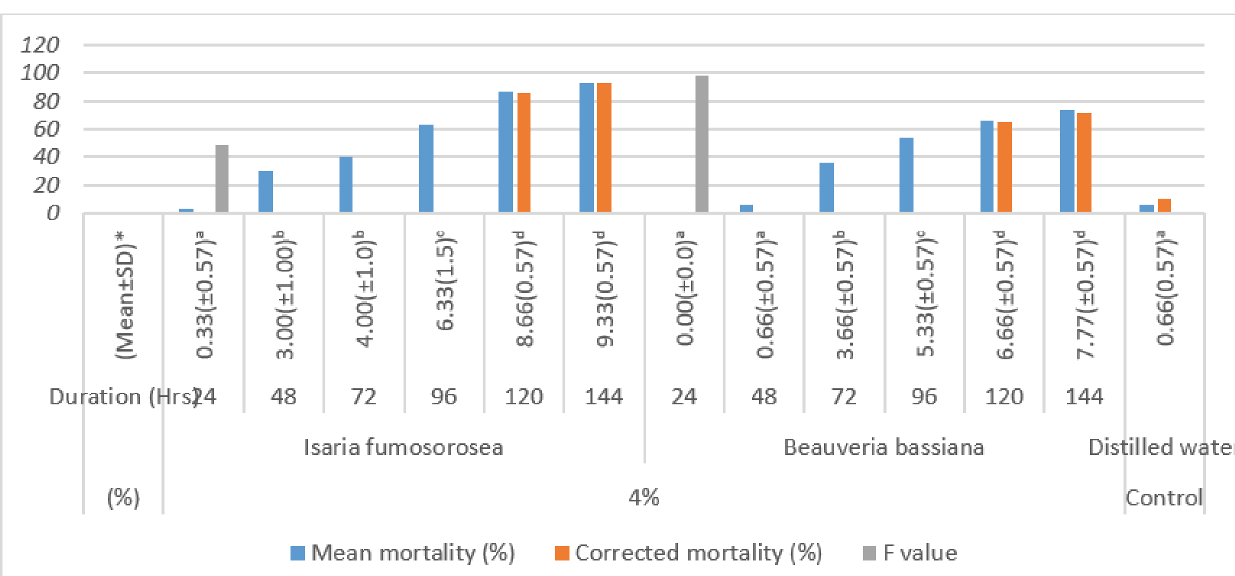


Figure 9: Corrected mortality percent and toxic effects of entomopathogenic fungi (4 % suspension) against second larval instar of *Chaetoproct aodata*

*Mean of 10 second instar larvae of *Chaetoprocta odata* /replication/ treatment; means followed by identical letters in lower case each column are not significantly different by Duncan's test at 5 %; Mean mortality % of individuals at the end of experiment corrected for mortality in control using Abbott formula between concentration and mortality i.e., with an increase in the independent variable, the dependent variable also increases. The derived R^2 values range from 0 to 1 which signifies 0 to 100 % and the derived values in the experiment were close to 1 which depicted that increasing concentration of *B. bassiana* caused more larvicidal activity. The results of LC_{50} values calculated through Probit analysis are given in Fig. 5. Among the two entomopathogenic fungi *viz.,I. fumosorosea* and *B. bassiana*, *I. fumosorosea* was recorded to have maximum mean percent mortalities at different concentrations (Fig. 5). The lowest LC_{50} value (3.34) was calculated for *I. fumosorosea* at 4 % concentration which indicated its high toxicity to kill the larvae as lower LC_{50} values show acute pathogenicity. All the concentrations caused significantly higher mortality rates compared to control (distilled water). However, during the present study, no concentration of both entomopathogens caused 100 % mortality to larvae although; there was an upsurge in the mortality rates when concentration increased.

4 DISCUSSION

C. odata is one of the potential pests defoliating walnut trees in Kashmir valley (Abass, 2013). The present work is the first attempt to know the pathogenicity caused by two entomopathogenic fungi *viz., I. fumosorosea* and

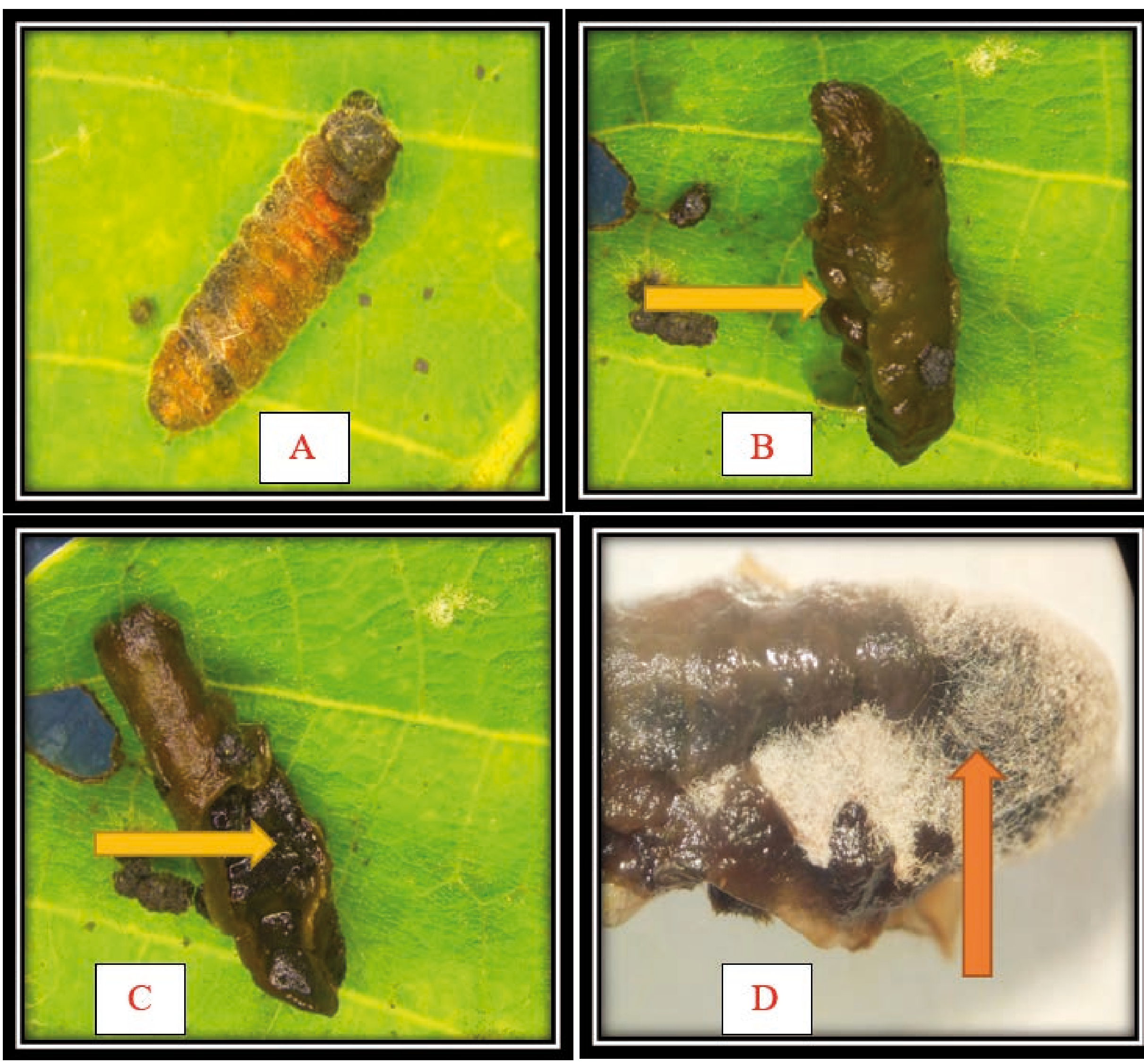


Figure 10: Entomopathogenic fungi *Isaria fumosorosea* and *Beauveria bassiana infesting* larvae of *Chaetoprocta odata* (A and B) Disruption of insect body tissue due to mycelial growth after infestation (C) Infestation by *Beauveria bassiana* (D) Infestation by *Isaria fumosorosea*

B. bassiana against second larval instar of *C. odata* (Fig. 10). Various entomopathogens have been used to control Lepidopteran pests especially *I. fumosorosea, B. bassiana* and *Metarhizium robertsii* (Metchnikoff) Sorokin (1883) (Hussain et al., 2009). Present results were in line with the observations of Gopalakrishnan & Narayan (1988) who evaluated the mortality rate of different larval instars of *Helicoverpa armigera* (Hübner, 1808), at different concentrations ranging from 1×10^{10} to 1×10^{7} with a mortality rate ranging from 60-100 %. During the experimental study, no emergence of adult took place from the treated larvae which was in agreement with the study carried by Hafez et al.(1994) who observed various life stage parameters in potato tuber moth (*Phthorimaea operculella* [Zeller, 1837] when treated with *B. bassiana* and found no emerge of adults at a concentration from 1×10^{4} to 1×10^{10} sporesml^{-1}.Entomopathogens are larvicidal as they contain extra-cellular secondary metabolites that have biocidal properties (Vey et al., 1985;Omura, 2011). As reported by other workers, the metabolites of entomopathogens use glycogen and lipid reserves of insects resulting in disruption of insect tissue due to mycelial growth that further leads to loss of appetite in them (Thomas et al., 1997) (Fig. 5). Similar observations were recorded during the experimental study when larvae were treated with entomopathogenic fungi and they refrained from the food as no notches were observed on the fresh walnut leaves. Our results were further affirmed with the findings of Shelton et al. (1998) who treated 2nd larval instar of diamondback moth, *Plutella xylostella* [Linnaeus, 1758] with entomopathogenic fungi, *Beauveria* spp. And found similar results. In addition, Hatting (2012) determined the pathogenicity of three entomopathogens when treated against ball worm, *Helicoverpa armigera* and found that *Nomuraea rileyi* (Farl.) Samson showed the highest toxicity followed by *I. fumosorosea* and *B. bassiana* respectively.

During the present investigation, it was observed that mortality due to entomopathogens was time and dose dependent. Initially, mortality was low after treatment which then gradually augmented from day 3 to day 6. Similarly, the finding of Wright et al. (2005) found that pathogenicity of the infected insect is dose and time dependent. Further, the study conducted by Bashir et al. (2018) reported that B. bassiana leads to 79 % mortality of Corcyra cephalonica [Stainton, 1866] larvae in in-vitro conditions. Likewise, our observations concurred with the finding of Nguyen et al. (2007) who found that the treatment of Helicoverpa armigera with M. anisopliae, B. bassiana and P. fumosoroseus, resulted in 68 to 100 % mortality,in laboratory investigations. Besides, it was observed that increased concentration showed higher mortality rates with amplified conidial growth which was in line with the findings of Safaviet al. (2007) who found that the virulence of entomopathogenic fungi is dependent on concentration, conidial growth and mostly on the nutritional content of pest especially carbon: nitrogen content. Thus, the availability of host food is one of the prime factors for the development of fungal pathogens (Tefera & Pringle, 2004). On the other hand, pathogenicity of I. farinosa against larval stages of Harmonia axyridis [Pallas, 1773] was studied by Steenberg & Harding (2009) and found that larval stages were most vulnerable and resulted in high mortality rates. In the current study, it was recorded that the highest mortality occurred due to I. fumosorosea which was in corroboration with findings of Zimmermam (2008) who found that I. fumosorosea has a broad host range and is highly infective to several insect orders especially Lepidoptera. Sabbour (2015) revealed that I. fumosorosea played a significant role in controlling the pests of the corn crop. Likewise, Schemmer et al. (2016) found I. fumosorosea as most pathogenetic fungi with a median lethal concentration of 0.09×104 conidiaml-1 against Camerariao hridella (Deschka & Dimic, 1986) (Lepidoptera: Gracillariidae). Thus from the above inferences and the results of the current study, it can be concluded that I. fumosorosea contains pathogenic characters that can be used as a bio-control agent against larvae of C. odata and may provide a practicable alternative to commercial insecticides used to control larvae in the early season.

5 CONCLUSION

Chemical insecticides are being used since ages to protect plants from pest attacks which resulted in insect resistance, environmental pollution and various health issues. Nowadays emphasis on biological control methods are increasing to manage insect pest population mostly entomopathogenic fungi. Current study demonstrated that, most important percent mortality was found in *I. fumosorosea* followed by *B. Bassiana* at all concentrations although both could be utilized as alternate tools to control population of *C. odata*. Among different concentrations of *I. fumosorosea*, 4 % concentration was the most effective with the lowest LC_{50} values. Therefore, further field study is necessary to evaluate the effectiveness of entomopathogenic fungal formulations in the management of *C. odata* in walnut orchards that can prove the most effective strategy in integrated pest management programs.

5.1 CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

5.2 ACKNOWLEDGEMENT

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Predatory insects as biological control agents against walnut aphids in Kashmir, India

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Abstract: Management of pests by biological control agents is a natural and environmental friendly method. It is an important part of integrated pest management programs where biological predators play a significant role in controlling various pests. During the present study, different insect pests were observed infesting walnut trees and fruits causing significant damage to the walnut orchards. Among these pests, sap suckers were found to cause notable damage out of which aphids were most dominating pests**.** High population of walnut aphids causes leaf drop, reduced tree vigour, nut quality and size. In addition to this, some insect predators were also observed on these walnut aphids. Six species of predaceous coccinellid beetles were found on walnut leaves in interaction with walnut aphids and they were identified belonging to sub family Coccinellinae. Among which *Calvia punctata* (Mulsant, 1853) and *Harmonia dimidiata* (Fabricius, 1781) were the most encountered species. Besides, one species from syrphidae family *Metasyrphus latifasciatus* (Macquart, 1859) was also observed. Moreover, extreme feeding potential was noted among these predators.

Key words: pest management, sap suckers; coccinellids, walnut aphids, *Calvia punctata*, *Harmonia dimidiata*, *Metasyrphus latifasciatus*, predatory potential

Plenilske žuželke kot biološki nadzor listnih uši na navadnem orehu v Kašmirju, Indija

Izvleček: Upravljanje škodljivcev z biološkimi sredstvi je naravna in okolju prijazna metoda. Je pomemben del integriranega varstva rastlin, v katerem imajo plenilski organizmi pomembno vlogo pri nadzorovanju različnih škodljivcev. V tej raziskavi so bile ugotovljene različne škodljive žuželke, ki napadajo navadni oreh in povzročajo znatno škodo v njegovih nasadih. Med temi škodljivci povzročajo znatno škodo sesajoče žuželke, med katerimi prevladujejo listne uši. Velike populacije listnih uši povzročajo na orehu odpadanje listja, zmanjšujejo vitalnost dreves, velikost in kakovost orehov. V raziskavi so bile opažene tudi plenilske žuželke, ki se hranijo z listnimi ušmi. Na listju navadnega oreha je bilo ugotovljeno šest vrst plenilskih polonic, ki se hranijo z listnimi ušmi in pripadajo poddružini Coccinellinae. Med njimi sta bili najbolj pogosti vrsti *Calvia punctata* (Mulsant, 1853) in *Harmonia dimidiata* (Fabricius, 1781). Med temi plenilskimi žuželkami je bila najdena tudi vrsta muh iz družine trepetalk, vrsta *Metasyrphus latifasciatus* (Macquart, 1859). Pri vseh teh plenilskih žuželkah je bil ugotovljen velik potencial hranjenja z listnimi ušmi.

Ključne besede: upravljanje s škodljivci**,** sesajoče žuželke, polonice, listne uši navadnega oreha, *Calvia punctata*, *Harmonia dimidiate*, *Metasyrphus latifasciatus*, plenilski potenciala

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1 INTRODUCTION

Kashmiri walnut known as *Juglans regia* L. is one of the prime industry of the valley with numerous cultivars of about 199 indigenous and exotic (Verma et al., 2009). After apple industry, it is the second major fruit covering an area of 26.91 % and is important source of state economy. Walnut has enormous health benefits as it is rich in vitamin B, omega-3, omega-6, essential fatty acids, sterols and phenolic substance (Davis et al., 2007; Vigneshwara, 2011). Irrespective of these health benefits walnut is highly prone to pest attacks and harbours diversity of pests which cause deterioration in its quality and huge losses occur every year. Among the different pests, aphids act as the major pests (Sharma et al., 2012). Aphids deteriorate the plant health by changing the nutrient amount leading to reduced photosynthetic capacity resulting in leaf yellowing, premature death and spotting (Dedryver et al., 2010). Incessant feeding of walnut aphid leads to reduced tree vigour, quality, size of nut and mostly the yield (Aliniazee & Hagen, 1995; Cecilio & Illharco, 1997). However, the overall population is restrained due to the impact of natural enemies mostly predators and parasitoids (Gaston & Lawton,1988; Storck-Weyhermüller,1988). Different biological agents feed on these aphids and acted as management tool for their control to reduce outburst of population. Some of the important group of predators belong to the family Coccinellidae, Syrphidae, Mantidae and Chrysopidae. Coccinellid beetles are most abundant biological control agents against aphids. They are of great economic importance as predators both in their larval and adult stages on various important crop pests such as aphids, coccids and other soft bodied insects (Hippa et al., 1978; Kringet et al., 1985). The coccinellid beetles are considered to be of great economic importance in agro-ecosystem due to their successful use in the biological control of many injurious insects (Agarwala & Dixon, 1992). After coccinellids, syrphidae family are also active group of predators. The present work is the attempt to find the different species of predators feeding on walnut aphids and the feeding potential of dominating species so as to develop biological control for aphids feeding on sap of walnut leaves.

2 MATERIALS AND METHODS

2.1 STUDY AREA

Studies were carried out in three different districts of Kashmir namely Ganderbal (34° 13′ 39.11″ N, 74° 46′ 19.78″ E), Anantnag (33.7311° N, 75.1487° E) and Kupwara (34° 32′ 14.85″ N 74° 13′ 50.81″ E). In each district one site was monitored throughout the year 2018. The three sites include Kangan from district Ganderbal, Kelam from Anantnag and Hatmulla from Kupwara district. In each site two orchards were selected.

2.2 SAMPLING METHODS

Each site was repeatedly sampled after two week interval for three hours throughout the year 2018. Sampling method for collection of predatory insects involved collection by one man one hour swept net collection method from ten trees selected randomly from each walnut orchard (Khairmode, 2014). For active fliers sampling was based on sweeping with insect collecting net which was repeated after a gap of 10 minutes with 10 sweeps each time. However, some beetles were collected by net sweeping method and hand picking method (Jonathan, 1995). The collected specimens were kept in collecting jars than transferred to vials containing 70 % alcohol for wet preservation and number was counted. The specimens were then brought to entomological laboratory for further studies.The branches harbouring insects were randomly selected for quantification and maintenance of record. The pests were also collected from different walnut orchards by using the various research equipment's like hand lens, forceps, soft camel hair brush, pair of scissors, vials (5 × 3 cm), polythene bags, specimen tubes containing 90 % alcohol as a preservative, insect killing bottles and Insect collecting net.

2.3 IDENTIFICATION

The collected specimens of each species were carefully studied for all details under binocular microscope and were identified by running keys and taxonomic work of different workers. The different taxonomic keys used to identify these species include Kapur (1958, 1956b); Kuznetsov (1997); Gordon (1970b, 1970e, 1985, 1987); Ahmad and Ghani (1966); Poorani (2002a, 2002b); Slipinski (2007); Slipinski et al. (2005); Slipinski and Giorgi (2006). Some specimens which were not identified were sent to Zoological survey of India (ZSI), Kolkata for taxonomic characterization.

2.4 FEEDING POTENTIAL

For evalutation of the feeding potential of predators feeding on walnut aphids, adults from field were collected and reared under laboratory conditions (26 ± 2 ˚C and 65 ± 5 % R.H.). Newly emerged adults and immature stages were used for the study. Different predatory species were kept individually in separate petri dishes (9 cm in diameter). They were provided with known number of adult walnut aphids (*Chromaphis juglandicola* (Kaltenbach, 1843)*)* and covered with muslin cloth. In order to record consumption rate, the number of aphids left out after 24 hr were counted. The number of aphids consumed during 24 hr was recorded and whole experiment was repeated three times but with increased number of known aphids. The data obtained during the present study was tabulated and presented as per the required statistical methods. One-way ANOVA at 0.05 % level of significance (Ducans test) was done. Analysis was done by using SPSS (Version 16.00) software.

3 RESULTS AND DISCUSSION

The results attained during the present study showed several insect pests infesting walnut trees causing significant damage. Sap suckers were observed to cause notable damage out of which aphids were most dominating pests viz., *Chromaphis juglandicola* (Kaltenbach, 1843) and *Panaphis juglandis* (Goeze, 1778). *C. juglandicola* is commonly called as walnut aphid and *P. juglandis* is called as dusky -veined aphid. Among these aphid species, infestation caused by *C. juglandicola* was observed very high on walnut trees. Both the species are sap suckers and are serious pests, feeding on phloem. The infestation starts from April and remains till October with the peak infestation in the month of June. Mir & Wani (2005) also reported that aphids attack walnut during April–October, causing stunted growth in nursery plantation. Each pest has different distribution on leaves. *C. juglandicola* was found scattered on the underside of leaves while *P. juglandis* was present on the upper side of leaves feeding near the mid rib of leaves although it is a minor pest in comparison to *C. juglandicola*. Both aphid species maintained distance and never fed on same leaf and strongly reinforces the Gause's Competition Exclusive Principle, which states that no two species having same ecological niche can coexist together (Wani & Ahmad, 2014). *C. juglandicola* and *P. juglandis* were never seen feeding on same leaf during the study period as *C.juglandicola* is important factor for limiting the population of latter as it excretes acidic honeydew (Olson 1974).

Both the aphids over winter as egg stage hatch out in early spring and produce young ones without mating. Thus, have many generations per year resulting in development of many colonies in summer season. In case of dusky-veined aphid (*P. juglandis*), winged males and wingless females appear in the month of September and cause general blackening of leaves while walnut aphid (*C. juglandicola*) cause early leaf drop and reduced nut size. During the present investigation, high infestation of walnut aphid population per leaflet was observed which showed close congruity with the results of UCIPM (2011) who had evaluated population of walnut aphid beyond 15 aphids per leaflet can reduce nut yield in terms of quality and quantity. Our results are in accordance with the studies carried out by Ginzel (2010) who reported both these aphid species are contributing factor for reduced tree vigour, nut size and yield.

Besides the pests on walnut trees different predators were also observed. A total of 7 predatory species were observed on the walnut leaves in interaction with walnut aphids. Six species were identified belonging to the family Coccinellidae and one from Syrphidae family. The species belonging to Coccinellidae include *Coccinella septempunctata* (Linnaeus, 1758) *Calvia punctata* (Mulsant, 1853), *Harmonia dimidiata* (Fabricius, 1781), *Oenopia conglobata* (Linnaeus, 1758), *Macroilleis hauseri* (Mader, 1930) and *Adalia tetraspilota* (Hope, 1831). These findings are in accordance with Khan et al. (2009, 2017) and Sluss (1967). From Syrphidae family only one species, *Metasyrphus latifasciatus* (Macquart, 1859) was observed. The relative abundance of these collected predators was observed in all the three districts and is shown in Table 1. *C. punctata* was the most abundant predator prevailing in all walnut orchards. It showed 19.83 % relative abundance at district Anantnag, followed by 18.3 % in district Ganderbal and 14.29 % in district Kupwara. The lowest occurrence was of *H. eucharis* (5.11 %) in district Ganderbal although, it was absent in district Kupwara. *M. latifasciatus* showed it's the highest occurrence in district Ganderbal with a relative abundance of 17.87 %. Likewise, low population of predators was observed in early March but later it gradually increased during the month of June, July and August (Fig.1).

3.1 FEEDING POTENTIAL OF *Calvia punctata* AND *Harmonia dimidiata*

The feeding potential of *Calvia punctata* and *Harmonia dimidiata* on adults of walnut aphid, *Chromaphis juglandicola* under laboratory conditions was observed. On analyzing the data, results revealed that the adults of *C.punctata* consumed on an average of 12.00 (± 1.0), 29.33 (± 1.52), 45.66 (± 2.51) and 69.00 (± 20) aphids when fed with 20, 40, 60 and 80 aphids respectively (Table 2). The consumption ranged from 60 to 86.25 % compared to *H. dimidiata* which on an average consumed 8.00 (± 1.0), 23.33 (± 4.0), 36.00 (± 3.0) and 47.33 (± 3.7) aphids respectively, with a consumption percentage of 40 to 59.16 % (Table 3). Both beetles fed on aphids

Table 1: Percentage relative abundance and Mean (± SE) of predatory insects collected in walnut orchards of three districts of Kashmir valley during the year 2018

Districts	Kangan (Ganderbal)		Kelam (Anantnag)		Hatmulla (Kupwara)	
Predators	Relative Abundance %	Mean (± SE)	Relative Abundance %	Mean (± SE)	Relative Abundance %	Mean (± SE)
Coccinella septempunctata	11.06	2.17 (± 0.76)	12.81	2.58 (± 0.85)	8.76	1.58 (± 0.50)
Calvia punctata	18.30	3.58 (± 1.23)	19.83	4.00 (± 1.28)	14.29	2.58 (± 0.98)
Adalia tetraspilota	7.23	1.42 (± 0.60)	10.33	2.08 (± 0.73)	6.45	1.17 (± 0.41)
Oenopia conglobata	6.81	1.33 (± 0.53)	8.26	1.67 (± 0.69)	10.14	1.83 (± 0.72)
Harmonia dimidiata	12.34	2.42 (± 0.96)	14.88	3.00 (± 1.09)	19.82	3.58 (± 1.36)
Harmonia eucharis	5.11	1.00 (± 0.41)	4.55	0.92 (± 0.42)	0.00	0.00 (± 0.00)
Metasyrphus latifasciatus	17.87	3.50 (± 1.23)	17.77	3.58 (± 1.20)	8.76	3.33 (± 1.29)

but *C. punctata* devoured more number of aphids in all four given treatments. On comparing the mean consumption rate of both the beetles by one-way ANOVA, data revealed that all the four treatments were significantly different from each other at $p < 0.05$ (Fig.2), with a high consumption rate (40-60 %) during the first few hours, followed by an appreciable reduction in later stages. Significant differences in mean consumption rate of aphids were also observed between the two coccinellid beetles in terms of mean consumption rate of aphids. Similarly percent mean aphid consumption under host density of 20 aphids was 40 % and 60 % for *H. dimidiata* and *C. punctata* respectively while it increased to 58.32 % in *H. dimidiata* and 73.32 % in *C. punctata* when host density was 40. The data showed an increasing trend with a host density of 60 and 80 with a percent mean consumption of 76.1 % and 86.25 % in *C. punctata* while 60 % and 59.16 % in *H. dimidiata* respectively. Overall results depicted that host density played a significant role in the aphid consumption rate (Table 2 & 3).

The results showed both the coccinellid beetles are voracious feeders on walnut aphids. These findings are in corroboration with Khan et al. (2007) who also reported *C. punctata* feeding on aphids and scale insects on walnut trees and other wild vegetation. Khamashon et al. (2018) who studied feeding behavior and consumption rate of *C. punctata* found that the consumption rate of adult females and males ranged from 86.9-72.9 % respectively. Sathe and Bhosale (2001) who reported around 21 species of coccinellid beetles feeding on aphids and other soft-bodied pests feeding on agricultural and forest plants.

Table 2: Feeding potential of *Calvia punctata* on *Chromaphis juglandicola* under laboratory conditions

				95 % confidence limits	
Treatments	No. of Aphids offered	Aphids consumed* (Mean ± SD)	Mean Consumption (%)	Lower	Upper
I	20	12.00 ^{d} (± 1.0)	60	9.51	14.48
II	40	29.33 ^{c} (± 1.52)	73.32	25.53	33.12
III	60	45.66 ^{b} (± 2.51)	76.10	39.41	51.91
IV	80	69.00 ^{a} (± 2.0)	86.25	64.03	73.96

*Mean of 3 replications/treatment; figures in parenthesis are standard deviation; means followed by different letters indicate that the means are significantly different ($p < 0.05$) by Duncan's test

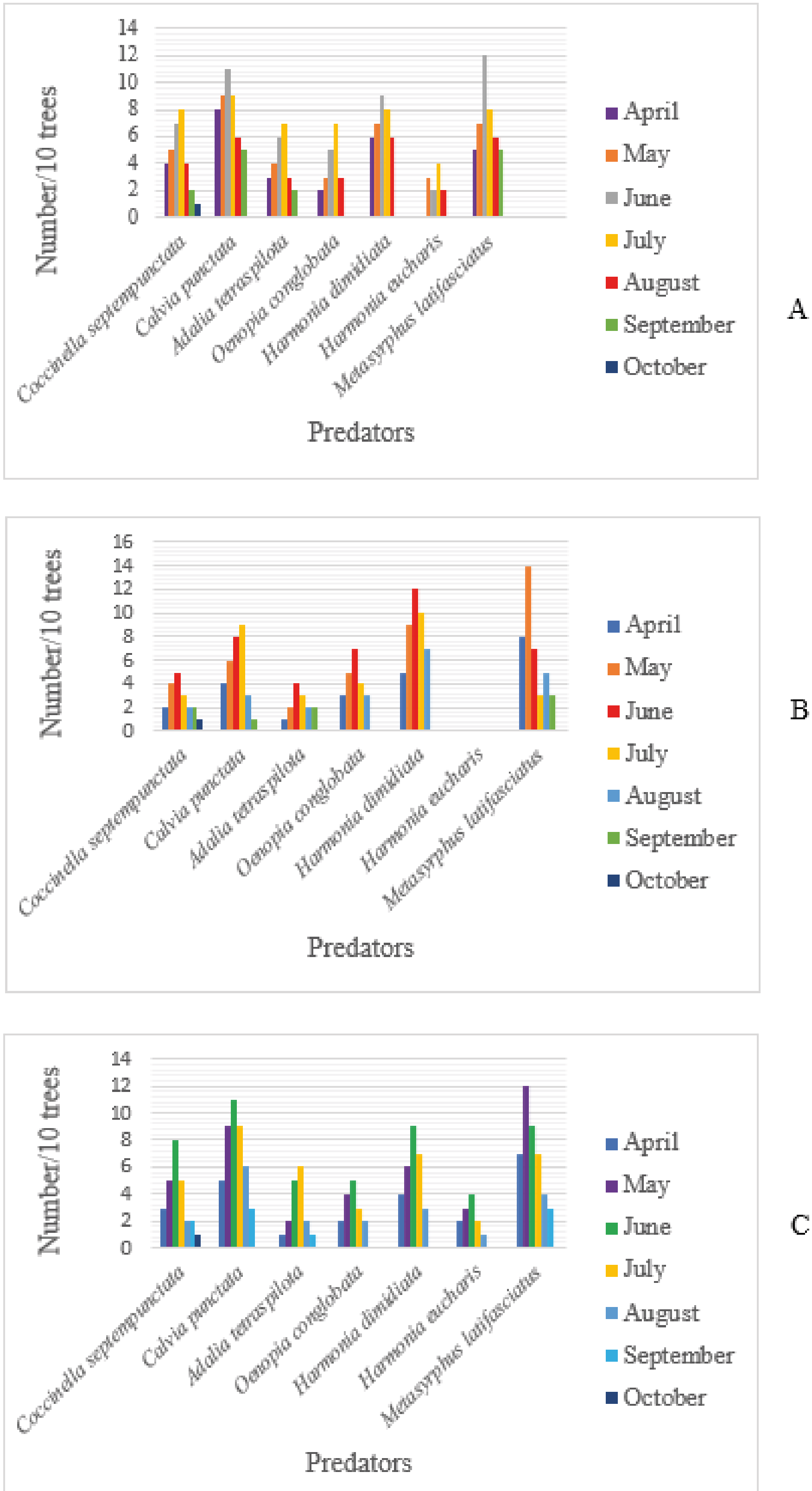

Figure 1: Monthly variation of predators throughout the sampling period at different sites. (A) Kelam (B) Hatmulla and (C) Kangan

Table 3: Feeding potential of *Harmonia dimidiata* on *Chromaphis juglandicola* under laboratory conditions

Treatments	No. of Aphids offered	Aphids consumed* (Mean ± SD)	Mean Consumption (%)	95 % confidence limits	
				Lower	Upper
I	20	8.00 d (± 1.0)	40	5.51	10.48
II	40	23.33 c (± 4.0)	58.32	13.29	33.37
III	60	36.00 b (± 3.0)	60	28.54	43.45
IV	80	47.33 a (± 3.7)	59.16	37.92	56.73

*Mean of 3 replications/treatment; in parenthesis are standard deviation; means followed by different letters indicate that the means are significantly different ($p < 0.05$) by Duncan's test

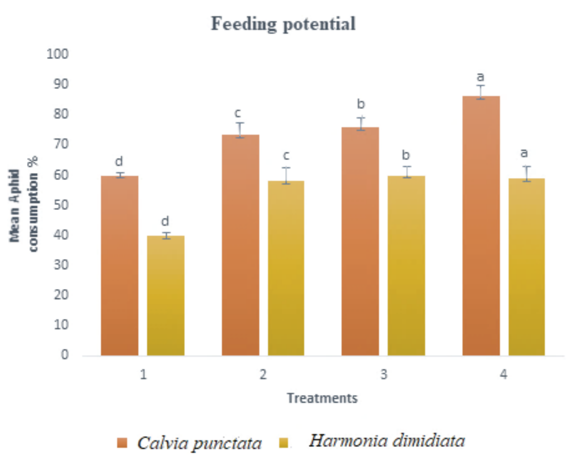

Figure 2: Graph depicting feeding potential of two coccinellid beetles on walnut aphid

3.2 FEEDING POTENTIAL OF SYRPHID FLY, *Metasyrphus latifasciatus*

Similarly, the feeding potential of different larval instars of syrphid fly, *Metasyrphus latifasciatus* was observed on adult walnut aphid, *Chromaphis juglandicola* under laboratory conditions. Feeding potential of syrphid fly on walnut aphid at different larval instars was recorded, although each larval instar tended to reduce the aphid population. The experimental data showed that as the number of instars upsurge, feeding potential also enhances. The mean consumption of first instar larvae was 23.25 %, second instar larvae was 48.25 % and the highest was in third instar larvae reaching 75.83 %. When the data was subjected to one-way ANOVA, it was depicted that the feeding potential of all three larval instars was significantly different with each other at $p < 0.05$ (Fig.3). Among all treatments, 3rd larval instar was found to be voracious feeder and devoured more number of aphids with a mean value of 30.3 (± 3.05). Thus, it can be concluded that the maximum predation rate was for the third larval instar followed by the second larval instar and first larval instar. The feeding rate of each larval instars of *M. latifasciatus* is presented in Table 4. Larval instars showed a different rate of feeding during the experimental conditions. The rate of aphid consumption increased with each developing stage. The present findings are in line with the observations carried by Baskaran et al. (2009) who recorded that the third larval instar consumed the highest number of aphids and had high feeding potential compared to first and second larval instars. The observed results, showed that feeding potential increased with an increase in larval stages from first to third which was in corroboration with the study carried by Ankersmit et al. (1986) who found that the age of larvae played a significant role in the consumption of aphids and high activity was observed at the end of larval development. Pascual-Villalobas et al. (2006) while working on aphids feeding on lettuce found that the syrphid population showed high predation on aphid colonies having 1.1 to 1.9 syrphid larvae/head. Hasken and Poehling, (1994) also observed that the aphid population was controlled by syrphids in all farming systems. Ghorpade (1981) also

Table 4: Feeding potential of syrphid fly larvae on walnut aphid under laboratory conditions

Larval Instar	No. of Aphids offered	Aphids consumed* (Mean ± SD)	Mean Consumption (%)	95% confidence limits	
				Lower	Upper
I	40	9.33 c (± 2.08)	23.25	4.16	14.50
II	40	19.6 b (± 3.51)	48.25	10.94	28.39
III	40	30.3a (± 3.05)	75.83	22.74	37.92

*Mean of 3 replications/treatment; figures in parenthesis are standard deviation; means followed by different letters indicate that the means are significantly different ($p < 0.05$) by Duncan's test

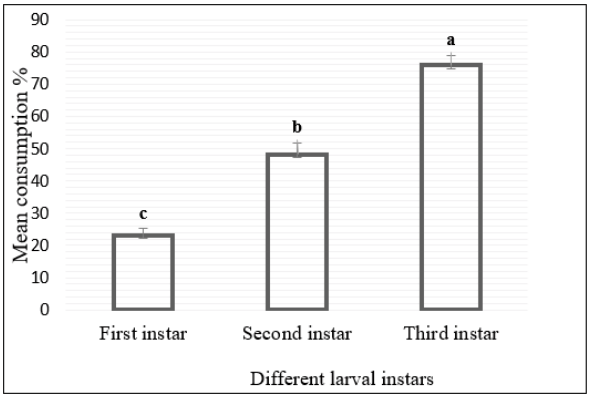

Figure 3: Graph depicting mean percentage of aphid consumption by different larval instars of syrphid fly

observed that syrphids act as one of the major bio-control agents of aphids.

4 CONCLUSIONS

On concluding the present results, it can be enumerated that these predators voraciously feed on aphids and can play a significant role in controlling the walnut aphid, *C. juglandicola* in field conditions. These predators proved their efficiency as an important bio control agent against walnut aphids. Due to their adeptness of having wide range of habitats and diversity, they can be successfully used for mass rearing and then its establishment in pest prevalent regions. Also the aphid density greatly affected the feeding potential. At higher density, predation was higher and at lower density the rate of predation was also observed low. This seems to be a good quality of the predator to feed more at higher prey density and less at lower prey density. This quality of predator not only allows the predators to survive at low prey density but also helps in reducing pest population at higher density.

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Study on physiological and morphological traits of purple shamrock (*Oxalis triangularis* A. St.-Hil.) as affected by humic acid under salinity stress

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Study on physiological and morphological traits of purple shamrock (*Oxalis triangularis* A. St.-Hil.) as affected by humic acid under salinity stress

Abstract: Salinity is one of the main environmental factors that limit plant growth and productivity. Humic acid (HA) can directly have positive effects on plant growth, and absorption of nitrogen, potassium, calcium, magnesium, and phosphorus by plants. This study aimed to concentrate on the impact of HA under salinity stress on *Oxalis triangularis* to use it as an ornamental plant in green spaces and landscaping. Treatments included three salinity stress levels (0, 40 and 80 mM NaCl) and four concentrations of HA (0, 200, 400, and 600 mg l^{-1}) on *O. triangularis* with four replicates. Applying HA under salinity stress at 40 and 80 mM increased stem length compared to the control, but this increase was lower at medium and high salinity levels. HA at 400 mg l^{-1} had the best effect on rhizome length under salinity stress at 40 and 80 mM. The reduced trend on proline content in HA at 0, 200, 400, and 600 mg l^{-1} under salinity stress at 40 mM was 19.65, 18.45, 16.92, and 13.57 %, respectively. By increasing the concentrations of HA, anthocyanin content was raised when compared to the control. Leaf sodium and potassium decreased by applying HA with or without salinity stress.

Key words: anthocyanin, leaf, proline, rhizome

Preučevanje vplivov huminske kisline na fiziološke in morfološke lastnosti škrlatne zajčje deteljice (*Oxalis triangularis* A.St.-Hil.) v razmerah slanostnega stresa

Izvleček: Slanost je eden izmed glavnih okoljskih dejavnikov, ki omejujejo rast in produktivnost rastlin. Huminska kislina (HA) ima lahko neposreden pozitiven učinek na rast rastlin in na absorbcijo dušika, kalija, kalcija, magnezija in fosforja. Namen te raziskave je bil preučiti vpliv huminske kisline v razmerah slanostnega stresa na škrlatno zajčjo deteljico (*Oxalis triangularis*) pri uporabah kot okrasna rastlina in za ozelenitve v krajini. Obravnavanja so obsegala tri ravni slanostnega stresa (0, 40 and 80 mM NaCl) in štiri koncentracije huminske kisline (0, 200, 400, and 600 mg l^{-1}) s štirimi ponovitvami. Uporaba huminske kisline je pri slanostnem stresu 40 and 80 mM povečala dolžino stebel v primerjavi s kontrolo a to povečanje je bilo manjše pri večjem slanostnem stresu. Uporaba huminske kisline v koncentraciji 400 mg l^{-1} je imela najboljši učinek na dolžino korenike pri slanostnem stresu 40 and 80 mM. Pri obravnavi s koncentracijami 0, 200, 400 in 600 mg l^{-1} huminske kisline pri slanostnem stresu 40 mM se je vsebnost prolina v rastlini zmanjševala od 19,65; 18,45; 16,92 do 13,57 %. S povečevanjem koncentracije huminske kisline se je povečevala vsebnost antocianina v primerjavi s kontrolo. Vsebnosti natrija in kalija v listih sta se pri uporabi huminske kisline zmanjševali pri ali odsotnosti slanostnega stresa.

Ključne besede: antocianin, korenika, list, prolin

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1 INTRODUCTION

Plants are faced with different types of environmental stresses, such as salinity, which can decrease plant growth and yield (Zamani et al., 2020). Salinity is an abiotic stress, which usually occurs in arid and semi-arid regions influencing plant growth and productivity (Porcel et al., 2012). Salt stress leads to disruption of physiological and biochemical activities such as ion balance, seed germination, osmotic regulation, photosynthesis, respiration, and disturbance in the absorption of potassium, phosphorus, calcium, and nitrogen ions leading to insufficient levels of those elements in the plant (Porcel et al., 2012; Ulczycka-Walorska et al., 2020).

Humic acid (HA) is extracted from different sources such as soil, humus, peat, oxidized lignite, and coal. HA can directly increase the growth of shoots and roots and the absorption of nitrogen, potassium, calcium, magnesium, and phosphorus by plants. HA as a naturally occurring polymeric organic compound is not dangerous for the plant and environment and contains elements that improve soil fertility, reduce soil nutrient deficiency and increase water and nutrient availability by forming chelates of various chemical elements (Motaghi and Sakinejad, 2014). HA increases plant growth through chelating different elements to overcome the lack of nutrients and has valuable effects on growth increase, production, and quality improvement of agricultural products due to having hormonal compounds (Ahmad et al., 2013; Motaghi and Sakinejad, 2014. HA can directly release different elements from minerals, absorb and provide them to the roots at the right time. Also, HA is a feed and growth stimulant for beneficial soil microorganisms, which help to release elements in the soil in various methods and can break aluminum-phosphorus or iron-phosphorus bonds in acidic soils and calcium-phosphorus bonds in alkaline soils and release phosphorus into the soil solution. It has been reported that due to the competitive adsorption between organic acids and phosphate, the adsorption of phosphorus on active surfaces is reduced, which is the reason for increasing the efficiency of phosphorus fertilizer in soils amended with organic materials. Sepehr and Zebardast (2013) reported that humic substances and phosphate fertilizers through competition for absorption sites and as a result reducing phosphorus stabilization in the soil, can reduce the consumption of phosphorus fertilizers as a result of reducing its environmental effects in the soil (Sepehr and Zebardast, 2013).

Oxalis triangularis A. St.-Hil. (syn. *O. regnellii*) is a rhizomateus perennial with a low, moderate growth habit commonly known as “False Shamrock”, is an edible perennial plant belonging to the Oxalidaceae family. The cultivated variety *O. triangularis* subsp. *papilionaceae* ‘Atropurpurea’ with purple-black, triangular-shaped leaves is highly popular as an ornamental pot plant (Šafrankova, 2014). It is native to Brazil and tropical Mexico and propagated by division of the rhizomes (Abd El-Razek et al., 2014). This ornamental plant in purple color can serve as a decoration for salads or foods. It is resistant to high temperature and draught due to tuberous rhizome below the ground (Kim et al., 2018). Thus, this plant can also use in landscape and green spaces. Regarding these advantages, there is no literature on the effect of salinity stress on this plant. Therefore, this study aimed to concentrate on the impact of salinity stress with or without HA on *O. triangularis* to use it as an ornamental plant in green spaces and landscaping.

2 MATERIAL AND METHODS

The research was done at the Research Greenhouse of the Horticultural Science Department of the Agricultural Faculty of Zanjan University. At first, the rhizomes of *O. triangularis* were cultivated in a mixture of sand and perlite and maintained in greenhouse for 4 months and then the salinity stress was applied for 6 weeks. During the cultivation process, the greenhouse temperature was adjusted to 18 ± 2 °C as the daytime temperature and 15 ± 2 °C as the nighttime temperature, and relative humidity was set to 60–70 % (Figure 1). Treatments included three salinity stress levels (0, 40, and 80 mM of NaCl) and four concentrations of HA (0, 200, 400, and 600 mg l^{-1}). HA (HA) was applied by foliar application at six times (once a week). Control treatment was the treatment without salinity stress and HA.

The morphological traits including stem, root, and rhizome lengths and the number of flowers leaves were measured at the end of the experiment. The biochemical traits like ion leakage, proline, anthocyanin, sodium (Na^+) and potassium (K^+) and color indices were investigated in this study Anthocyanin content was assayed according to (Wagner, 1979). Briefly, the mixed methanol and chloridric acid solution with the ratio of 99:1 was applied and the absorption was read by spectrophotometer (model SAFAS MONACO (RS 232)) at 550 nm. Proline content was measured using the methods described by Bates et al. (1973). The leaf extract was homogenized in 10 ml of 30 ml L^{-1} of sulfosalicylic acid, used for the measurement of proline by spectrophotometer at 520 nm. Ion leakage was measured according to Bideshki and Arvin, 2010. To determine potential EC (Electrolyte conductivity) 1, the samples were autoclaved for 20 min at 121 °C, and maintaining the samples at 21 °C overnight, EC2 was measured. The percentage of ion leakage was determined for each treatment according to the following formula:

Figure 1: The pictures of HA under salinity stress

Ion leakage (%) = *EC1* / *EC2* × 100

Sodium (Na^+) and potassium (K^+) were measured by flame photometer (model M410, Corning, Palo Alto, CA, USA) (Tekaya et al. 2014).

2.1 LEAF COLOR PARAMETERS

Leaf color parameters were assessed using Photoshop software, L*, a*, and b*color spaces, and Image J software with Color Space Converter to convert RGB color space to L*, a*, and b* spaces. The colors (L*, a*, and b*) were measured for each image according to the methods of Jajarimi and Taghizadeh, 2015, where L* is 100, it represents a perfect reflecting diffuser, in contrast to zero, which represents black. Positive a* values represent the red color, while the negative is green. Positive b* is yellow and the negative value is blue (Hosseini et al., 2021).

2.2 STATISTICAL ANALYSIS

This experiment was conducted on 3×4 factorial design based on a completely randomized design with four replications. Data were analyzed by SAS software version 9.1 and the means were compared by the Duncan test at 0.05 (p value < 0.05). Additionally, R software version 4.3.2 was used to draw clustering heatmap in multivariate analysis and correlation graph.

3 RESULTS

The maximum rhizome length (6 cm) was observed at 200, 400 and 600 mg l^{-1} of HA at 40 mM salinity. The minimum rhizome length (2.5 cm) was obtained in plants treated to 80 mM NaCl alone. Salinity stress decreased the rhizome length by 5 % and 41 % at 40 and 80 mM compared to the control (without HA and salinity stress), respectively (Figure 2A). Plants treated by HA at 400 mg l^{-1} alone and salinity stress at 80 mM alone had the highest (5 cm) and lowest (3 cm) rhizome length, respectively (Figure 2B). Salinity stresses at 40 and 80 mM reduced rhizome length by 22 % and 33 % when compared with the control. Although HA at 200 and 400 mg l^{-1} alone increased this trait by 5 % and 11 % as compared to control, respectively. The decreased trend in rhizome length at all concentrations of HA under salinity was found, but this decrease was lower than salinity stress alone. The highest (17.5 cm) and lowest (11.5 cm) stem lengths were observed in plants treated with HA at 400 mg l^{-1} and salinity stress at 80 mM alone, respectively (Figure 2C). Increased salinity decreased stem length. This decrease at 40 and 80 mM was 11.3 and 13.2 % as compared to the control, respectively. Application of HA alone led to increasing of stem length by 24, 32 and 13 % at 200, 400, and 600 mg l^{-1} in comparison with control. Application of HA under salinity stress also increased stem length compared to control, respectively, but this increase was lower at high levels of salinity. HA at 200 mg l^{-1} had the highest number of leaves (16.5) and followed by 400 mg l^{-1} (Figure 2D). HA (16.25). By applying HA at 200 and 400 mg l^{-1}, the number of leaves increased by 11 % and 19 % under salinity stress at 40 mM and by 1.9 % and 1.9 % at 80 mM salinity stress. Although, HA alone at 600 mg l^{-1} led to an increase (7.6 %) in number of leaves, but it had no effect at 40 mM of NaCl and even it had a negative impact (23 %) at 80 mM NaCl on this trait (Figure 2).

The lowest and the highest ion leakage was related to HA at 600 mg l^{-1} (10.05 %) and 80 mM salinity stress (56.55 %), respectively (Figure 2E). By raising salinity stress ion leakage increased by 225 % and 341 % at 40 mM and 80 mM levels as compared to control, respectively. Ion leakage increased by HA application under salinity stress, but this increase was lower at all concentrations of HA than salinity stress alone. Ion leakage decreased by 17, 19, and 21 % compared to the control at concentrations 200, 400, and 600 of HA, respectively.

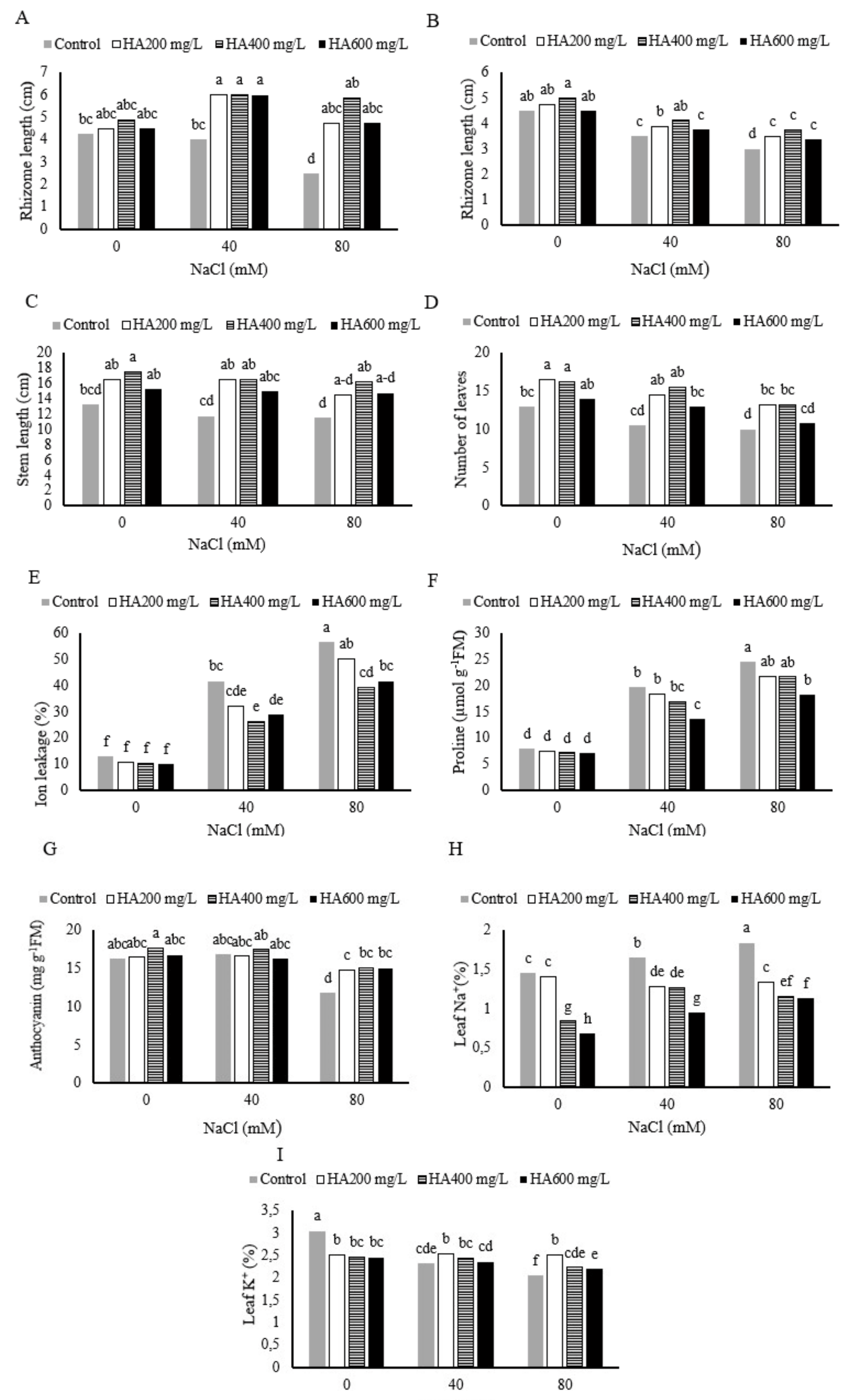

Figure 2: The effect of HA on *Oxalis triangularis* growth parameters, leaf sodium (Na^+) and potassium (K^+), proline and anthocyanin and ion leakage under salinity stress. HA : HA, the columns with the same letter are not significantly different at 0.05 level

The lowest and the highest proline content were observed in HA at 600 mg l^{-1} (7 μmol g^{-1}FM) and severe salinity stress (24.5 μmol g^{-1}FM), respectively (Figure 2F). By applying HA and salinity stress, proline content decreased and increased in comparison with the control, respectively. The reduced trend on proline content was 19.65, 18.45, 16.92, and 13.57 % under salinity stress at 40 mM plus 0, 200, 400, and 600 of HA in comparison with control. The same trend was found under salinity stress at 80 mM at all of the mentioned HA treatments by 24.5, 21.75, 21.65, and 18.25 % compared to the control, respectively.

Considering anthocyanin content, the highest and the lowest ones were demonstrated in HA at 400 mg l^{-1} (17.68 mg g^{-1}FM) and NaCl at 80 mM alone (11.77 mg g^{-1}FM). Salinity stress increased and decreased anthocyanin by 3.55 % and 27 % at 40 mM and 80 mM levels in comparison with control, respectively (Figure 2G). By increasing the concentrations of HA, anthocyanin content raised by 0.9, 8.33, and 2.4 % at 200, 400, and 600 mg l^{-1} when it compared to the control, respectively. Considering HA, anthocyanin content increased by 0.9 % and 1.71 % at 200 mg l^{-1} and 8.3 % and 6.98 % at 400 mg l^{-1} under 0 and 40 mM of NaCl when it compared to control, respectively. On the other hand, this antioxidant compound reduced by 9 %, 7.35 %, and 7.66 % at 200, 400, and 600 mg l^{-1} under 80 mM salinity in comparison with the control, respectively.

Leaf Na^+ showed the highest content (1.84 %) in plants treated with 80 mM salinity. HA at 600 mg l^- alone had the lowest leaf Na^+ (Figure 2H). Salinity stress increased leaf Na^+ by 13 and 26 % as compared to control, respectively. Leaf Na^+ decreased by applying HA with or without salinity stress. By applying HA at 200, 400, and 600 mg l^{-1}, leaf Na^+ decreased by 11, 12, and 34 % under 40 mM NaCl in comparison with control, respectively. This decrease was higher in 80 mM NaCl plus HA at all concentrations.

Plants under control and salinity stress treatments at 80 mM had the highest (3.05 %) and the lowest (2.05 %) leaf K^+, respectively (Figure 2I). Salinity stress decreased leaf K^+ by 23 % and 32 % as compared to control, respectively. Although, leaf K^+ decreased by applying HA with or without salinity stress in comparison with control, but this content was higher than salinity stress alone. By applying HA at 200, 400, and 600 mg l^-, leaf K^+ decreased by 17 %, 20 %, and 22 % under 40 mM NaCl in comparison with control, respectively. This decrease was lower in 80 mM NaCl plus HA at 400 mg l^{-1} and 600 mg l^{-1}.

3.1 MULTIVARIATE ANALYSIS

Cluster I, which included salinity levels at 40 and 80 mM with or without HA at 200, 400, and 600 mg l^{-1}. Within this cluster salinity at 40 and 80 mM alone showed very low Na^+ and K^+ (Figure 3).

Cluster II was controlled through HA alone, which had high anthocyanin.

To determine the dispersion of treatments, principle components analysis (PCA) was used (Figure 4A). According to the first two components, the variances explained by the first two components were 40.5 and 21.30 %, respectively. In addition to the positive correlations with stem and rhizome length, PC1 also showed the strongest positive correlations with proline, leaf Na^+, and ion leakage. Anthocyanin and leaf K^+ were strongly and negatively correlated with PC2 (Figure aA). Regarding the principle cluster analysis on treatment showed that control had a negative correlation with PC1 and PC2. As shown in Figure 4B, stem length had a positive correlation with rhizome length (r = 0.38), rhizome length (r = 0.23), and anthocyanin (r = 0.29), while the negative correlation with K^+ (r = 0.37). There was a strong negative correlation between ion leakage and anthocyanin (r = 0.7).

There were differences in leaf color among treatments (Figure 5). Control and HA at 200, 400, and 600 mM belong to a group with the same values for a and b color indices. The highest b value was observed in salinity at 40 mM alone. It was also found that salinity at 40 mM with HA in all used concentrations was placed in the same group.

4 DISCUSSION

Salinity is one of the main environmental factors that limits plant growth (Enteshari and Sharifian, 2012)**.** Saline conditions decrease height, shoot length, leaf area, fresh mass, and dry mass in many plants (Kang et al., 2021). When the concentration of NaCl rises, growth parameters like plant height biomass and uptake of nutrients decreased (Khaled and Fawy, 2011). The length of rhizome and roots are the most important indicators to measure the effect of salinity, because the roots are in direct contact with the soil and provide water transfer from the soil to the shoot. By reducing absorption and reducing the development of roots, salinity reduces the accumulation of dry matter in the roots and reduces

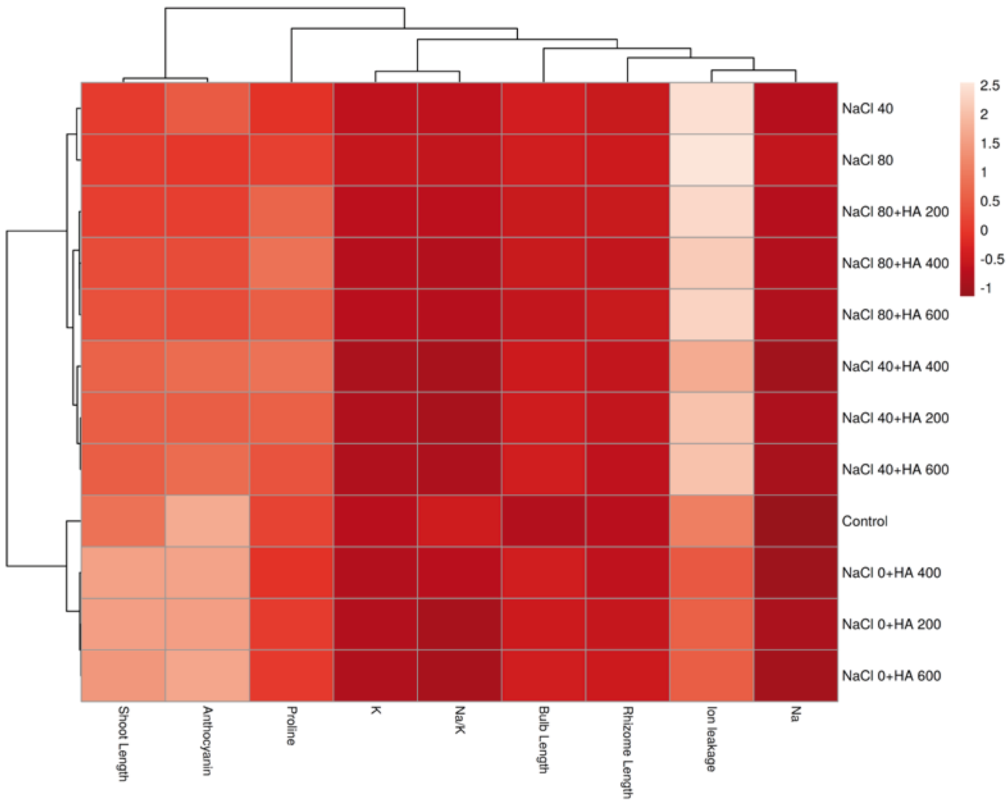

Figure 3: Cluster analysis of *Oxalis triangularis* based on physical and chemical properties of leaf under salinity stress by application of HA (gradient from low (dark red), white (red) to high (pink)). Abbreviations: sodium (Na^+) and potassium (K^+), Na, K = Na^+/K^+

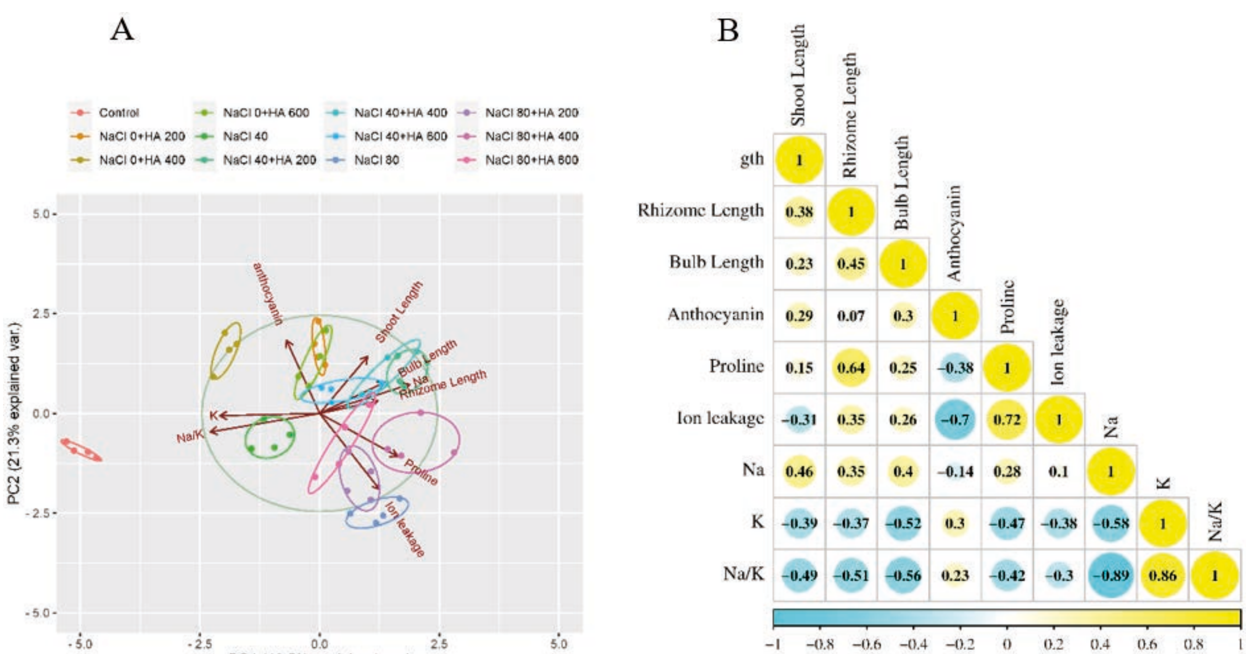

Figure 4: The dispersion of *Oxalis triangularis* based on physical and chemical properties of leaf under salinity stress by application of HA according to the first and the second principal components analysis (PC1/PC2)

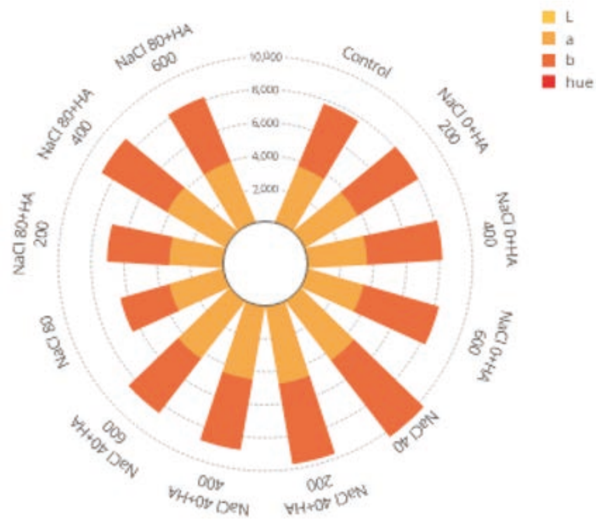

Figure 5: Polar bar chart of color parameters of *Oxalis triangularis* based on physical and chemical properties of leaf under salinity stress by application of HA

root performance. Under the conditions of stress, the air stomata are closed and the rate of photosynthesis is reduced, and as a result, the transfer of photosynthetic materials to the roots is reduced, and finally, salinity can reduce the growth of the roots, and thus the capacity to absorb and transfer water and nutrients from the soil to the aerial parts (Khorasaninejad et al., 2010). Improving the absorption of nutrients such as phosphorus through the roots, humic substances lead to an increase in the longitudinal growth of roots due to the auxin-like effect that affects root growth. The results of the present study are consistent with the results reported in *Poa pratensis* L. and *Capsicum annum* L. that HA effectively increases root length in these plants (Ervin et al., 2008; Çimrin et al., 2010)

Reducing the growth of shoot length is the most obvious effect of salinity on plants. Prevention of plant growth under salinity stress can be due to reduced cell division, ion imbalance, reduced water absorption, impaired absorption of elements, the effect of toxic ions, especially sodium, impaired absorption, recovery, and metabolism of nitrogen and protein, the closing of stomata and reduced by the efficiency of photosynthesis (Oueslati et al., 2010). And as a result, by reducing the height of the shoot, it is possible to transfer a smaller amount of carbohydrates, especially in stressful conditions.

Plant growth of *Lolium perenne* L increased in response to treatments with low and medium concentrations of HA (Nikbakht et al., 2014). HAs stimulate the activity and synthesis of H^+-ATPase enzymes in the plasma membrane of plant cells, which is similar to the auxin effect (Baldotto et al., 2013). In addition, the plasma membrane (PM) proton H^+-ATPase activity in root cells is induced by HAs (Jindo et al., 2012) and promote rooting of ornamental plants (Baldotto et al., 2013). In general, the effect of HAs on plant physiology is recognized with regard to enhancement of root growth and nutrient uptake (Jindo et al., 2012; Khattab et al., 2014 and Kazemi et al., 2019). HA stimulated root growth increased proliferation of root hairs. Enhancing root initiation by HAs, which promotes a guarantee for the growth of the above ground parts of the plant (Fan et al., 2014). Our results on the growth parameters by applying HA under salinity stress were in agreement with Saeedi Pooya et al. (2018) on turfgrass and Jozay et al. (2021) on *Syngonium*.

Also, the decrease in the growth of shoot length under salt stress conditions is due to excessive absorption of sodium chloride in the tissues and around the root, which leads to an increase in osmotic potential and a decrease in water absorption by the plant and a decrease in tissue water, which leads to the lack of cell elongation. Other reasons for growth reduction due to salinity stress include the reduction of water absorption and the inhibition of photosynthetic products, carbohydrate synthesis and the toxicity caused by the high amount of sodium in the cell (Bayat et al., 2012; Taiz et al., 2015). There have been several reports on the reduction of plant shoot length under salt stress. One of the mechanisms that humic substances lead to an increase in longitudinal growth is related to its gibberellin-like compounds. In a report, the effect of HA on stem growth was due to its effect on root H-ATPase activity and the distribution of root nitrate in the stem, which in turn led to changes in the specific distribution of cytokinins, polyamines and ATP, therefore, it had an effect on stem growth (Rubio et al., 2009). On the other hand, HA forms stable and dissolved complexes that increase the absorption of elements, soil fertility, and production in plants (Sajadian and Hokmabadi, 2015). The main effect of HAs on plant physiology includes enhanced root growth (Jindo et al., 2012). Enhanced growth was due to better-developed root systems by applying humic substances, may affect membrane permeability. It also interacts with the phospholipid structures of the cell membranes and react as carriers of nutrients through them. As a result, the plants take more mineral elements (Khaled and Fawy, 2011).

The decrease in the number of leaves as a result of salinity treatment in our experiments is due to the inhibitory effect of salinity stress on the absorption and transfer of photosynthetic materials, followed by a reduction in the level of photosynthesis and photosynthetic pigments such as chlorophyll a and b, net absorption of CO_2 and stomatal conduction and stomatal closure. Also, the decrease in the number of leaves due to salinity stress can be caused by other factors such as osmotic stress, ionic toxicity, nutrient imbalance, and oxidative stress (Anser et al., 2012). In a research, Kumar et al. (2009) stated that salinity by increasing the osmotic pressure of the soil solution leads to a decrease in water absorption and as a result to a decrease in cell division, elongation and differentiation and as a result to a decrease in the number of leaves. The decrease in chemical potential in saline environments initially causes an imbalance of water potential between the apoplast and symplast, which itself causes a decrease in the cell pressure potential, which ultimately leads to a decrease in vegetative growth and the number of leaves. Therefore, it seems that in the present study, salinity caused a decrease in the number of leaves in *O. triangularis* by causing ionic toxicity, osmotic stress, disrupting the balance of nutrients, reducing photosynthesis, and reducing water absorption (Said-Al et al., 2011). Therefore, the results of the present research are consistent with the results of the research conducted on *Spinacea oleracea* L. (Sogoni et al., 2021) and *Rosa canina* L. (Bilal et al., 2020). The positive effects of HA application may be due to the presence of hormone-like compounds from the group of auxins, cytokinins and gibberellins in the plant (Abel-Mawgoand et al., 2007). HA is a polymer that can increase the metabolism of micro-organisms in the soil by increasing the absorption of nutrients through chelating and regenerating properties and maintaining membrane permeability (indirect effect) physical soil and increasing the number of leaves (Ahmad et al.. 2013). The results of this research are consistent with the results of the effect of HA on *Solanum melongena* L., *Capsicum annum* L. and gladiolus, that spraying HA on eggplant and pepper plants (Ahmad et al., 2013) increases the number of leaves in these plants.

Membrane stability is among the physiological characteristics that are affected by environmental stresses. At high concentrations of salinity, the permeability of the cell membrane increases and as result the stability of the membrane decreases and finally leads to leakage of electrolytes. The amount of ion leakage of the membrane is one of the factors that can show the intensity of damage caused by stress in the plant. Membrane leakage can be used as an indicator to measure the level of resistance to salt stress in different plants and even different organs of the same plant. In response to oxidative damage caused by salt stress, plants use different enzymatic and non-enzymatic antioxidant systems. Antioxidant enzymes such as catalase and peroxidase remove free radicals. HA increases the activity of antioxidant enzymes under salinity stress conditions, increasing the level of antioxidant enzymes, causes plants to better tolerate stress conditions and reduce ion leakage (Garcia et al., 2016).

Proline is a storage amino acid in the cytoplasm and plays an effective role in protecting the structure of macromolecules inside the cell during stress conditions. In other words, proline is an indicator in determining the sensitivity to stress in plants. When the plant is exposed to stress, the breakdown of proteins and, as a result, the increase of amino acids and amides is accelerated, one of these amino acids is proline. It is osmotic and osmotic protective and proline is one of these compounds. Proline increase in plants during stress is a kind of defense mechanism (Lotfi et al., 2015). Proline increases plant tolerance to stress through several mechanisms such as osmotic regulation and preventing enzyme degradation. Proline prevents oxidation inside plant cells under stress conditions. The use of organic compounds such as HA is one of the methods that may improve the efficiency of water and food consumption and reduce the effect of salinity stress in plants. Similar to present results, HA can be increase the activity of antioxidants and decrease proline in rapeseed (Lotfi et al., 2015).

Salinity stress increased ion leakage, proline content which was in agreement with Tiwari et al. 2010; Chen et al. (2013) and García-Caparrós et al. (2016) due to enhanced membrane damage, cell membrane permeability and oxidative damage. They also stated that ion leakage from the plasma membrane and proline accumulation has been reported as one of the most important selection criteria for the identification of salt-tolerant plants. Total anthocyanin in pomegranate trees was significantly improved by increasing the amount of humic (Khattab and Shaban, 2014), which was in agreement with our results. The activation of H^+-ATPase improves the uptake of plant nutrients by enhancing electrochemical proton gradient that drives ion transport across cell membranes via secondary transport systems (Jindo et al.; 2012). Potassium accumulation decreased at higher levels of HA (500 and 1000 mg l^{-1}), but it increased at 100 mg l^{-1}. This is in accordance with our results. The possible reason for increasing the absorption of nutrients by plants can be attributed to the increase in root growth. (Nikbakht et al., 2008).

Anthocyanins are one of the phenolic compounds that play an important role in flower color. One of the biochemical changes that occur in environmental stress, including salinity stress, is the production of ROS. Plants must use antioxidant systems such as anthocyanin to resist the oxidative stress. Several reports show that salinity stress reduces physiological indicators such as anthocyanin. The reduction of anthocyanin in salinity stress is due to receiving more photosynthetically active rays, ultraviolet rays cause the reduction of anthocyanin because most of the anthocyanin substances are located in the surface layers of the mesophyll and the epidermis of the leaf. The use of HA increases the amount of anthocyanin in saffron (Ahmadi et al., 2017). It has been reported that HA can cause the synthesis of phenolic compounds such as anthocyanins by stimulating alpha-amylase. The results of this research are consistent with the results obtained in lilium and *Borago officinalis* L., that the application of different concentrations of HA has effectively increased the amount of anthocyanin in these plants (Amiri et al., 2017; Parandian and Samavat, 2012)

Sodium is not present as a necessary element of the plant and its accumulation in the plant in saline conditions leads to the reduction of calcium and potassium ions. Although sodium can help to increase the turgor pressure, it cannot replace potassium ion in special activities such as the activation of enzymes and protein synthesis. The reduction of potassium absorption as a result of the increase of sodium is a competitive process and has nothing to do with the predominant type of salt in the soil (Nikbakht et al., 2014). Potassium is the most prominent dissolved element to keep down the osmotic potential of root cells and is a prerequisite for cell turgescence, and it is necessary to maintain a sufficient level of potassium and plant survival in saline environments. Under saline and alkaline conditions, the high concentration of sodium not only causes disturbances in the absorption of potassium by the root, but also affects the membrane of the root cells and its selective properties (Nikbakht et al., 2014). Due to its strong chelating property, HA keeps sodium ions on itself and prevents the absorption of this ion by the plant. Humic compounds indirectly by providing high-consumption and low-consumption mineral elements for the root, improving the soil structure, increasing the permeability of the substrate to water and air, increasing the soil microbial population and beneficial microorganisms, increasing the cation exchange capacity and the ability to buffer the pH of the substrate or solution. Food, providing some materials for plant roots such as nucleic acids, acetamides and providing HA as carriers of low consumption elements and other growth factors, increase soil fertility.

Salinity not only causes osmotic stress but also leads to disturbances in the absorption of other elements and transfer to the plant's aerial organs and nutritional disturbances, followed by a decrease in plant growth (Said-Al et al., 2011). The activation of PM H^+-ATPase via HA application improves the uptake of plant nutrients by enhancing electrochemical proton gradient that drives ion transport across cell membranes via secondary transport systems (Jindo et al., 2012). Salinity stress decreased leaf K^+ in our experiment which were in accordance with Jaleel et al.(2008) and Khaled and Fawy (2011). Ali et al. (2012) stated that high sodium levels disturb potassium (K^+) nutrition and the reduction of K percentage could be attributed to the competition that exists between Na^+ and K^+, especially at high external NaCl concentrations. Foliar application in 0.1 % HA treatment increased N, P, K, Ca, Mg, Na, Fe, Zn, and Mn amounts under salinity stress at 60 mM when compared with the control and 0.2 % HA treatment (Khaled et al., 2013).

5 CONCLUSION

Plant growth parameters and leaf K^+ decreased under salinity stress, but the content of leaf proline, anthocyanin, and Na^+ increased. The application of HA helped to decrease ion leakage and proline content and improved leaf anthocyanin and growth parameters. In general, the results of this research showed that salinity stress has a negative effect on the growth and performance of *O. triangularis*. High levels of salinity are more affected the growth and performance of the plant. The application of HA was able to partially reduce the harmful effects of salinity on the *O. triangularis* due to its adjustment effect on the physiological parameters of the plant, such as the amount of proline, the ratio of sodium to potassium, and ion leakage. The results of this research showed that the application of HA, especially at a concentration of 600 mg l^{-1}, can moderate the negative effects of salinity on the morphological and physiological characteristics of *O. triangularis* and increase the resistance to salinity stress.

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Influence of seeding density on seed and oil yield, and fatty acid composition of white mustard (*Sinapis alba* L.)

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Influence of seeding density on seed and oil yield, and fatty acid composition of white mustard (*Sinapis alba* L.)

Abstract: The aim of this study was to determine the effects of seeding density on the seed yield of white mustard, the oil yield and fatty acid composition under the agroecological conditions of the northwestern Republic of Croatia. The field trials were conducted at the experimental station of the Faculty of Agriculture, University of Zagreb (45° 48' N, 16° 05' E) during two growing seasons. The trial included four seeding densities of white mustard: 50, 70, 90 and 110 germinable seeds m^{-2}. The trial was set up using a randomized block design with five replications. The highest seed yield was obtained with a seeding density of 110 germinable seeds m^{-2}, with no significant differences between seeding rates of 70 and 90 germinable seeds m^{-2}. The average oil content during researched years varied from 23.97 % in the seeding density of 50 germinable seeds m^{-2} to 24.37 % in the seeding density of 90 germinable seeds m^{-2}. The higher oil yield was achieved in 2014 due to the higher oil content in the seed that year. Regarding fatty acid composition, erucic acid was dominant along with oleic acid, linoleic acid, and linolenic acid.

Key words: alternative oilseeds, mustard, oil content, erucic acid, biodiesel, lubricants

Vpliv gostote setve na pridelek semena in olja ter sestavo maščobnih kislin pri beli gorjušici (*Sinapis alba* L.)

Izvleček: Namen raziskave je bil določiti vpliv gostote setve na pridelk olja in semena ter sestavo maščobnih kislin bele gorjušice v agroekoloških razmerah severozahodne Hrvaške. Poljski poskus je potekal na poskusni postaji Agronomske fakultete Univerze v Zagrebu (45° 48' N, 16° 05' E) v dveh rastnih sezonah. Poskus je obsegal štiri gostote semen bele gorjušice in sicer 50, 70, 90 in 110 kaljivih semen m^{-2}. Poskus je bilo zasnovan kot naključni bločni poskus s petimi ponovitvami. Največji pridelek semen je bil dosežen z gostoto setve 110 kaljivih semen m^{-2}, vendar brez značilnih razlik z gostotama setve 70 in 90 kaljivih semen m^{-2}. Poprečna vsebnost olja je v letih poskusa variirala od 23,97 % pri gostoti setve 50 kaljivih semen m^{-2} do 24,37 % pri gostoti setve 90 kaljivih semen m^{-2}. Največja vsebnost olja je bila dosežena v letu 2014 zaradi nasplošno največje vsebnosti olja v tem letu. Glede sestave maščobnih kislin je bila dominantna erucična kislina, ki so ji sledile oleinska, linoleična in linolenska kislina.

Ključne besede: alternativne oljarice, gorjušica, vsebnost olja, erucična kislina, biodiezel, lubricants

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1 INTRODUCTION

White mustard (*Sinapis alba* L.) is an annual plant from the Brassicae family that originates from the Mediterranean region (Sawicka & Kotiuk, 2007). It is an oilseed crop with great potential as a spring alternative crop in Europe as it has greater tolerance to stressful environments than rapeseed (Gunasekera et al., 2006a).

Besides seed production for processing to oil, white mustard has significant agronomy importance due to its ability to improve soil structure, its fertilizing and phytosanitary effects (Toboła, 2010) and its ability of heavy metal phytoextraction from the soil (Evangelou et al., 2007). Therefore, it is often used as green manure and intercrop.

White mustard seeds are also used as a condiment, hot dog mustard, salad dressing, natural food preservative, and food additive in the food processing industry (Rahman et al., 2018). In addition, the seeds are used as drug components in phytotherapy due to their analgesic, antiproliferative, antiviral and antimicrobial properties (Peng et al., 2013; Boscaro et al., 2018).

According to Ciubota-Rosie et al. (2013), mustard seeds have a high energy content with an oil content of 28–45 % and a relatively high protein content. Due to the high concentrations of erucic acid and the high content of glucosinolates in the fat-free seed residues, mainly sinalbin, traditional white mustard cultivars are not widely used in the production of foodstuffs (Jankowski et al., 2015). The strongest objection to the use of high erucic oil has been linked with its cardiotoxic potential (Galanty et al., 2023). High glucosinolates in livestock feed have adverse effects including reduced feed intake and growth, gastrointestinal irritation, goiter, anaemia, and hepatic and renal lesions (Bischoff, 2021).

Erucic acid is an oleochemical feedstock which is converted into erucamide and behenyl alcohol. Erucamide is used as a processing aid in the manufacture of polyethylene film, while behenyl alcohol is an emulsifier, viscosity regulator, or emollient in many cosmetic products (Hebard, 2016). Therefore, the oil extracted from the white mustard seed is used for industrial purposes, usually as a lubricant (Falasca & Ulberich, 2011). The vegetable oils are preferred over mineral oil as lubricating base oil due to their high biodegradability, renewability and low toxicity (Sajeeb & Krishnan, 2019) In addition, high erucic acid oils have a high degree of lubricity (Aukema & Campbell, 2011).

Recent studies also indicate the possibility of using white mustard oil as a feedstock for biodiesel production (Ciubota-Rosie et al., 2013; Sultana et al., 2014; Ambrosewicz-Walacik et al., 2015). An important advantage of using non-edible white mustard oil as an alternative feedstock for biodiesel production is that it does not compete with its use as food.

Plant density is one of the most important agronomic measures that determine grain yield, as it effects plant growth and development. It is well known that evenly distributed plants use land, light and other resources evenly and efficiently. A higher plant population per unit area, beyond an optimal limit, leads to competition among plants for natural resources, which results in weaker plants and can cause severe lodging (Kumar et al., 2004). According to Shekhawat et al. (2012), the optimal plant population density per unit area varies with the environment, genotype, sowing date, and growing season.

Important agronomic properties for oilseed crops, besides seed yield, are also oil yield and oil quality. A great influence on oil content and fatty acid profile has genetic base and environment and their interaction (Gunasekera et al., 2006b; Zhang et al., 2015), as an agronomic management (Shekhawat et al., 2012).

Therefore, the objective of this study was to determine the effects of seeding density on the yield potential of white mustard and to determine oil yield and quality under agroecological conditions in northwestern Croatia.

2 MATERIALS AND METHODS

The field trials were conducted at the experimental station of the Faculty of Agriculture, University of Zagreb (45° 48′ N, 16° 05′ E) during two growing seasons (2014 and 2017). The trial involved four seeding densities of white mustard: 50, 70, 90, and 110 germinable seeds m^{-2} i. e. 3 kg ha^{-1}, 4 kg ha^{-1}, 5 kg ha^{-1} and 6 kg ha^{-1} were used. A local landrace grown in the Brod-Posavina Country, Croatia was used for sowing. The trial was set up using a randomized block design with five replications. The plot size was 6.6 m^2 (5.5 m x 6 rows x 20 cm).

Sowing was performed on April 14, 2014, and March 23, 2017 by the "Wintersteiger" plot seeder. The previous crop in 2014 was spelt, and in 2017 year was a mixture of wheat and peas. Fertilization was carried out with basic tillage by application of 400 kg ha^{-1} NPK 7:20:30 fertilizer (28 kg ha^{-1} N, 80 kg ha^{-1} P_2O_5 and 120 kg ha^{-1} K_2O). Topdressing was applied at the stage of six leaves with 100 kg ha^{-1} CAN (27 % calcium ammonium nitrate).

The weed control was carried out with 1.3 l ha^{-1} Butisan (active substance metazaklor 500 g l^{-1}) and with 0.8 l ha^{-1} Agil 100 EC (active substance propaqizafop 100 g l^{-1}).

Control of flea beetles (*Phyllotreta* spp.) was performed three times in vegetation with insecticides Chromorel D (active substance chlorpyrifos 500 g l^{-1} + cypermethrin 50 g l^{-1}) in the doze of 0,5 1 ha $^{-1}$, Karate Zeon (active substance lambda-cyhalothrin 50 g l^{-1}) in the dose of 0,15 1 ha $^{-1}$ and Rotor 1.25 EC (active substance deltamethrin 25 g l^{-1}) in the dose of 1 ha $^{-1}$.

The harvests were carried out with harvester "Wintersteiger" at the stage of horticultural maturity (July 24, 2014, and July 10, 2017) when seed moisture was below 12 %. The seed yield was calculated based on 9 % moisture and 2 % impurity content.

The oil content was determined in the Laboratory for Oil and Fat Technology at the Faculty of Food Technology and Biotechnology, University of Zagreb, on an average sample of five repetitions according to standard ISO 659:2009, the method according to Soxhlet (International Organization for Standards , 2009). Oil content was reported on a dry matter basis.

Fatty acids were determined by their methyl esters according to standard ISO 5509:2000 (International Organization for Standards , 2000) using a gas chromatograph (ATI Unicam 610, Cambridge, England) with capillary column TR-FAME (Thermo Scientific, Waltham, MA, USA) (30 m x 0.22 mm thickness the film of 0.25 µm; stationary phase: 70 % cyanopropyl-polisilfenilen siloxane) and FID detector (flow rate of 0.7 ml min^{-1}, helium carrier gas, injector temperature 250 °C, split: 1:75, detector temperature: 280 °C, the amount of sample injected: 1.0 µl) with the programmed column temperature 120 °C to 160 °C-4°C min^{-1}, 160 °C to 190 °C–10 °C min^{-1} at 190 °C was maintained for 10 min. Identifying individual fatty acids was carried out by comparing the retention time of methyl esters of certain fatty acids with the retention times of a standard mixture of methyl esters of fatty acids (F.A.M.E.) of known composition. Computer-selected method of normalization of peak areas was used to calculate the quantitative composition of fatty acids. Obtained data for fatty acid composition were analysed by descriptive statistics.

The obtained data for seed yield and oil yield were statistically analyzed through variance analysis using DSAASTAT (Onofri, 2007). The statistically significant differences were tested by the LSD test at 5 % probability.

2.1 SOIL CHARACTERISTICS

The trials were conducted on an anthropogenic eutric cambisol. The upper soil layer was neutral (pH in 1M KCl = 7.09), poorly supplied with humus (2.34 %), and well supplied with nitrogen (0.12 %) The soil was richly supplied with the plant-available phosphorous (AL-P_2O_5 = 37.03 mg 100 g soil^{-1}) and potassium (AL-K_2O = 16.20 mg 100 g soil $^{-1}$).

2.2 WEATHER CONDITIONS

A mean decade and monthly air temperatures and precipitation from March to July (during the growing season of mustard) in the years of research and a long-term average (1981–2010) for weather station Zagreb–Maksimir are given in Table 1.

In 2014 year, the total amount of precipitation during the growing season was higher by 54.2 % and the mean monthly temperature was higher by 1.2 °C than the long-term average.

The deficiency of precipitation in this growing season was noted only in March when precipitation was lower by 61 % than the long-term average. In the same month, the air temperature was higher by 3.7 °C than the long-term average. The higher air temperature was prolonged in April but with a sufficient amount of precipitation, which positively influenced germination and emergence. During the sensitive stages of flowering and seed development i. e. in May, July and July precipitations were higher by 112 %, 52 % and 121 % than the long- term average, respectively. On 26 May 2014 when the crop was at the stage of late flowering, hail occurred. During these months air temperatures were similar to the long-term average.

In 2017 year, the total amount of precipitation during the growing season was lower by 24.5 % and the air temperature was higher by 2.2 °C than the long-term average. Besides June, in all other months during the vegetation period was noted deficiency of precipitation. Where March, April, May and July were noted 63.4 %, 25.5 %, 48.7 % and 18.8 % lower precipitation than the long-term average, respectively. The mean monthly air temperatures during all months were higher than the long-term average, especially in June (by 2.9 °C) and July (by 2.5 °C) i.e., during stages of seed development and oil synthesis.

3 RESULTS AND DISCUSSION

3.1 SEED AND OIL YIELD

The average seed yields of white mustard obtained in the study are in agreement with the research of Harasimowicz-Hermann et al. (2019) and Serafin-Andrzejewska et al. (2020) under agroecological conditions in Poland. Higher yields, up to 2.0 t ha^{-1}, were reported under favourable environmental and cultivation conditions in Pakistan by Hassan & Arif (2012). No significant differences were found in seed yield between years. No signifi-

Table 1: Decade and monthly air temperatures and precipitation in 2014, and 2017, and long-term average

Months/decades		Air temperature, °C		Long-term average	Precipitation, mm		Long-term average
		2014	2017	1981-2010	2014	2017	1981-2010
March	I	8.3	8.6		6.3	19.6	
	II	11.4	8.9		0.1	0.1	
	III	11.7	12.4		14.6	0.1	
	I - III	10.5	10.0	6.8	21.0	19.8	54.1
April	I	14.1	14.5		13.8	0.7	
	II	10.8	10.5		19.2	21.3	
	III	15.0	12.1		37.4	22.3	
	I - III	13.3	12.4	11.4	70.4	44.3	59.5
May	I	14.3	14.0		33.3	22.4	
	II	13.5	19.0		55.0	12.0	
	III	19.2	19.9		56.7	0.8	
	I - III	15.7	17.7	16.5	145.0	35.2	68.6
June	I	20.2	20.6		5.0	38.6	
	II	20.3	22.3		42.1	2.2	
	III	20.2	24.7		99.9	67.0	
	I - III	20.2	22.5	19.6	147.0	107.8	97.4
July	I	20.7	24.6		14.9	18.8	
	II	23.1	23.7		44.0	0.4	
	III	21.6	23.8		98.9	38.8	
	I - III	21.8	24.0	21.5	157.8	58.0	71.4
Average/Total		16.3	17.3	15.1	541.2	265.1	351.0

cant interactions between years and seeding density were found for seed yield either. (Table 2).

The analysis of variance showed a significant influence of seeding density on seed yield. The highest seed yield was obtained with a seeding density of 110 germinable seeds m^{-2}, with no significant differences between seeding density of 70 and 90 germinable seeds m^{-2} (Table 3). Similarly, Sáez-Bastante et al. (2016) reported that under Mediterranean rainfed conditions in a semi-arid area in southern Spain, higher plant density of white mustard resulted in higher seed yield, but not proportionally. They found that seed yield was slightly lower at a density of 40 plants m^{-2} than when plant density was doubled (80 plants m^{-2}). Keivanrad & Zandi (2012) reported that a plant density of Indian mustard in Iran greater than 80 plants m^{-2} did not result in a significant increase in seed yield.

The oil content of white mustard varied across years and sowing rates from 23.97 % (50 germinable seeds m^{-2}) to 24.37 % (90 germinable seeds m^{-2}). The average oil contents are comparable to the results obtained by Stamenković et al. (2018) in Serbia and Sáez-Bastante et al. (2016) in Spain. While Ciubota - Rosie et al. (2013) in Romania found an oil content of 28 %.

Another important parameter in this study was the oil yield per hectare. The analysis of variance showed a significant difference in oil yield between the years. A higher oil yield was obtained in 2014. The higher oil yield in 2014 was due to the higher oil content of seeds in 2014 (25.25 %) compared to 2017 (22.97 %). Water availability and cooler temperature during the seed development stage are the main determinants of seed and oil yields

Table 2: Results of analysis of variance for researched properties of white mustard

Source of variation	Seed yield (kg ha^{-1})	Oil yield (kg ha^{-1})
Year	ns	*
Seeding density	*	ns
Year x seeding density	ns	ns

ns–not significant; * significant for $p < 0.05$

Table 3: The influence of seeding density on seed yield and oil yield of white mustard in 2014 and 2017 year

Source of variation	Seed yield (kg ha^{-1})	Oil content (% d. m)	Oil yield kg ha^{-1})
Year			
2014	1064	25.25	237 a
2017	1052	22.97	205 b
Seeding density (germinable seeds m^{-2})			
50	933 b	23.97	197
70	1028 ab	24.01	217
90	1081 ab	24.37	232
110	1168 a	24.10	238

Different letters significant for $p < 0.05$
d. m.–dry matter

(Marjanović-Jeromela et al., 2019). It can be concluded that the weather conditions in 2014 were more favourable for achieving higher oil content in the seeds. In 2014, the amount of precipitation during seed development (June and July to harvest) was higher by 62 % than during the same period in 2017. The air temperature was also 2.3 and 2.2 °C lower in June and July 2014 than in the same months in 2017 (Table 1).

Higher seeding density increased oil yield, but the difference wasn't statistically significant (Table 3). These results are in agreement with those of Sáez-Bastante et al. (2016), who also found no effect of plant density (16, 26, 40 and 80 plants per m^2) on the oil yield of white mustard.

3.2 FATTY ACID COMPOSITION

The fatty acid composition of white mustard oil in dependency on seeding density during 2014 and 2017 is shown in Table 4 and Table 5. The analysis of the fatty acid composition of white mustard oil shows that erucic acid (C22:1), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) are dominant fatty acids in this oil.

The analysed oil of white mustard also contained palmitic acid (C16:0), stearic acid (C18:0), arachidonic acid (C20:0) and gadoleic acid (C20:1). In addition to these fatty acids, eicosatrienoic acid (C20:2) was present in 2014, and palmitoleic acid (C16:1), heptadecanoic acid (C17:0) and behenic acid (C22:0) were present in 2017 (Table 4 and Table 5). According to the Official law regulation of R. Croatia (Official Gazette, 2019), only in 2017, the average content of oleic acid was slightly above the prescribed value, as was the presence of heptadecanoic acid. The content of another fatty acid in white mustard oil was within the prescribed values.

In general, the fatty acid composition of any type of oil significantly influences the physical properties, nutritional value, and oxidative stability of the oil. As erucic acid was dominant in the oil of white mustard in this study and reached a limit of more than 5 % required by the European Directive for human consumption and for foodstuffs containing added fats or oils Directive 76/621/EEC (Council of the European Union, 1976), this oil is not recommended for human consumption, but it is well suited for industrial purposes. Erucic acid (C22:1) is an important oleochemical product that has many uses in metallurgy, machinery, rubber, chemical industry, and other fields due to its hydrophobicity and water resistance (Wang et al., 2022). In addition, this oil can be used as a feedstock to produce biodiesel (Ciubota-Rosie et. al., 2013). According to Pinzi et al. (2009), a higher content of monounsaturated fatty acids and saturated fatty acids (such as oleic and palmitic acids) is considered more desirable than polyunsaturated fatty acids (linoleic and linolenic acids) in terms of biodiesel oxidation stability, cetane number and fuel cold weather performance.

In the 2014 year by increasing the seeding density from 50 to 110 germinable seeds m^{-2}, the content of erucic acid was gradually increased by 2.8 %, 3.6 % and 4.4 % compared to the seeding density of 50 germinable seeds m^{-2}. In 2017 it varied from 37.10% (70 germinable seeds m^{-2}) to 38.29 (90 germinable seeds m^{-2}).

The oleic acid content in 2014 varied between 20.90 % (50 germinable seeds m^{-2}) and 21.40 % (90 germinable seeds m^{-2}) depending on seeding density. In 2017, the average content of oleic acid was 3.10 % higher than in 2014, ranging from 23.55 % (110 germinable seeds m^{-2}) to 24.50 % (50 germinable seeds m^{-2}).

The linoleic acid content in 2014 with an increase in seeding density from 50 germinable seeds m^{-2} to 90 and 110 germinable seeds m^{-2} decreased from 10.30 % to 9.70 %. In 2017, the average content of linoleic acid was slightly higher compared to 2014 (by 1.3 %), with a variation from 11.02 % (70 germinable seeds m^{-2}) to 11.41% (50 germinable seeds m^{-2}).

The content of linolenic acid in 2014 with increasing seeding density from 50 to 110 germinable seeds m^{-2} resulted in decreases of 3.4 %, 4.4% and 4.6 % compared to the lowest seeding density. In 2017, linolenic acid content was on average 2.23 % lower than in 2014, with a variation from 9.41 % (70 germinable seeds m^{-2}) to 9.85 % (110 germinable seeds m^{-2}) depending on seeding density.

Fatty acid profile in mustard depends on genetic base (Sawicka et al., 2020) but also on weather conditions (Ciubota-Rosie et al., 2013). The analysis of white mustard oil showed differentiation of fatty acid profile among researched years. Therefore, in the 2017 growing season when air temperature during stages of seed development and maturation (May and June) was higher and precipitations lower, the content of oleic and linoleic acid increased while linolenic and erucic acid decreased. Wilkes et al. (2013) also observed the influence of the growing season on the content of oleic, linoleic and erucic acids in the oil of Indian mustard (*Brassica juncea* (L.) Czem.). According to these authors, oleic and linoleic acids were inversely correlated with the content of erucic acid, which tended to be higher in cooler growing conditions. Similarly, Pospišil et al. (2007) reported for rapeseed, where the highest content of oleic acid and the lowest content of linoleic and linolenic acid in the oil were observed in the growing season with higher monthly air temperatures and lower precipitation in May and June.

Temperature is a major environmental factor that regulates fatty acid desaturation in plants (Dar et al., 2017). Lower temperatures generally favour the accumulation of polyunsaturated fatty acids (PUFA), such as linolenic acid (Ciubota-Rosie et al., 2009). The results of Hou et al. (2006) indicate that the content of linolenic acid (18:3) in soybean seed oil is the most sensitive to environmental changes. According to Menard et al. (2017), plants modify the content of polyunsaturated fatty acids in their membranes and storage lipids to adapt to temperature changes. In developing seeds, this response is largely controlled by the activities of the microsomal

Table 4: Fatty acids composition of white mustard in dependency on seeding density, 2014 year

Fatty acid (% of total)		Seeding density (germinable seed m^{-2}) 50	70	90	110	Mean	SD	CV	Official Regulation of R. Croatia*
C16:0	Palmitic	3.20	3.00	3.00	3.00	3.00	0.10	3.28	0.5-4.5
C18:0	Stearic	1.30	1.00	0.90	0.90	1.03	0.19	18.47	0.5-2.0
C18:1	Oleic	20.90	21.30	21.40	21.00	21.15	0.24	1.13	8.0-23.0
C18:2	Linoleic (ώ-6)	10.30	10.00	9.70	9.70	9.93	0.29	2.89	10.0-24.0
C18:3	Linolenic (ώ-3)	15.00	11.60	10.6	10.40	11.90	2.13	17.92	6.0-18.0
C20:0	Arachidinic	0.60	0.60	0.60	0.60	0.60	0.00	0.00	ND-1.5
C20:1	Gadoleic	9.10	9.8	10.1	10.1	9.78	0.47	4.83	5.0-13.0
C20:2	Eicosatrienoic	0.7	0.7	0.7	0.7	0.70	0.00	0.00	ND-1.0
C22:1	Erucic	35.60	38.4	39.20	40.0	38.30	1.91	5.00	38.30

ND-Not detected; SD–standard deviation; CV–coefficient of variation
* Official Gazette-NN 11/2019 (2019)

Table 5: Fatty acids composition of white mustard in dependency on seeding density, 2017 year

Fatty acid (% of total)		Seeding density (germinable seed m^{-2}) 50	70	90	110	Mean	SD	CV	Official Regulation of R. Croatia*
C16:0	Palmitic	3.22	3.29	3.14	2.94	3.15	0.15	4.81	0.5-4.5
C16:1	Palmitoleic	0.17	0.17	0.16	0.19	0.17	0.01	7.29	ND-0.5
C17:0	Heptadecanoic	0.99	2.35	0.00	0.07	0.85	1.10	128.51	ND
C18:0	Stearic	0.92	0.95	0.99	0.90	0.94	0.02	1.85	0.5-2.0
C18:1	Oleic	24.50	24.45	24.35	23.55	24.21	0.45	1.84	8.0-23.0
C18:2	Linoleic (ώ-6)	11.41	11.02	11.17	11.29	11.22	0.17	1.49	10-24
C18:3	Linolenic (ώ-3)	9.74	9.41	9.66	9.85	9.67	0.19	1.93	6-18
C20:0	Arachidonic	0.60	0.61	0.61	0.60	0.61	0.01	0.95	ND-1.5
C20:1	Gadoleic	9.19	9.26	9.40	9.33	9.30	0.09	0.97	5.0-13.0
C22:0	Behenic	0.49	0.54	0.51	0.51	0.51	0.02	4.02	0.2-2.5
C22:1	Erucic	37.85	37.10	38.29	37.56	37.70	0.50	1.33	22.0-55.0

ND-Not detected; SD–standard deviation; CV–coefficient of variation
* Official Gazette-NN 11/2019 (2019)

ω-6- and ω-3-fatty acid desaturases FAD2 and FAD3. The enzyme fatty acid desaturase 2 (FAD2) catalyses the conversion of oleic acid (C18:1) to linoleic acid (18:2), which is further desaturated to linolenic acid (18:3) by the enzyme FAD3 (Dar et al., 2017). With increasing temperatures, the activity of FAD2 and FAD3 decreases and with it the content of polyunsaturated fatty acids in the seeds (Alsajri et al., 2020).

Also, it can be observed that in 2014 by increasing seeding density from 50 to 110 germinable seeds m^{-2} erucic acid was increased while linoleic and linolenic gradually decreased. These findings are in line with Karydogianni et al (2022) in Greece who found higher quantities of polyunsaturated fatty acid at low lower plant density (46 plants m^{-2}) in comparison to higher plant density (76 plants m^{-2}). While Sáez-Bastante et al. (2016) in Spain found that increased plant density had a positive effect on the content of linoleic and linolenic acid. The negative correlation between erucic acid (C22:1) and oleic acid (C18:1) and linoleic acid (C18:2) in oil of Indian mustard (*Brassica juncea*) according to Wilkes et al. (2013) reflects the biosynthetic pathway of these fatty acids and the amount and/or activity of enzymes involved in each step of the pathway. It is a well-known substrate competition in the catalysis of C18:1 CoA to erucic acid or PUFA biosynthesis (i.e. linoleic acid and linolenic acid). In the cytoplasm, C18:1 can either be elongated to C22:1 in a reaction involving a β-ketoacyl CoA synthase (also known as fatty acid elongase 1, FAE1) or to linoleic acid (C18:2) and subsequently to linolenic acid (C18:3) through the action of the membrane-bound, microsomal enzymes desaturase FAD2 (omega-6 desaturase) and FAD3 (omega-3 desaturase) (Lu et al., 2011).

4 CONCLUSIONS

In conclusion, under agroecological conditions in north-west Croatia, 70 germinable seeds m^{-2} seeding density is sufficient to achieve a high seed yield.

Higher oil yield was achieved in 2014 due to lower air temperatures and higher precipitation during the stage of seed formation and maturation. The high content of erucic acid in white mustard oil makes it suitable for industrial purposes, such as the production of lubricants and biodiesel.

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The growth of plants containing pyrrolizidine alkaloids (PAs) in plots cultivated with medicinal aromatic plants (MAPs) and in their natural wild habitats in Kosovo

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The growth of plants containing pyrrolizidine alkaloids (PAs) in plots cultivated with medicinal aromatic plants (MAPs) and in their natural wild habitats in Kosovo

Abstract: Thousands of plant species worldwide produce about 600 different pyrrolizidine alkaloids (PAs), which are known to cause disease in humans and animals. The plants known for their PAs content were investigated in 70 plots cultivated with 19 MAPs species and the natural habitats of 20 wild MAPs species. Most of the poisonous plants found in cultivated and the natural habitats of wild MAPs belong to the families of Asteraceae and Boraginaceae. In the cultivated MAPs plots, 22 plant species known for their PAs content were identified, including 7 from the Asteraceae, 13 from the Boraginaceae, and 1 species from the Convolvulaceae and Solanaceae families. 34 species known for their PAs content were identified in natural habitats, 17 of which belonged to the Boraginaceae and 15 to the Asteraceae families. Convolvulaceae and Solanaceae families were represented by only one species each. Most species from the Asteraceae family known for their PAs content identified in cultivated fields and natural habitats were from the genera *Senecio* and *Jacobaea*, while genera *Myosotis*, *Pulmonaria* and *Symphytum* from Boraginaceae family. In the plots cultivated with MAPs, *Convolvulus arvensis* L. known for its PAs content and tropane alkaloids (TAs) was the most prevalent.

Key words: plant species, pyrrolizidine alkaloids, medicinal plants, cultivated, wild

Prisotnost rastlin, ki vsebujejo pirolizidinske alkaloide na rastiščih gojenih in samoniklih zdravilnih in aromatičnih rastlin na Kosovu

Izvleček: Več tisoč rastlinskih vrst , razširjenih širom po svetu, tvori okrog 600 različnih pirolizidinskih alkaloidov (PAs), ki povzročajo bolezni pri ljudeh in živalih. V raziskavi so bile preučevane rastline, ki vsebujejo PAs na 70 ploskvah, kjer se goji 19 zdravilnih in aromatičnih rastlin in na naravnih rastiščih 20 samoniklih vrst teh rastlin. Večina strupenih rastlin, najdenih na rastiščih gojenih in samoniklih zdravilnih in aromatičnih rastlin pripada družinama Asteraceae in Boraginaceae. Na ploskvah gojenih zdravilnih in aromatičnih rastlin je bilo najdeno 22 vrst, ki vsebujejo pirolizidinske alakaloide, od tega 7 iz družine Asteraceae, 13 iz družine Boraginaceae in po 1 vrsta iz družin Convolvulaceae in Solanaceae. Na naravnih rastiščih samoniklih zdravilnih aromatičnih rastlin je bilo najdenih 34 vrst, ki vsebujejo pirolizidinske alkaloide, od katerih je 17 vrst pripadalo družini Boraginaceae, 15 vrst družini Asteraceae. Družini Convolvulaceae in Solanaceae sta bili zastopani s po eno vrsto. Večina vrst iz družine Asteraceae, ki vsebujejo pirolizidinske alkaloide na rastiščih gojenih in samoniklih zdravilnih in aromatičnih rastlin je bilo iz rodov *Senecio* in *Jacobaea* medtem, ko so vrste iz družine Boraginaceae pripadale rodovom *Myosotis*, *Pulmonaria* in *Symphytum*. Na rastiščih gojenih zdravilnih in aromatičnih rastlin je bil najbolj pogost njivski slak (*Convolvulus arvensis* L.), znan po vsebnosti pirolizidinskih in tropanskih alkaloidov.

Ključne besede: rastlinske vrste, pirolizidinski alkaloidi, zdravilne rastline, gojene, samonikle

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1 INTRODUCTION

Pyrrolizidine alkaloids (PAs) are active substances of secondary metabolism in many plant species that belong to the families Asteraceae, Boraginaceae, and Fabaceae. Different authors confirm that more than 6000 vascular plant species in the world produce pyrrolizidine alkaloids (Macel, 2010; Schrenk, 2020). Based on EFSA's opinion on pyrrolizidine alkaloids in food and feed, there are approximately 600 different PAs (EFSA 2011). The potentially toxic PAs, with their N-oxides, are identified in 3-5 % of flowering plants (Smith & Culvenor, 1981). The plants known for their PAs content can contaminate soil and water as well as non-PAs-producing plants (Günthardt et al. 2020). PAs-producing plants are considered to be among the most frequent natural toxins that can endanger the health of people, animals, and wildlife, especially people who use herbal medicines (Longhurst et al. 2019). There is evidence that PAs caused many outbreaks of poisoning cases, which resulted in thousands of deaths, documented in many countries, (Chauvin et al., 1994). PAs contamination has been reported also in honey in various countries (Kempf et al., 2009, Griffin et al., 2014, He et al., 2017). Many authors found the presence of pyrrolizidine alkaloids (PAs) in the species belonging to the family Asteraceae, especially in the genera: *Senecio, Jacobaea, Tanacetum, Adenostyles, Eupatorium*, and *Tussilago* (Pestchanker & Giordano, 1986; Witte et al., 1992; Suau et al., 2002, Christov & Evstatieva, 2003; Hol et al., 2003; Macel et al., 2004; Pelser et al., 2005;, Kostova et al., 2006; Kirk et al., 2010; Cheng et al., 2017; Smyrska-Wieleba et al., 2017; Lebada et al., 2000; Adamczak et al., 2013; Nedelcheva et al., 2015; Klevenhusen et al., 2022). In addition to the species of the Asteraceae family, PAs were also found in the genera and species belonging to the Boraginaceae Family; in particular, the species of the genera *Anchusa, Anchusella, Cynoglosum, Echium, Heliotropium, Lithospermum, Myosotis, Pulmonaria* and *Symphytum* (Hendriks et al., 1988 Pfister et al., 1992; Van Dam et al., 1994; El-Shazly et al., 1996; Oberlies et al., 2004; O'Dowd and Edgar, 2006). Different researches show that the concentration of PAs in plant organs differs depending on their age; the young shoots of *Heliotropium europaeum* L. had more PAs than older ones, while the youngest leaves of *Cynoglossum officinale* L. contained up to 190 times higher levels of PAs than older leaves (Van Dam et al., 1994; O'Dowd & Edgar, 2006). Since both, wild and cultivated MAPs may grow in association with PAs containing plants, the risk of their mixing during the harvesting time is high. The risk of occurrence of PAs in herbal products used in medicine or as medicinal tea may be a consequence of contamination caused by weeds (German Federal Institute for Risk Assessment, 2013).

For medicinal plants that naturally contain PAs, there are regulations and recommendations on contamination, including the EMA's (EMEA, 2006) guidance document „Good Agricultural and Collection Practice".

After the Kosovo war, the MAPs sector became a source of income for the rural population and has developed into a sector that exports semi-finished products to international markets. About 3000 taxa of vascular plants grow in the Republic of Kosovo, (Millaku et al., 2013), of which about 300 (10 %) are wild MAPs. Currently, about 70 species of wild plants are collected in Kosovo, 20 of which have high economic potential. According to a report from 2022 (Anonymous, 2022), in addition to the collection of wild plants, there are also 928 hectares in Kosovo where MAPs are cultivated. About 90 % of these wild and cultivated products are exported mainly as semi-processed products to the EU and the USA, where they are used as raw materials in the pharmaceutical, food, and cosmetic industries.

Recently, some exporters on MAPs from Kosovo have faced export difficulties, because in some cases the analyzed MAPs samples exceeded the standards or the maximum permitted levels of EU Regulation 2020/2040 (Commission Regulation (EU) 2020/2040), which regulates the maximum content of pyrrolizidine alkaloids in certain food products. The detection of PAs levels above the maximum permitted levels mobilized the Association „Organika" (Kosovar Association of Processors and Exporters of NWFP) and the exporters of these products, who called for a survey of toxic plants containing PAs in the plots cultivated with MAPs, as well as in the natural habitats of wild medicinal and aromatic plants (MAPs). The objective of this study was the inventory of PAs plants known for their PAs content in cultivated as well as in natural MAPs growing habitats.

2 MATERIALS AND METHODS

2.1 METHODOLOGY FOR THE INVENTORY OF PLANTS CONTAINING PAS IN PLOTS CULTIVATED WITH MAPS

During the vegetation period 2021-2022, the plants known for their PAs content were investigated in 7 regions of the Republic of Kosovo, in plots cultivated with 19 species of MAPs. Those were: *Allium ampeloprasum* L., *Alcea rosea* L., *Althaeae officinalis* L., *Calendula officinalis* L., *Centaurea cyanus* L., *Lavandula angustifolia* Mill., *Levisticum officinale* W.D.J. Koch, *Malva sylvestris* L., *Matricaria chamomilla* L., *Melissa officinalis* L., *Mentha* x *piperita* L., *Mentha spicata* L., *Ocimum basilicum* L., *Origanum onites* L., *Origanum vulgare* L., *Rosmarinus* *officinalis* L., *Salvia officinalis* L., *Thymus vulgaris* L. and *Urtica dioica* L.). A total of 70 cultivated plots with MAPs in 7 regions (10 plots for each region). were studied. Of 70 researched plots, 50 were organically certified, while the other 20 plots were undergoing the certification. The identification of PAs-containing plants in plots with an area of less than 1 ha was done on the whole plot, while in plots with an area of more than 1 ha, 10 survey points with an area of 10 x 10 m^2 were randomly selected. The inventory of PAs-containing plants in plots with annual MAPs started after germination and lasted until the time of harvest to eliminate them after identification. On the other hand, in plots with perennial herbaceous MAPs, the inventory of plants known for their PAs content was made before the harvest to prevent their mixing with MAPs.

2.2 METHODOLOGY FOR THE INVENTORY OF PLANTS CONTAINING PAS IN NATURAL HABITATS

The inventory of the plants known for their PAs content was also conducted in naturaly growing habitats of 20 MAPs with high economic potential (*Achillea millefolium* L., *Alchemilla vulgaris* L., *Althaea officinalis* L., *Allium ursinum* L., *Artemisia absinthium* L., *Bellis perennis* L., *Capsella bursa-pastoris* (L.) Medik., *Centaurium erythraea* Rafn., *Epilobium angustifolium* L., *Hypericum perforatum* L., *Origanum vulgare* L., *Plantago lanceolata* L., *Primula veris* L., *Rubus plicatus* Weihe & Nees, *Rubus idaeus* L., *Satureja montana* L., *Thymus pulegioides* L., *Urtica dioica* L., *Verbascum thapsus* L., and *Viola tricolor* L.). The inventory included 5 areas in the Republic of Kosovo certified as areas of organic MAPs cultivation. The plants known for their PAs content in zone I include Istog mountains (Alps 1) (34,850 ha), zone II includes Shala e Bajgorës mountains (55,000 ha), zone III Lipë - Gjakovë/Alps 2 (62,488 ha), zone IV - Sharri mountains (110,000 ha) and zone V - Gollaku 3 (Novobërde, Gjilan, Viti, Kamenica). Maps for certified organic areas of wild MAPs and data for cultivated and certified organic plots were obtained from the Association „Organika". Figure 1 shows an example of a part of a zone map (zone IV /5 - Sharri Mountains).

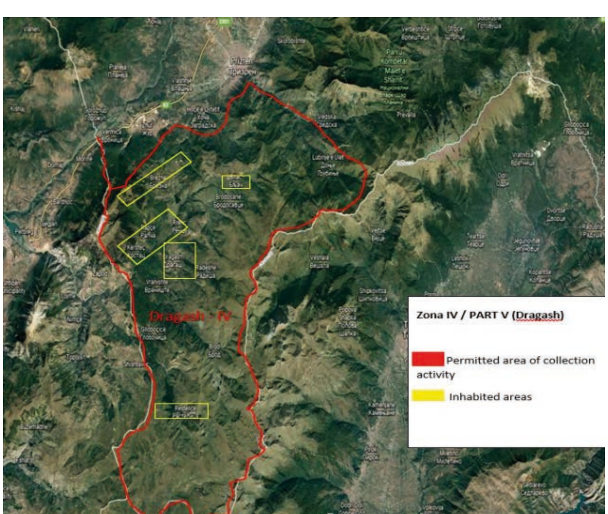

Figure 1: Map of area IV/5 certified for harvesting MAPs as organic products

In the areas surrounded by a red line, harvesting of wild MAPs is allowed, while in the areas surrounded by a yellow line, harvesting is prohibited (zonal map IV/5). Plant taxa identification and nomenclature were based mostly on Flora Europaea (Tutin & al., 1968-1980, 1993), Flora of Serbia (Josifović, 1970-1977; Sarić & Diklić, 1986; Stevanović, 2012), Flora of Macedonia (Micevski, 1985-2005; Matevski, 2010) and Flora of Albania (Barina et al., 2018; Paparisto et al., 1988; Qosja et al., 1992, 1996; Vangjeli et al., 2000), while finally their nomenclature was updated according to Euro+Med Plantbase (Euro+Med 2006+). Jaccard similarity index (Jaccard, 1912) was used to compare the similarities and diversity of plants containing PAs in cultivated plots of 7 regions of Kosovo.

3 RESULTS AND DISCUSSION

3.1 PLANT SPECIES CONTAINING PYRROLIZIDINE ALKALOIDS (PAS) FOUND IN CULTIVATED PLOTS

The inventory of the plants known for their PAs content in 70 plots cultivated with 19 species of MAPs plants resulted in the identification of 22 plant species known to contain PAs.

The following 7 species were identified from the Asteraceae (Compositae) Family: *Eupatorium cannabinum* L., *Jacobaea erratica* (Bertol.) Fourr., *Jacobaea vulgaris* Gaertn., *Senecio leucanthemifolius* subs. *vernalis* (Waldst. & Kit.) Greuter, *Senecio vulgaris* L, *Tanacetum vulgare* L. and *Tussilago farfara* L.. The plants known for their PAs content from the Asteraceae family were present in certain periods within the year. The species *Senecio vulgaris* was present in the cultivated areas from late February to December. *Tussilago farfara* was present from early March to November. The species *Jacobaea erratica* and *Eupatorium cannabinum* occurred from June to September, especially in the plots planted with mint

(*Mentha* x *piperita* L. and *Mentha spicata* L.), which were located near rivers and had a lot of moisture.

The Boraginaceae family was represented by the following 13 species: *Anchusa azurea* Mill., *Anchusela cretica* (Mill.) Bigazzi & al., *Anchusa officinalis* L., *Lycopsis arvensis* L., *Cynoglossum officinale* L., *Echium italicum* L., *Echium vulgare* L., *Echium rubrum* Forssk., *Heliotropium europaeum* L., *Lithospermum arvense* L., *Myosotis arvensis* L. (Hill), *Myosotis stricta* Roem. & Schult. and *Symphytum officinale* L..

The most frequent species were: *Buglossoides arvensis* (L.) I. M. Johnst. recorded in 19 plots, *Anchusa officinalis* L. in 18 plots, *Cynoglossum officinale* L. in 17 plots, *Echium vulgare* L. in 15 plots, while the species *Heliotropium europaeum* L. and *Myosotis arvensis* (L.) Hill, were present in 10 plots. The species *Symphytum officinale* L. was present in 2 plots cultivated with *Mentha* x *piperita* L. in the Kamenica region and 2 plots cultivated with *Mentha spicata* L. in the Dukagjini region (Istog locality). The species *Echium rubrum* Forssk. was found only in one locality (Kishnica) on soils and rocks of volcanic origin (serpentine) planted with *Thymus vulgaris* L. and *Melissa officinalis* L..

In addition to the identified species from the fami-

Figure 2: *Convolvulus arvensis* L. in plots cultivated with *Origanum onites* (A) and *Urtica dioica* (B)

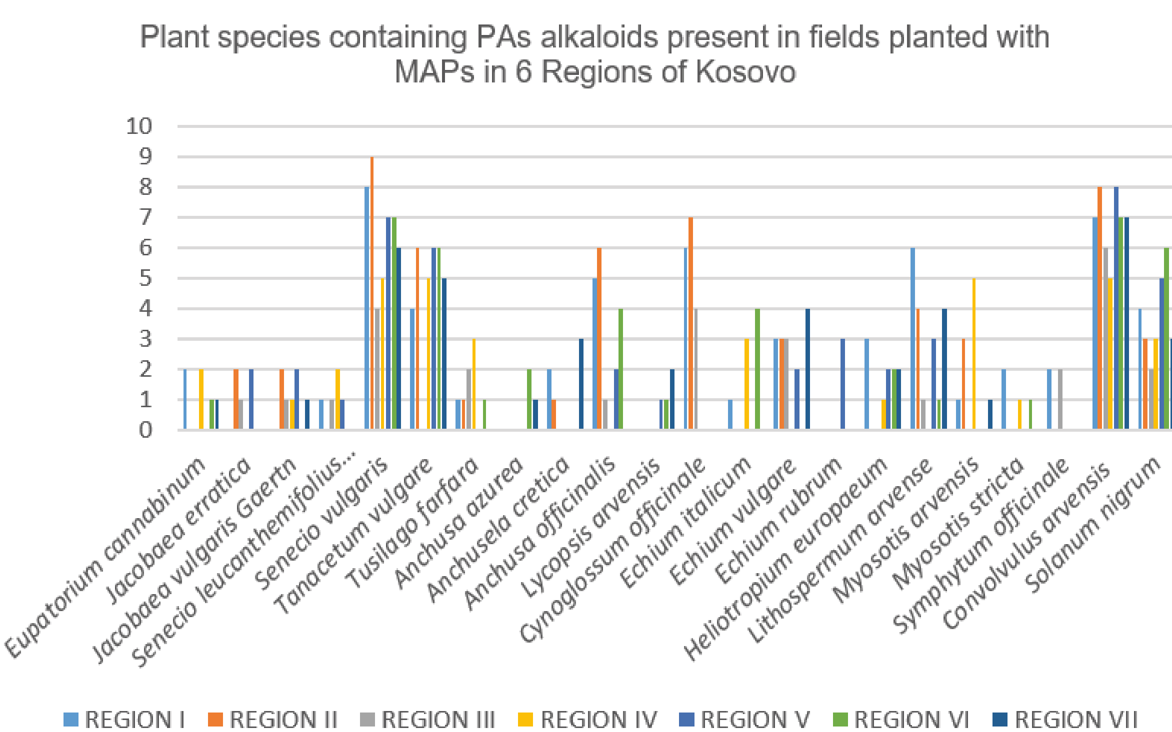

Figure 3: The identified plants known for their PAs content in plots cultivated with MAPs in 7 regions

lies Asteraceae and Boraginaceae, one species from the family Convolvulaceae and the family Solanaceae was also identified.

Convolvulus arvensis L., which is known for its production of pyrrolidine alkaloids (Todd et al. 1995) was the most frequent in plots cultivated with MAPs and was found from June to November in 48 cultivated plots (Figure 2). Mechanical removal was most difficult in the plots cultivated with MAPs because the underground part (rhizome) was deeply rooted in the soil, while the aboveground part enveloped the body of the medicinal plants. Over the harvest period of MAPs (*Mentha* x *piperita*, *M. spicata*, *Melisa officinalis*, *Urtica dioica*, *Levisticum officinale*, *Origanum onites*, *Origanum vulgare*, *Thymus vulgaris* and *Salvia officinalis*), this species was recorded during the harvesting and drying process.

The summary results of identified plants known for their PAs content in the MAPs-cultivated plots are shown in Figure 3.

The similarities and diversity of PAs-containing plants in plots cultivated with MAPs in the 7 regions of Kosovo were analyzed using the Jaccard similarity index (Table 1).

The greatest similarities in the composition of plant species appeared between regions II and III with an index value of 0.66 (66 %); the biggest differences appeared between regions III and VI where the Jacquard index is 0.31 or 31 %.

3.2 PLANT SPECIES CONTAINING PYRROLIZIDINE ALKALOIDS (PAS) FOUND IN NATURAL HABITATS RICH IN MAPS

During the research of plant species known for their PAs content in natural habitats rich in MAPs in the 5 surveyed areas, 34 such species were identified.

The following 17 plant species known for their PAs

Table 1: Jaccard's similarity index for plants containing PAs in 7 regions of Kosovo

	II	III	IV	V	VI	VII
I	0.611111	0.555556	0.555556	0.450000	0.526316	0.526316
II		0.666667	0.388889	0.529412	0.368421	0.529412
III			0.333333	0.562500	0.315789	0.315789
IV				0.388889	0.562500	0.470588
V					0.444444	0.529412
VI						0.529412

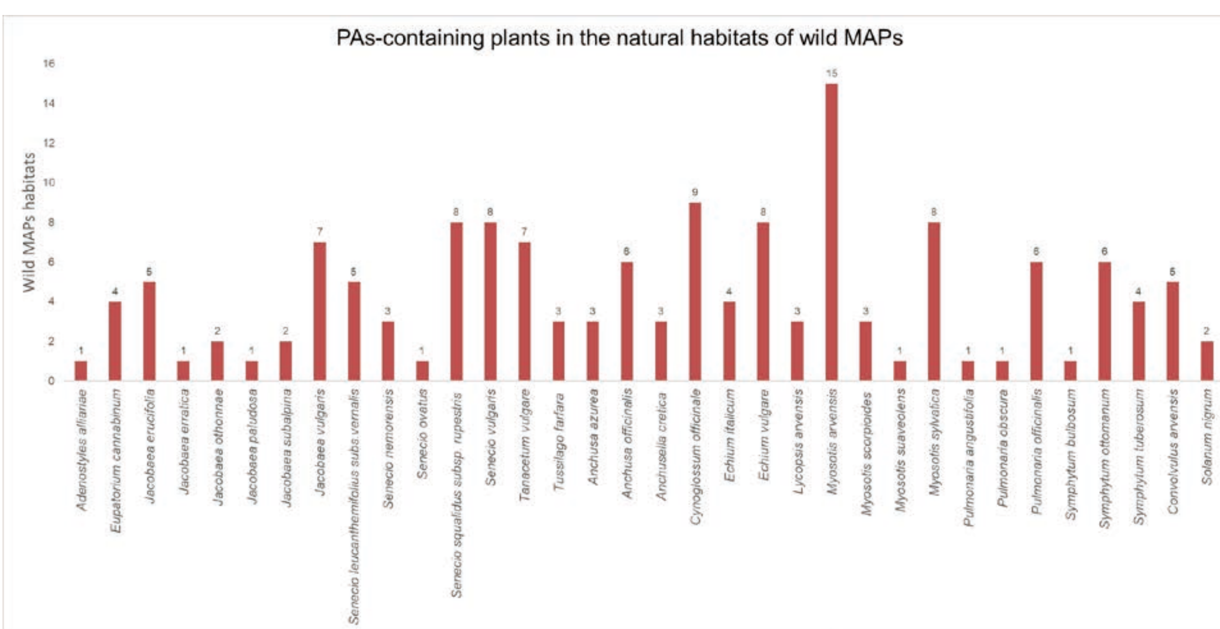

Figure 4: The presence of plants known for their PAs content in the natural habitats of wild MAPs in the researched regions of Kosovo

Figure 5: (A): *Senecio nemorensis* L. in the habitat of wild nettle (*Urtica dioica* L.), (B): *Eupatorium cannabinum* L. with *Rubus idaeus* L

content were identified from the Boraginaceae family: *Anchusa azurea*, *Anchusa officinalis*, *Anchusella cretica*, *Cynoglosum officinale*, *Echium italicum*, *Echium vulgare*, *Lycopsis arvensis*, *Myosotis arvensis*, *Myosotis scorpioides*, *Myosotis suaveolens*, *Myosotis sylvatica*, *Pulmonaria angustifolia*, *Pulmonaria obscura*, *Pulmonaria officinalis*, *Symphytum bulbosum*, *Symphytum ottomanum* and *Symphytum tuberosum*. From the Asteraceae family, 15 plants known for their PAs content were identified, as following: *Adenostyles alliariae, Eupatorium cannabinum, Jacobaea erucifolia, Jacobaea erratica, Jacobaea othonnae, Jacobaea paludosa, Jacobaea subalpina, Jacobaea vulgaris, Senecio leucanthemifolius* subs.*vernalis, Senecio nemorensis, Senecio ovatus, Senecio squalidus* subsp. *rupestris, Senecio vulgaris, Tanacetum vulgare* and *Tusilago farfara*.

Figure 4 shows the occurrence of species known for their PAs content in the natural habitats where certain MAPs grow.

Most plants known for their PAs content (14 species) occurred in natural habitats where *Achillea millefolium* and *Artemisia absinthium* species grow. High numbers of the plants known for their PAs content were also found in the natural habitats where the following species grow: *Urtica dioica* (12 species-Fig.5A), *Verbascum thapsus* (11 species), *Hypericum perforatum* (10 species), *Rubus idaeus* (9 species-Fig.B), *Epilobium angustifolium* (8 species).

This study showed that 18 plants known for their PAs content (*Eupatorium cannabinum*, *Jacobaea erratica*, *Jacobaea vulgaris*, *Senecio leucanthemifolius* subs. *vernalis*, *Senecio vulgaris*, *Tanacetum vulgare*, *Tussilago farfara*, *Anchusa azurea, Anchusa officinalis*, *Anchusella cretica*, *Cynoglossum officinale*, *Echium italicum*, *Echium vulgare*, *Myosotis arvensis*, *Symphytum officinale*, *Convolvulus arvensis, Solanum nigrum*) were found in both, plots cultivated with MAPs and in natural habitats where MAPs grow.

4 DISCUSSION

Analysis of data from 70 plots cultivated with MAPs showed that there were 7 species known for their PAs content from the Asteraceae family, of which the most common was the species *Senecio vulgaris*, present in 46 plots, followed by the species: *Tanacetum vulgare*, *Tussilago farfara* and *Jacobaea vulgaris*.

Guidelines for dietary supplements in Europe state that plant species from the genus *Senecio* may have significant concentrations of PAs. In an area of one hectare with 60,000 plants, only 6 plants with a PAs load of 1,310 mg kg^{-1} yield 0.1310 mg kg^{-1} of the dried harvested crop.

The presence of the species *Senecio vulgaris* in 66 % of the plots cultivated with MAPs, as well as other PAs-c known for their PAs content from this family, indicates the risk of their co-harvesting with MAPs if not removed before harvesting. Of the 13 plants known for their PAs content from the Boraginaceae family, *Lithospermum arvense, Anchusa officinalis*, *Cynoglossum officinale*, *Echium vulgare* and *Heliotropium europaeum* were the most common species.

The results of a study (Abd El-Razik et al., 2019) of toxic plants growing in association with medicinal plant fields in Egypt are similar to our findings where the *Senecio* was most problematic precisely because of its content of PAs. Their findings are similar to the results of this study regarding the presence of the species *Convolvulus arvensis*. Another study (Ljevnaić-Mašić et al., 2022) of weed occurrence in conventionally and organically grown MAPs shows that *Senecio vulgaris* was among the most common weed species in both MAPs cropping systems.

The presence of 22 plant species known for their PAs content in plots managed with MAPs is a strong indication that there is a risk of inadvertent co-harvesting of PAs-producing weeds if the PAs-containing species are not removed prior to harvest or during MAPs harvest. Species of the genera *Senecio*, *Jacobaea*, *Tussilago*, *Tanacetum*, *Anchusa*, *Echium*, *Heliotropium*, *Myosotis* and *Symphytum* grew not only in MAPs-managed areas but also in large fields and in uncultivated areas adjacent to cultivated areas. Although these species were not found in some croplands, there is a possibility that PAs-containing plant seeds are already present in the soil or are introduced from adjacent fields or roadsides. (Anonymous, 2020). Therefore, the selection of land for the cultivation of MAPs is of particular importance. In the natural habitats of 20 wild-harvested herbaceous MAPs from the family Asteraceae, most species known for their PAs content were from the genera *Senecio* (5 species) and *Jacobaea* (4 species). The most common species were *Senecio vulgaris*, *Senecio squalidus* subsp. *rupestris*, *Jacobaea vulgaris*, and *Tanacetum vulgare*. The species *Eupatorium cannabinum* occurred in the habitats of the medicinal plant species *Althaeae officinalis*, *Rubus plicatus*, *Rubus idaeus* and *Urtica dioica*, while the species *Adenostyles alliariae* was found only in the habitat of the species *Alchemilla vulgaris*, which grows in Kosovo mainly in the subalpine and alpine areas.

Species known for their PAs content [*Jacobaea pancicii* (Degen) Vladimirov & Raab-Straube (*Jacobaea abrotanifolia* subsp. *carpathica* (Herbich) B. Nord. & Greuter, *Jacobaea subalpina* (W. D. J. Koch) Pelser &, *Jacobaea othonnae* (M. Bieb.) C. A. Mey., *Tephroseris papposa* subsp. *wagnerii* (Degen) B. Nord. and *Senecio rupestris* Waldst. & Kit.], which are rare species for southeastern Europe (Christov & Evstatieva, 2003; Mandic et al., 2009) were not found in the natural habitats of the researched wild MAPs.

In the natural habitats of wild MAPs from the Boraginaceae family, *Myosotis arvensis* was the most common found species in 15 wild MAPs habitats whereas the species *Symphytum ottomanum* was found in 6 and *Pulmonaria officinalis* in 5 habitats of wild MAPs.

Most plant species (14 species) known for their PAs content were found in *Achillea millefolium* and *Artemisia absinthium* habitats, 12 in *Urtica dioica*, 11 in *Verbascum thapsus,* and 10 in *Hypericum perforatum* habitat. Only 3 species were found in the habitats of *Althaeae officinalis* and *Satureja montana*.

The fact that 34 plants known for their PAs content were present in the natural habitats of the 20 wild-harvested MAPs indicates the risk and possibility of the inadvertent mixing of collected MAPs material. Several publications (Letsyo et al., 2017; Chmit et al., 2019; Steinhoff, 2019; Suparmi et al., 2020) show that PAs-containing plants contaminate raw materials used in the production of food and herbal medicines.

5 CONCLUSIONS

As a conclusion from this study, we can say that a considerable number of plants known for their PAs content were found in the plots cultivated with MAPs and in the natural habitats of wild MAPs. The identification of 22 plant species known to contain PAs in the cultivated plots of MAPs and 34 species of such plants identified in their natural habitats indicates that there is a possibility that these plant species were not handled with due care during harvest. One way to avoid co-harvesting of plants known for their PAs content is to train collectors to visually identify plants that produce PAs and to provide catalogs or atlases of such plants. The identification of numerous plants species known for their PAs content, including the most common species *Convolvulus arvensis*, shows that the application of good agricultural practices on cultivated land is insufficient and that strict application of these rules is necessary.

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5.2 AUTHOR CONTRIBUTIONS

Conceptualization and research designing, F.M. (Fadil Millaku) and A.R.; methodology, F.M. and N.B.; writing—original draft F.M. (Fadil Millaku); A.R and N.B were involved in the revision of this paper. All three authors approved the present manuscript.

5.3 CONFLICTS OF INTEREST

No potential conflict of interest was reported by the authors.

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Behaviour of the main okra (*Abelmoschus* spp.) cultivars grown in Côte d'Ivoire to root-knot nematodes (*Meloidogyne incognita* (Kofoid & White, 1919)) under greenhouse conditions

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Behaviour of the main okra (*Abelmoschus* spp.) cultivars grown in Côte d'Ivoire to root-knot nematodes (*Meloidogyne incognita* (Kofoid & White, 1919)) under greenhouse conditions

Abstract: Root-knot nematodes are the main factor limiting okra production in Côte d'Ivoire. Using resistant cultivars appears to be one of the best strategies for managing root-knot nematodes. The aim of this study was to determine the behaviour of the main okra cultivars grown in Côte d'Ivoire against *Meloidogyne incognita*. Seeds of 20 okra cultivars were planted in pots under greenhouse conditions. Fourteen-day-old plants of okra cultivars were inoculated with 500 second-stage juveniles of *M. incognita*. Agronomic and pathological parameters were determined. The Basanti cultivar exhibited the highest gall index (5.0/plant), final population (7938 individuals/plant), and reproductive factor (15.88/plant) of *M. incognita*, whereas the Hiré cultivar showed one of the lowest gall indexes (3.0/plant), final population (912 individuals/plant), and reproductive factor (1.8/plant). Two groups of cultivars were identified based on their susceptibility to *M. incognita* and their agronomic performance. One group consisted of cultivars that were less susceptible to *M. incognita* and had better agronomic performance. Cultivars that were more susceptible to *M. incognita* and had poorer agronomic performance made up the other group. The Hiré cultivar was the least favourable to *M. incognita* development. Based on the current study, the Hiré cultivar may be a promising option for farmers in root-knot nematode-prone environments.

Key words: Côte d'Ivoire, cultivars, galls, nematodes, okra, susceptibility

Odziv glavnih sort jedilnega osleza (*Abelmoschus* spp.) gojenega v Slonokoščeni obali na ogorčico vozlanja korenin (*Meloidogyne incognita* (Kofoid & White, 1919)) v poskusu v rastlinjaku

Izvleček: Ogorčice vozlanja korenin so glavni omejujoči dejavnik pri gojenju jedilnega osleza v Slonokoščeni obali. Uporaba odpornih sort izgleda kot najboljša strategija za uravnavanje teh ogorčic. Namen raziskave je bil določiti odziv poglavitnih sort jedilnega osleza na tega škodljivca v Slonokoščeni obali. Semena 20 sort jedilnega osleza (bamije) so bila posajena v lonce v rastlinjaku. Šitirinajst dni stare sejanke so bile inokulirane z juvenilnimi ogorčicani v 500 sekundnem stadiju, kaneje so bili določeni agronomski in patološki parametri. Sorta Basanti je imela največji indeks šišk (5,0/rastlino), največjo velikost končne populacije ogorčic (7938 osebkov/rastlino) in največji reproduktivni faktor (15,88/rastlino), sorta Hiré je imela najmanjši indeks šišk (3,0/rastlino), najmanjšo končno populacijo ogorčic (912 osebkov/rastlino) in najmanjši reproduktivni faktor (1,8/rastlino). Prepoznani sta bili dve skupini sort glede na njihovo občutljivost na ogorčico in agronomske lastnosti. Prva skupina je bila sestavljena iz sort, ki so bile na ogorčico manj občutljive in so imele boljše agronomske lastnosti. Druga skupina sort je bila na ogorčico bolj občutljiva in je imela slabše agronomske lastnosti. Sorta Hiré je bila za razvoj ogorčice *M. incognita* najmanj primerna in bi lahko bila obetajoča izbira za kmete v okoljih okuženih s to ogorčico.

Ključne besede: Slonokoščena obala, sorte, šiške, nematodi, jedilni oslez, občutljivost

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1 INTRODUCTION

Okra is an annual plant of the Malvaceae family (Fondio et al., 2007), native to Ethiopia (Sathish & Eswar, 2013). It is one of the most valuable food crops in tropical and subtropical parts of the world (Oyelade et al., 2003). Mihretu et al. (2014) reported that okra is a plant with many uses due to its fresh leaves, buds, flowers, seeds, and young pods. Indeed, the young leaves of okra are consumed in many African countries because of their richness in vitamins A and C, proteins, calcium, and iron (Ngbede et al., 2014). Oil (Habtanu et al., 2014) and biofuel (Farroq et al., 2010) are produced using okra's mature seeds. Okra leaves and stems are used to make fibre and rope (Jideani & Adetula, 1993). Okra's immature pods are eaten as vegetables. They are used in salads, soups, and stews (Ndunguru & Rajabu, 2004). In addition, consumption of immature pods of okra provides 4,550 kcal kg^{-1} for humans (Edet & Etim, 2010).

In Côte d'Ivoire, all people cultivate and consume okra (Fondio et al., 2003). It is typically grown during the main rainy season on small plots near large areas of cereal crops such as rice or maize, as well as yams. It can also be found dispersed throughout these fields (Fondio et al., 2001). Currently, okra is cultivated in the off-season around urban areas, along with other vegetable crops. Two okra species are cultivated in Côte d'Ivoire: *Abelmoschus esculentus* L. and *A. caillei* (A.Chev.) Stevels (Fondio et al., 2007). Plants of *A. esculentus* produce ribbed pods, while those of *A. caillei* produce non-ribbed pods (Fondio et al., 2007). Several cultivars of each okra species are cultivated in Côte d'Ivoire. The Hiré, Kirikou, Emerald, Fatou, Tomi, Adjouablé, and Koto cultivars are popular. They are cultivated for their high yield and financial profitability. Some are local cultivars, while others are the property of European and American seed companies. Some cultivars are imported from neighbouring countries such as Burkina Faso, Mali, Ghana, and Niger. Several okra cultivars are adapted to Côte d'Ivoire's agro-ecological conditions.

However, plant-parasitic nematodes are a limiting factor in okra production. Root-knot nematodes are the most economically destructive (Mukhtar et al., 2013), including *Meloidogyne arenaria* (Neal, 1889), *M. javanica* (Treub, 1885), and *M. incognita* (Kofoid & White, 1919) (Moens et al., 2009). They cause plant wilting and stunting, leaf chlorosis, root gall formation, root reduction, and poor yields if their populations exceed the economic threshold (Sikora & Fernandez, 2005). Root-knot nematodes cause yield losses of 70-90 % (Safiuddin et al., 2011). The estimated economic losses in India due to *M. incognita* on okra are valued at US$ 8.7 million (Jain et al., 2007). Yield losses are the most significant if the root-knot nematode population is high when the crop is planted (Djian-Caporalino et al., 2009). However, if the initial population is low, the plant does not suffer considerable damage in the first year. However, the parasite's multiplication can be so extensive that it results in significant crop losses the following year (Djian-Caporalino et al., 2009).

Various management strategies can help reduce the losses caused by root-knot nematodes on okra (Kumar et al., 2020). Chemical nematicides, although effective, are expensive (Bell, 2000) and are not available to farmers (Alam, 1987). They can be harmful to humans, livestock, and the environment (Kumar et al., 2020). Resistant cultivars are one of the best alternatives to chemical nematicides. In fact, it is economical for farmers to cultivate resistant cultivars in crop rotations. It allows nematode populations to be gradually reduced in infested areas (Kumar et al., 2020). The aim of this study is to determine the behaviour of the main okra cultivars grown in Côte d'Ivoire against root-knot nematodes, specifically *Meloidogyne incognita*.

2 MATERIALS AND METHODS

2.1 CULTIVATION OF OKRA PLANTS

2.1.1 Acquisition of okra seeds

Seeds of 20 okra cultivars were collected from crop seed suppliers (formal structures and retailers) in the District of Abidjan. Seeds of the Clemson Spineless, Emerald, Hiré, Kirikou F1, Koda F1, and Rafiki F1 cultivars were purchased from the Semivoire company. However, seeds of the Caribou F1 cultivar were bought from the Callivoire company, whereas those of Adjouablé and Fatou were acquired from the Akomi company. Seeds of Basanti, Icrisat, Indiana, Koto, Paysan, Perkins, Raci, Tomi, Volta, Yeleen, and Yodana cultivars were obtained from seed retailers in urban markets. Adjouablé, Emerald, and Tomi are cultivars belonging to the *Abelmoschus caillei* species, while the other ones are from the *A. esculentus* species.

2.1.2 Soil preparation and okra planting

A topsoil sample was collected from a fallow plot at the experimental station of NANGUI ABROGOUA University. The soil sample was sterilised twice at 121 °C for 45 minutes and later distributed in perforated polythene bags (1 kg of soil per bag). Seeds of the okra cultivars were planted on the previously sterilised manure. Okra seedlings were watered at 48-hour intervals for one week. Twenty vigorous seedlings of each okra cultivar were transplanted onto the soil in bags.

2.2 INOCULATION OF OKRA PLANTS

2.2.1 Experimental design

The trial included two factors: okra cultivars (20 okra cultivars) and *M. incognita* inoculation (inoculated plants and non-inoculated plants). There were, therefore, 40 treatments in the trial. Okra plants were arranged in a completely randomised design with 10 plants per treatment. The experiment was conducted for three months (June to August). The plants were exposed to temperatures ranging from 20.9 to 29.5 °C, with relative humidity higher than 85 % and 13 to 14 hours of photoperiod.

2.2.2 Preparation of the inoculum

Second-stage juveniles of *M. incognita* were used in this trial. They were maintained on tomato plants, cultivar Cobra 26, at the experimental station of NANGUI ABROGOUA University. The tomato plants were uprooted and washed with tap water. *M. incognita* eggs were collected using the method of Hussey & Barker (1973). The egg suspensions were incubated at 26 °C for 72 hours. Second-stage juveniles from the eggs were concentrated at 500 individuals per ml of aliquot and later inoculated onto two-week-old okra plants.

2.2.3 Inoculation of okra plants

Four holes, approximately 1 cm in diameter and 5 cm deep, were made in the soil around each okra plant. A 1 ml aliquot containing 500 second-stage juveniles of *M. incognita* was distributed in the holes. Control plants were inoculated with distilled water. Four hundred okra plants were used in this trial, with 200 plants inoculated with *M. incognita* and 200 non-inoculated plants. The trial comprised 40 treatments, with 10 plants per treatment. Okra plants were watered at 72-hour intervals with 200 ml of water per plant.

2.3 EVALUATION OF THE EFFECTS OF TREATMENTS

2.3.1 Determination of okra agronomic parameters

The effects of the treatments on okra development were evaluated 60 days after inoculation using agronomic parameters such as plant height, leaf number, leaf area, stem diameter, pre-flowering, pre-emergence time, root, and shoot mass. Plant height was measured with a tape measure from the base of the stem to the apical end. Leaves were counted per okra plant. Root and shoot mass were measured using an electronic balance, whereas the leaf area of plants was measured using the Mesurim2 v1.63 application (Cosentino). The okra leaves were detached from the plants. A photograph of each leaf spread out on a horizontal support was made. The support was marked with a scale bar (1 cm). The photograph of each leaf was imported into the Mesurim2 application (available online), which analysed and determined the surface area of the leaf (cm^2). A slide gauge was used to measure the stem diameter of plants. The pre-emergence time of seeds (time between seed planting and the appearance of the first seedlings) and pre-flowering time (time between seed emergence and the appearance of the first flowers) were measured for each treatment.

2.3.2 Determination of nematode pathological parameters

Pathological parameters, such as gall index, final population, and reproductive factor of *M. incognita*, were used to evaluate the effects of treatments on galls development. Okra plants were uprooted and grouped per treatment. The root systems of the okra plants were rinsed with tap water to remove any remaining soil clods. Root systems were examined to record the severity of the galls using the Bridge & Page (1980) scale.

The gall index was assessed using the method of Zewain (2014). A pair of scissors was used to cut plant roots into explants. *M. incognita* population was extracted from each root system using two methods. The NaOCl method (Hussey and Barker, 1973) was used to collect eggs and swollen individuals. However, second-stage juveniles and adult males were collected from the NaOCl-derived root shred using the maceration method (Coyne et al., 2010). Whereas, individuals from soil samples of each plant were extracted using the Whitehead tray method (Coyne et al., 2010). Individuals of *M. incognita* were counted per root system. The final population of *M. incognita* for each plant was the sum of all individuals (eggs, second-stage juveniles, adult males, and swollen stages) collected per root system and soil samples after the three methods. The reproductive factor of *M. incognita* was computed using the formula of Rivoal et al. (2001).

2.3.3 Determination of the behaviour of cultivars

The behaviour of okra cultivars against *M. incognita* was determined using gall index and reproductive factor data. The evaluation was based on the Sasser et al. (1984) scale (Table 1).

2.4 STATISTICAL ANALYSIS

The final population of *M. incognita* and leaf number of okra plants were normalised using the Log10 (x + 1) function. The Shapiro and Wilk test was used to test the normal distribution of the data. The parameters were analysed with Statistica 7.1 software (StatSoft, Inc.) according to both factors and their interactions. If there was a significant difference at the 5 % level, a post-anova test was performed to identify the best cultivars. Multivariate analyses such as Principal Component Analysis (PCA) and Agglomerative Hierarchical Clustering (AHC) were performed to identify groups of okra cultivars based on their level of resistance to *M. incognita*. The data were scaled using the z-score normalization method before applying PCA.

Table 1: Scale of plant susceptibility to root-knot nematodes (Sasser et al., 1984)

Plant damage (Gall index)	Host efficiency (Reproductive factor)	Degree of resistance
≤ 2	≤ 1	Resistant
≤ 2	≥ 1	Tolerant
≥ 2	≤ 1	Hypersusceptible
≥ 2	≥ 1	Susceptible

3 RESULTS

3.1 VARIATION SOURCES IN AGRONOMIC AND PATHOLOGICAL PARAMETERS

Both factors (okra cultivar and *M. incognita* inoculation) influenced the agronomic and pathological parameters (Table 2). There was a highly significant difference between okra cultivars in terms of agronomic and pathological parameters ($p < 0.001$). The *M. incognita* inoculation factor did not influence the okra agronomic parameters, except for stem diameter and root mass. As expected, a highly significant difference was noted between inoculated and non-inoculated plants based on the gall index, the final population, and the reproductive

Table 2: Result of analysis of variance of data for agronomic and pathological parameters

Parameters		Sources of variations		
		Cultivar	Inoculation	Cultivar × Inoculation
Agronomic Parameters	Degrees of freedom	19	1	19
	Leaf Number	0.000***	0.791ns	0.323ns
	Pre-emergence Time	0.000***	0.183ns	0.013*
	Pre-flowering Time	0.000***	0.275ns	0.001***
	Stem Diameter	0.000***	0.001***	0.004**
	Plant Height	0.000***	0.104ns	0.129ns
	Leaf Area	0.000***	0.190ns	0.000***
	Shoot Mass	0.000***	0.837ns	0.001***
	Root Mass	0.000***	0.000***	0.000***
Pathological Parameters	Gall Index	0.000***	0.000***	0.000***
	Final population	0.000***	0.000***	0.000***
	Reproductive Factor	0.000***	0.000***	0.000***

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: Not significant probability factor of *M. incognita* ($p < 0.001$). There was an interaction between both factors on pre-emergence and pre-flowering times, stem diameter, leaf area, shoot and root mass, gall index, final population, and *M. incognita* reproductive factor ($p < 0.001$). In contrast, there was no significant interaction between both factors for leaf number and plant height ($p \geq 0.05$).

3.2 BEHAVIOUR OF OKRA CULTIVARS AGAINST *Meloidogyne incognita*

Each pathological parameter of *M. incognita* varied between okra cultivars (Table 3). The gall index varied from 2.00 to 5.00, depending on the okra cultivar. The final population and the reproductive factor of *M. incognita* varied, respectively, from 912 to 7938 individuals and from 1.82 to 15.88, depending on the okra cultivar. A highly significant difference was noted between okra cultivars based on each studied pathological parameter ($p < 0.001$). The gall index was the highest on the Basanti cultivar, with a value of 5. However, the lowest gall index was recorded for the Rafiki (2.00), Adjouablé (2.67), and Kirikou (2.67) cultivars. The final population of *M. incognita* was higher on the Basanti cultivar (7938 individuals per plant) compared to other cultivars. However, it was lowest on the Hiré cultivar (912 individuals per plant). The reproductive factor of *M. incognita* was higher on the Basanti cultivar (15.88) than on the other cultivars. However, it was the lowest on the Hiré cultivar, with a value of 1.82. In summary, the value of the gall index recorded for all okra cultivars was higher than 1, and the reproductive factor was higher than 2. The main okra cultivars cultivated in Côte d'Ivoire are, therefore, susceptible to *M. incognita*.

Table 3: Values of *M. incognita* pathological parameters between okra cultivars

	Pathological parameters			
Okra Cultivars	Gall index	Final population	Reproductive factor	Degree of Resistance
Adjouablé	2.67 ± 0.36e	2459 ± 149de	4.92 ± 0.30de	Susceptible
Tomi	4.83 ± 0.14ab	1900 ± 65de	3.80 ± 0.13de	Susceptible
Emerald	3.83 ± 0.40c	1606 ± 76de	3.21 ± 0.15de	Susceptible
Fatou	4.17 ± 0.14bc	1831 ± 18de	3.66 ± 0.04de	Susceptible
Koto	3.67 ± 0.36cd	1513 ± 32de	3.03 ± 0.06de	Susceptible
Basanti	5.00 ± 0.12a	7938 ± 297a	15.88 ± 0.59a	Susceptible
Caribou	3.40 ± 0.35cd	2734 ± 107de	5.47 ± 0.21de	Susceptible
Clemson	3.00 ± 0.22de	1964 ± 65de	3.93 ± 0.13de	Susceptible
Hiré	3.00 ± 0.19de	912 ± 53e	1.82 ± 0.11e	Susceptible
Icrisat	4.83 ± 0.14ab	5271 ± 304b	10.54 ± 0.61b	Susceptible
Indiana	4.00 ± 0.22c	1824 ± 64de	3.65 ± 0.1de	Susceptible
Kirikou	2.67 ± 0.28e	4494 ± 368c	8.99 ± 0.7c	Susceptible
Koda	4.83 ± 0.14ab	4527 ± 50c	9.05 ± 0.10c	Susceptible
Paysan	4.17 ± 0.26bc	2544 ± 428de	5.08 ± 0.86de	Susceptible
Perkins	4.00 ± 0.31c	3120 ± 212d	6.24 ± 0.42de	Susceptible
Raci	3.00 ± 0.31de	2218 ± 164de	4.44 ± 0.33de	Susceptible
Rafiki	2.00 ± 0.22e	2527 ± 112de	5.05 ± 0.22de	Susceptible
Yeleen	4.83 ± 0.14ab	4250 ± 94c	8.34 ± 0.10c	Susceptible
Yodana	3.83 ± .014c	4508 ± 172c	9.02 ± 0.7c	Susceptible
Volta	3.00 ± 0.31cd	2537 ± 46de	5.07 ± 0.9de	Susceptible
CV (%)	7.02	4.79	5.77	
p	0.000	0.000	0.000	

Average ± Standard deviation; Values with the same letter in each column are statistically identical at the 5% level; CV (%): Coefficient of variation; Fisher's statistic value, *p*: Probability value

3.3 GROUPS OF OKRA CULTIVARS

Principal component analysis of all parameters revealed two principal components accounting for 67.51 % of the variability in okra cultivars. Principal component 1 (PC1) explained 48.30 % of the total variability. PC1 was positively correlated (≥ 0.6) with all okra agronomic parameters (Table 4). The contributions of the parameters varied according to the principal components (Table 4). The parameters with the highest contribution to PC1 were shoot mass (16.92 %), plant height (13.33 %), leaf area (16.70 %), stem diameter (12.01%), and pre-emergence time (12.51 %). Therefore, PC1 was defined as the axis of okra cultivars resistant to *M. incognita*.

However, principal component 2 (PC2) represented 19.21 % of the total variability. It was positively correlated with pathological parameters (gall index and reproductive factor of *M. incognita*) and root mass (≥ 0.67) (Table 4). The contributions of the parameters varied according to the principal components (Table 4). The parameters

Table 4: Coefficient of correlation and contribution of parameters to principal components

	Coefficients of correlation		Contributions (%)	
Parameters	PC1 (48.30 %)	PC2 (19.21 %)	PC1 (48.30 %)	PC2 (19.21 %)
Shoot mass	0.90*	0.12	16.924*	0.718
Leaf number	0.60*	-0.31	7.077	5.007
Plant height	0.80*	0.05	13.328*	0.106
Leaf area	0.90*	0.15	16.702*	1.110
Stem diameter	0.77*	-0.01	12.101*	0.002
Pre-emergence time	0.78*	-0.21	12.514*	2.296
Pre-flowering time	0.60*	-0.12	7.346	0.776
Root mass	0.68*	0.70*	9.529	25.401*
Gall index	-0.02	0.89*	0.006	41.532*
Reproductive factor	-0.45	0.67*	4.473	23.051*

* = Parameters with the greatest contribution to the formation of Principal component 1 (PC1) and Principal component 2 (PC2)

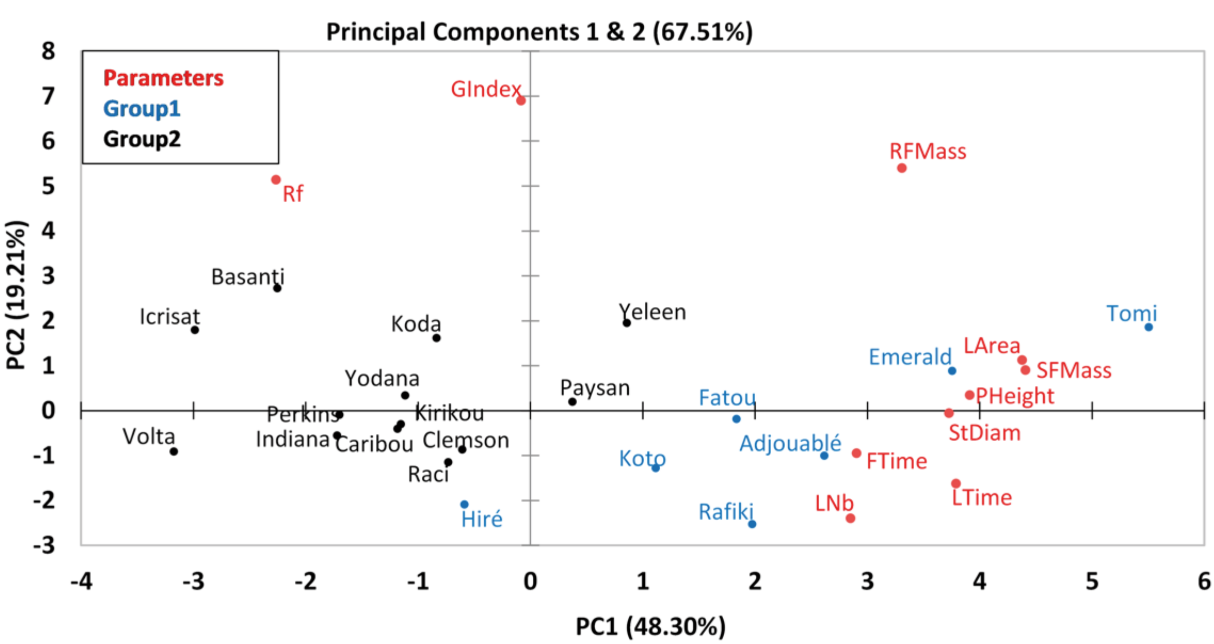

Figure 1: Factorial plan with groups of okra cultivars against *Meloidogyne incognita*
PC1: Principal component 1, PC2: Principal component 2, SFMass: Shoot mass, LNb: Leaf number, PHeight: Plant height, LArea: Leaf area, StDiam: Stem diameter, LTime: Pre-emergence time, FTime: Pre-flowering time, RFMass: Root mass, GIndex: Gall index, Rf: Reproductive factor

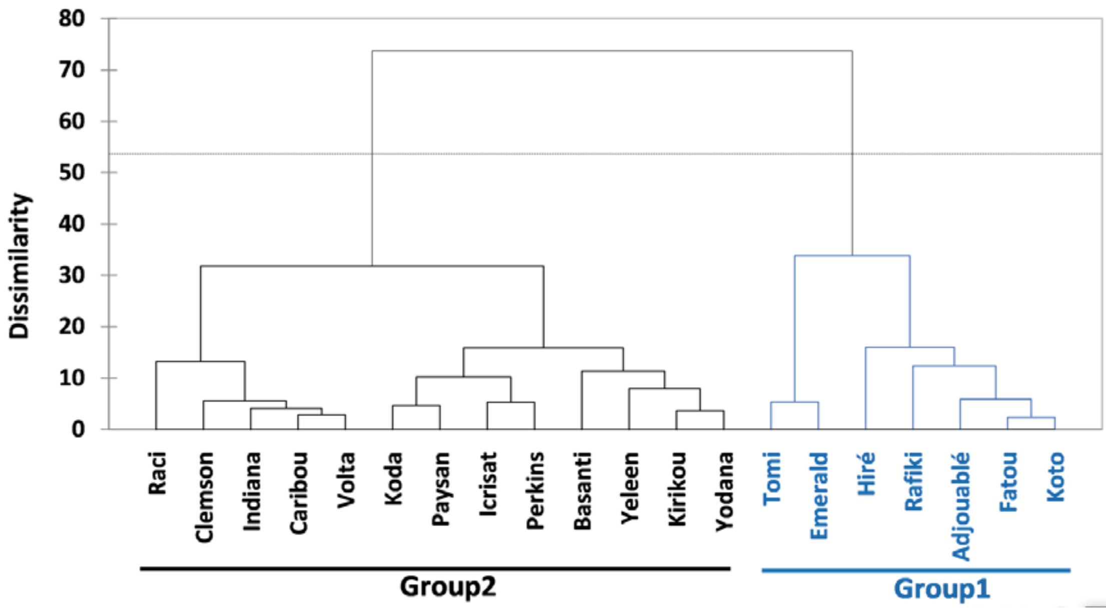

Figure 2: Similarity dendrogram of okra cultivar susceptibility to *Meloidogyne incognita*

with the highest contribution to PC2 were gall index (41.53 %), reproductive factor (25.05 %), and root mass (25.40 %) (Table 6). Thus, PC2 was defined as the axis of cultivars susceptible to *M. incognita*.

Projection of the parameters and individuals onto the plan defined by both principal components revealed two groups (Group 1 and Group 2) within the okra cultivars planted in Côte d'Ivoire against *M. incognita* (Figure 1). Agglomerative hierarchical classification (with a dissimilarity of 53) also confirmed both groups of okra cultivars (Figure 2).

3.4 CHARACTERISTICS OF GROUPS OF OKRA CULTIVARS

Group 1 comprised 35 % of the studied okra cultivars. It comprised three cultivars of *A. caillei* (Adjouablé, Tomi, and Emerald) and four cultivars of *A. esculentus* (Fatou, Hiré, Koto, and Rafiki). Group 1 cultivars were the least susceptible to *M. incognita* (Table 5). These cultivars had the lowest gall index (3.45 per plant) and reproductive factor of *M. incognita* (3.64 per plant). However, these cultivars showed the best agronomic performance. Their plants were the tallest (30.08 cm/plant), with the highest leaf number (6.31 leaves/plant) and leaf area (56.52 cm^2/plant). Shoot mass (13.07 g/plant) and root mass (3.73 g/plant) were the highest. Pre-emergence time (5.45 days/plant) and pre-flowering time (74.76 days/plant) were the longest.

The remaining 65 % of the studied cultivars formed Group 2. These were 13 cultivars of *A. esculentus*. Group 2 cultivars were the most susceptible to *M. incognita* (Table 5). Their plants showed the highest gall index (5.89 per plant) and reproductive factor (7.36 per plant). These cultivars had the poorest agronomic performance. Plant height was the lowest (25.53 cm/plant), as were leaf number (5.54 leaves/plant) and leaf area (32.37 cm^2/plant). Shoot mass (8.07 g/plant) and root mass (3.01 g/plant) were the lowest. Pre-emergence time (3.58 days/plant) and pre-flowering time (69.44 days/plant) were the shortest.

3.5 CORRELATION BETWEEN PARAMETERS

Coefficients of correlation ranging from -0.44 to 0.88 were noted between the okra agronomic parameters (Table 6). Okra plant height increased significantly with leaf area, stem diameter, shoot mass, and root mass ($0.47 \leq r \leq 0.82$; $p < 0.05$). Strong positive correlations existed between the leaf area and shoot mass ($r = 0.88$; $p < 0.001$) and between the leaf area and root mass ($r = 0.71$; $p < 0.001$). Root mass increased with shoot mass ($r = 0.64$; $p < 0.01$). The gall index increased significantly with root mass and the reproductive factor of *M. incognita* ($0.46 \leq r \leq 0.61$; $p < 0.05$).

Table 5: Characteristics of groups of okra cultivars in the presence of *Meloidogyne incognita*

	Cultivar groups			
Parameters	Group 1 (7 cultivars)	Group 2 (13 cultivars)	CV (%)	p
Shoot mass(g)	13.07 ± 2.02a	8.07 ± 0.78b	12.56	0.010
Leaf number	6.31 ± 0.21a	5.54 ± 0.11b	2.66	0.002
Plant height (cm)	30.08 ± 2.29a	25.53 ± 3.65b	10.95	0.020
Leaf area (cm^2)	56.52 ± 9.33a	32.37 ± 2.23b	11.70	0.004
Stem diameter (mm)	5.78 ± 0.24a	4.66 ± 0.18b	4.01	0.002
Pre-emergence time (day)	5.45 ± 0.36a	3.58 ± 0.24b	6.65	0.000
Pre-flowering time (day)	74.76 ± 0.67a	69.44 ± 0.99b	1.16	0.002
Root mass (g)	3.73 ± 0.59a	3.01 ± 0.25a	12.06	0.200
Gall index	3.45 ± 0.36b	5.89 ± 0.23a	7.17	0.020
Reproductive factor	3.64 ± 0.42b	7.36 ± 0.95a	12.22	0.010

Mean ± standard deviation; *p*: Probability value; CV (%): Coefficients of variation. In each line, values with the same letter are statistically identical at the 5 % level

Table 6: Matrix of correlations between okra agronomic and nematode pathological parameters

Parameter	PHeight	LNb	Larea	StDiam	LTime	FTime	SFMass	RFMass	GIndex	Rf
Pheight	1									
LNb	0.36ns	1								
Larea	0.82***	0.44ns	1							
StDiam	0.47*	0.33ns	0.52*	1						
Ltime	0.54*	0.59**	0.58**	0.59**	1					
Ftime	0.11ns	0.41ns	0.64**	0.64**	0.37ns	1				
SFMass	0.76***	0.36ns	0.88***	0.66**	0.54*	0.53*	1			
RFMass	0.59**	0.19ns	0.71***	0.47*	0.41ns	0.28ns	0.64**	1		
Gindex	-0.07ns	-0.44ns	0.00ns	-0.18ns	-0.1ns	-0.09ns	0.04ns	0.61**	1	
Rf	-0.41ns	-0.31ns	-0.28ns	-0.28ns	-0.50*	-0.23ns	-0.36ns	0.11ns	0.46*	1

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: Not significant probability; SFMass: Shoot mass, LNb: Leaf number, PHeight: Plant height, LArea: Leaf area, StDiam: Stem diameter, LTime: Pre-emergence time, FTime: Pre-flowering time, RFMass: Root mass, GIndex: Gall index, Rf: Reproductive factor; Values in bold are the most significant correlations ($|r| \geq 0.46$)

4 DISCUSSION

Agronomic parameters, such as plant height, leaf number, and pre-flowering time, and pathological parameters, such as gall index and reproductive factor, varied according to the okra cultivar. This agronomic variation may be attributable to differences in the genetic make-up of the okra cultivar genotypes (Jacquet et al., 2005). The collection of okra cultivars in Côte d'Ivoire includes two species of okra: *A. esculentus* and *A. caillei*. The difference in species could justify the agronomic variation between okra cultivars. In addition, some cultivars, such as Hiré, native to Côte d'Ivoire (Technisem, 2017a), seem to be adapted to the country's agroecological constraints. Adegbite (2011) found that maize cultivars varied in their agronomic performance in the presence of *M. incognita*.

The pathological difference between cultivars in the presence of *M. incognita* would be because of the specificity of the host-parasite interaction. Nematodes interact with their hosts in various ways (Jones et al., 2013). Host-parasite interaction depends on factors such as the nutritional and immune status of the host, the virulence and reproductive factors of the parasite, and edaphic factors (Castillo and Vovlas, 2007). For a successful host infection, the nematode must overcome the host's defence strategies (Kyndt et al., 2012). These strategies include the localised production of toxins such as phytoalexins

(Wuyts et al., 2006). The presence of such toxins results in an unfavourable environment for some nematodes. However, some nematodes can produce compounds such as glutathione-S-transferase that neutralize these toxins (Dubreuil et al., 2011). This suggests that *M. incognita* has a unique relationship with each okra cultivar.

Okra plants inoculated with *M. incognita* showed similar agronomic characteristics to non-inoculated plants. This similarity in agronomic characteristics is because of the low density of the inoculum, which corresponds to 500 individuals per kg of soil or 1 individual per gramme of soil. Under favourable conditions, root-knot nematodes can complete one development cycle per month (Khan et al., 2009). They would therefore need more time to invade and damage the root system, which would have a negative impact on okra development. According to Djian-Caporalino et al. (2009), the plant does not suffer any damage in the first year if the density of root-knot nematodes is low initially. However, the parasite's multiplication can be so extensive that it results in significant crop losses the following year if conditions are favourable.

Despite variations in pathological parameters, all okra cultivars were susceptible to *M. incognita* based on the Sasser et al. (1980) scale. This result reveals the high pathogenicity of *M. incognita* on okra cultivars. To achieve this, the second-stage juveniles of *M. incognita* overcame the physical and biochemical barriers of the okra plants. The differences in gall index and reproductive factor between cultivars could be because of the differences in genetic make-up between okra genotypes (Jacquet et al., 2005). These results contradict those of Sujatha et al. (2017), who found different responses in tomato cultivars to *M. incognita*. The difference in findings may be due to the use of different evaluation methods. Sujatha et al. (2017) used the percentage of galled roots and the gall index, while the current study used the gall index and reproductive factor according to the Sasser et al. (1984) scale.

Two groups of okra cultivars were identified using multivariate analyses. One group comprised 13 cultivars (Basanti 447, Caribou F1, Clemson Spineless, Icrisat, Indiana, Kirikou F1, Koda F1, Paysan, Perkins, Raci, Volta, Yeleen, and Yodana F1) that are more susceptible to *M. incognita*. The Kirikou F1 cultivar, which belongs to this group, is one of the most widely planted okra cultivars in Côte d'Ivoire. It is planted in season or off-season in several agroecological zones. So, its precocity of 40 to 45 days (Technisem, 2017b) and its susceptibility to *M. incognita* could help to increase and perpetuate the *M. incognita* population on farms. According to Mukhtar and Kayani (2020), *M. incognita* and *M. javanica* are the most destructive root-knot nematode species. This could justify the near-impossibility of planting okra in the country for the previous two years.

In addition, the second group of seven cultivars least susceptible to *M. incognita* was identified. It comprised three cultivars (Adjouablé, Tomi, and Emerald) of *A. caillei* and four cultivars (Fatou, Hiré, Koto, and Rafiki F1) of *A. esculentus*. This group of cultivars had the lowest values for gall index and reproductive factor. The Hiré cultivar had the lowest reproductive factor of *M. incognita* in this group. The Hiré cultivar is one of the most popular and widely planted okra cultivars in Côte d'Ivoire. It is native to Côte d'Ivoire (Technisem, 2017a) and appears to be adapted to the country's agro-ecological constraints. Indeed, the Hiré cultivar is very productive in tropical conditions (Technisem, 2017a). Thus, the implementation of a cropping system with several okra cultivars would make it possible to increase the level of resistance of this cultivar to *M. incognita*. Indeed, Bridge (1996) suggested that nematode control could be achieved by enhancing the biodiversity inherent in traditional multi-crop or multi-cultivar cropping systems to increase nematode resistance or tolerance.

5 CONCLUSIONS

The current study showed that *M. incognita* infects the main okra cultivars planted in Côte d'Ivoire. All okra cultivars are susceptible to *M. incognita*. However, the Hiré, Koto, Fatou, Adjouablé, Tomi, Emerald, and Rafiki cultivars showed potential for tolerance to *M. incognita*. Among these, the Hiré cultivar could be a promising cultivar in an environment prone to root-knot nematodes. Its tolerance to *M. incognita* and favourable agronomic performance make it a potential solution for reducing yield losses caused by the nematode. Further research and field trials should be conducted to validate these findings and determine the best management strategies for incorporating the Hiré cultivar into okra production.

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Seed composition, physical characteristics and mineral content of Sudanese landraces of pumpkin

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Seed composition, physical characteristics and mineral content of Sudanese landraces of pumpkin

Abstract: The study examined 23 landrace pumpkin seed samples from six Sudanese states (South Kordofan (SK), North Kordofan (NK), Gazira (GZ), Gadarif (GF), White Nile (WN), and Blue Nile (BN)), focusing on their composition and physical characteristics. The results showed significant differences in oil percentages, with SK having the highest percentage. Protein content varied between 16.91 % and 26.13 % in NK. The proximate composition of pumpkin seeds also varied significantly. The study found a significant difference in fatty acids, with polyunsaturated fatty acids having 37.02 % and monounsaturated fatty acids having 27.50 %. The total unsaturated fatty acids ranged from 55.2 % to 70.2 %, while the total saturated fatty acids ranged from 29.45 % to 45.27 %. Seed length, width, and thickness varied, with WN having the highest kernel percentage (78.45 %). Several minerals were extracted from the seeds, with potassium being the most abundant element, ranging from 946 to 1100 mg 100 g^{-1}. Analysis revealed that the seeds contained a rich source of zinc (Zn), ranging from 8.4 mg 100 g^{-1} to 16.21 mg 100 g^{-1}. The study concluded that Sudanese landrace pumpkin seeds are valuable for edible oil and protein fortification due to their high levels of oil, protein, and minerals.

Key words: landrace, North Kordofan, pumpkin seeds, South Kordofan

Zgradba semen, fizikalne lastnosti in vsebnost mineralov v sudanskih deželnih rasah buč

Izvleček: V raziskavi so bila preučene semena 23 deželnih ras buč, vzorčenih v šestih sudanskih državah (Južni Kordofan (SK), Severni Kordofan (NK), Gazira (GZ), Gadarif (GF), White Nile (WN) in Blue Nile (BN)), s poudarkom na njihovi zgradbi in fizikalnih lastnostih. Rezultati so pokazali značilne razlike v odstotkih vsebnosti olja, kjer ga je akcesija SK vsebovala največ. Vsebnost beljakovin je bila pri akcesiji NK med 16,91 % in 26,13 %. Vsebnost za prehrano pomembnih sestavin se je med semeni buč značilno spreminajala. Ugotovljene so bile tudi značilne razlike pri vsebnostih maščobnih kislin, kjer je vsebnost večkrat nenasičenih maščobnih kislin znašala 37,02 %, enkrat nenasičenih maščobnih kislin pa 27,50 %. Celokupna vsebnost nenasičenih maščobnih kislin je bila v razponu od 55,2 % do 70,2 %, celokupna vsebnost nasičenih maščobnih kislin pa med 29,45 % in 45,27 %. Dolžine, širine in debeline semena so bile spremenljive, akcesija WN je imela največji odstotek jedrca (78,45 %). V analizi semen so bili določeni številni elementi, kjer je bil kalij najpogostejši, z vsebnostjo od 946 do 1100 mg 100 g^{-1}. Analiza je tudi odkrila, da so semena bogat vir cinka z vsebnostjo od 8.4 mg 100 g^{-1} do 16.21 mg 100 g^{-1}. Zaključek raziskave je, da so semena lokalnih sudanskih ras buč zaradi njihove sestave primerna za jedilno olje in za obogatenje hrane z beljakovinami in minerali.

Ključne besede: deželne rase, Severni Kordofan, semena buč, Južni Kordofan

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1 INTRODUCTION

Among the most important cucurbits grown in Sudan are pumpkins, which belong to the genus *Cucurbita* and are economically and nutritionally important members of the Cucurbitaceae family, known locally as "Graa assaly". With respect to regional distribution, the central state is by far the most important production area of variable landraces of pumpkins, followed by the southern and western states where these landraces are extensively grown during the rainy season in Kordofan. Pumpkins are usually produced in Sudan by small farmers in rainfed areas, irrigated private farms, and big government schemes. Compared with cash crops like cotton, little attention has been paid so far to pumpkin production. Therefore, reliable data on the area and production of pumpkins is challenging to obtain. Also, there is no improved cultivar of pumpkin for commercial cultivation in Sudan, and the production of cucurbits is based on local accessions and landraces. According to FAOSTAT (2020), pumpkin production in Sudan has fluctuated substantially in the recent year. It tended to increase through the 2014–2020 period, ending at 33,396 tons in 2020 on an area of 201 hectares.

Although the pumpkin itself has various benefits, the pumpkin seeds have been the focus of interest in the last few years in the field of diet and disease research due to their various active components and chemical composition as well as the health benefits. Studies by Odoemelam (2005) and Ardabili et al. (2011) indicated that pumpkin seeds have nutritive and calorific values and are also a rich source of edible oils and fats. Stevenson et al. (2007) studied that pumpkin seeds have high nutritional value and rich in nutraceutical components such as unsaturated fatty acids, especially oleic acid and linoleic acid, palmitic acid, and stearic acid.

Landraces provide genetic diversity and are important genetic resources for plant breeders. Reza et al. (2018) investigated twenty-one accessions of *Cucurbita pepo* L. and eleven accessions of *Cucurbita moschata* Duchesne for their fruit and seed characteristics, which differed significantly ($p < 0.001$) among accessions in terms of mass, width, length, thickness, and 100 seed mass. Balkaya et al. (2010) studied 40 populations of *C. moschata* and showed a wide diversity of seed characteristics. For example, a range of 13.8–24.3 mm for seed length, 7.5–15.3 mm for seed width, and 1.6–4.7 mm for seed thickness.

In Sudan, the major sources of oil seeds are groundnut (*Arachis hypogoea* L.), cotton seeds (*Gossypium barbadense* L.), sesame seeds (*Sesamum indicum* L.) and sunflower seeds (*Helianthus annuus* L.). Ziyada and El-Hussien (2008) suggested looking for new sources of oil-bearing seeds. The most promising, unconventional, and new sources of seed oil in the Sudan are the available species of the family Cucurbitaceae. To date, little research has been carried out on the physicochemical properties and mineral content of pumpkin seeds obtained from different states in Sudan. In this study, the proximate composition, physical characteristics, and mineral content of pumpkin seeds collected from six states in Sudan are examined. The results of this study are expected to improve the documentation of landrace pumpkin seeds collected from Sudan, thereby enhancing their utilization by different users, especially oil producers.

2 MATERIALS AND METHODS

2.1 PLANT MATERİAL

Twenty-three samples of landrace pumpkin seeds (approximately 500 g of each) were collected from the six major pumpkin-producing states in Sudan. The states included South Kordofan (ten samples 1–10), North Kordofan (two samples 11–12), Gazira (four samples 13–16), Gadarif (three samples 17–19), White Nile (two samples 20–21), and Blue Nile (two samples 22–23). Pumpkin landraces and areas abbreviated (SK, NK, GZ, and GF), (A), and number of fallow areas: (SK, NK, GZ, and GF / A1, 23). Dried pumpkin seeds were obtained from each state's local market.They were manually sorted to remove damaged seeds, undersized, immature seeds, and other extraneous materials. Then they were dehulled manually. The samples were then sealed in airtight plastic bags and kept in the refrigerator at 4 °C for analysis.

2.2 PROXIMATE ANALYSIS

Moisture content was determined at 105 °C. Ash content was determined at 550 °C. Crude protein, lipid, and fiber were also determined according to the procedures of AOAC, (1990).

2.3 PHYSICAL CHARACTERISTICS

The length, width, and thickness of whole pumpkin seeds were measured with an accuracy of 0.01 mm (0.25 mm) using a Vernier EBH (Germany). The measurement was performed on 100 randomly selected seeds from the test samples.

2.4 FATTY ACID COMPOSITION

The fatty acid composition (FA) of oil samples was determined according to AOCA method no Ce 1-62 (1991) by using (GC/MS) technique model (GC/MS-QP 2010- Ultra, Shimadzu, Japan), with capillary column (RTx-5 ms-30 m × 0.25 mm × 0.25 µm), 2 ml of sample was mixed thoroughly with 7 ml of alcoholic sodium hydroxide (NaOH) that prepared by dissolving 2 g in 100 ml methanol. 7 ml from alcoholic sulfuric acid (1 ml H_2SO_4 to 100 ml methanol) was then added. The mixture was then shaked for 5 minutes. The content of the test tube was left to stand overnight. 1 ml of super saturated sodium chloride (NaCl) was then added and contents being shaken. 2 ml of normal hexane was added and the contents were shaked throughly for three minutes. Then the *n*-hexane layer (the upper layer of the test tube) was taken using disposable syringe.5 µl from the n-hexane extract was diluted with 5 ml of diethyl ether. Then the mixture was filtered through syring filter 0.45 µm and dried with 1g of anhydrous sodium sulphate as drying agent 1 µl of the diluted sample was injected in the GCMS instrument.

2.5 SEED INDEX

From each pumpkin seed sample, 100 seeds were weighted using an electronic balance (Shimadzu, Japan, p = 0.001 g). This was repeated three times with the same number of seeds. An average of these mass was recorded. This method was applied to determine the mass of the whole seed, kernel, hull, and mass of 100 whole seeds (g) according to the method described by Jafari et al. (2012).

2.6 HUSK RATE

Husk content was estimated by dehulling 100 g of pumpkin seed manually and weighted according to Manda (2018). The percentage of mass of the whole pumpkin seed and kernel was used to figure out how much husk as per the following expression:

Husk rate (%) = (*mass of hull*) / (*mass of whole pumpkinseed and kernel*) ×100

2.7 DETERMINATİON OF MINERAL CONTENT

AOAC method no. 986.24 (1995) and the Atomic Absorption Spectrophotometer (AAS model GBC 932 plus, Dandenong/Australia) were used to figure out the mineral content. One gram of the sample was put into a porcelain crucible and heated in a muffle furnace to 550 °C. The ash was treated with 10 ml of concentrated hydrochloric acid, then made up to 100 ml.

2.8 STATISTICAL ANALYSIS

The data generated were subjected to Statistical Analysis System (SAS) software (GenStat, 2014). The mean ± SE were tested using one-factor analysis of variance (ANOVA), and the means were separated using Duncan's multiple range test (DMRT).

3 RESULTS AND DISCUSSION

Table 1 illustrates the ash content of the 23 landraces of pumpkin seeds, which ranged from 1.537 % reported by S. Kordofan (PR/A10) to 3.300 % reported by GZ (PR/A13). The differences observed could be explained by soil conditions, seed status, climate, and mineral presence. These values were close to the 4.64 ± 0.04 % reported by Mohammed (2015) and to the 4.87 % and 4.93 % reported by Can-Cauich et al. (2021), but lower than those obtained by Ardabili et al. (2011), who reported 5.34 %.

The protein content ranged from 16.91 % to 26.13 %. Landrace from NK (PR/A12) had the highest values; these values are within the range of 23.70–30.68 % reported by Olaofe et al. (1994) for melon, pumpkin, and gourd seeds. The crude protein in the pumpkin seed compared favorably with high protein seeds and nuts like cowpeas (22.7 %) and soybeans (35 %). The protein content of the pumpkin seed suggests that it can contribute to the daily protein need of 23–100 g for adults, as recommended by some authorities (Ajayi et al., 2006).

The total crude oil content of pumpkin seeds varied between localities and ranged from 17.77 % to 49.91 %; landraces from SK (PR/A10) had the highest yield of seed oil with 49.91 %, while landraces from GZ (PR/A13) had the lowest value with 17.77 %. These values fell in the range reported for different species of Cucurbita (9.8–52.1 %), different varieties of *C. pepo* (31.2–51.0 %) reported by Stevenson et al. (2007), and Egyptian varieties (50.1–51.01 %) reported by El-Adawy and Taha (2001). They were also like those reported by Amin et al. (2019) for native and hybrid pumpkin seeds, which were 23.5 and 17.6 %, respectively, and to those reported for Eritrean pumpkin seeds (22.2–35 %) (Younis et al., 2000). These findings show that landrace pumpkin seeds can be considered a potential source of vegetable oil for domestic and industrial purposes. The value of crude fiber ranged from 5.17 % to 15.745 %; the landrace from SK (PR/A3) had the lower value, while the landrace from SK (PR/A10) had the highest. This result was in accordance with the findings published by Al-Anoos et al. (2015) for three pumpkin varieties (*C. Maxima* Duchesne), which ranged from 4.12–9.69 %, and Alfawaz (2004), who reported 2.13 ± 0.57 16.48 ± 0.81 for kernel and whole seed, respectively. These values were lower than those obtained by Rezig et al. (2012), who reported 21.97 %, and higher than the value of 2.49 % reported by Ardabili et al. (2011), but moderately less compared to the result reported by Steiner-Asiedu et al. (2014), which was less than 2.5 %.

The total carbohydrate content of landrace seeds ranged from 2.56 % to 45.98 %. The SK landrace (PR/

Table 1: Proximate analysis of 23 pumpkin landraces seeds collected from different areas in Sudan*

		Proximate Analysis					
Location	Pumpkin Race per Area	Moisture (%)	Ash (%)	Oil content (%)	Protein (%)	Fiber (%)	Carbohydrate (%)
S.Kordfan	SK/A1	6.947^{ij}	2.417^{df}	24.55^{g}	20.70 ^{f}	7.48 ^{hi}	37.91 ^{bcd}
S.Kordfan	SK/A2	7.307 ^{i}	1.743^{ij}	37.04^{bc}	22.60 ^{bd}	5.92 ^{jk}	25.49 ^{h}
S.Kordfan	SK/A3	6.967^{ij}	1.950^{gi}	37.08^{bc}	25.56 ^{a}	5.17 ^{k}	23.28 ^{h}
S.Kordfan	SK/A4	8.940^{dh}	2.833^{bc}	28.37^{e}	21.65 ^{de}	8.36 ^{gh}	29.85 ^{g}
S.Kordfan	SK/A5	9.487^{cd}	2.773^{bd}	20.77^{kl}	18.60 ^{gh}	13.20 ^{bc}	35.17 ^{ef}
S.Kordfan	SK/A6	8.793^{eh}	2.143^{fh}	24.53^{g}	18.91 ^{g}	12.07 ^{ce}	33.56 ^{f}
S.Kordfan	SK/A7	8.510g^{h}	2.283^{eg}	36.13^{cd}	22.49 ^{bd}	12.68 ^{bd}	17.91 ^{ij}
S.Kordfan	SK/A8	9.370^{ce}	2.350^{eg}	22.52^{hi}	18.50 ^{gh}	13.50 ^{b}	33.76 ^{f}
S.Kordfan	SK/A9	9.270^{cf}	2.163^{fh}	36.70^{bd}	22.28 ^{cd}	11.26 ^{df}	18.32 ^{i}
S.Kordfan	SK/A10	12.000^{a}	1.537^{j}	49.91^{a}	23.37 ^{b}	15.74 ^{a}	2.56 ^{l}
Mean (SK)		8.76	2.23	31.76	21.466	10.538	25.781
N.Kordfan	NK/A11	8.550^{gh}	2.220^{fh}	37.26^{bc}	25.25^{a}	10.82^{ef}	15.91^{j}
N.Kordfan	NK/A12	9.510^{cd}	2.283^{eg}	38.01^{b}	26.13^{a}	10.35^{f}	13.71^{k}
Mean (NK)		9.030	2.2515	37.635	25.69	10.585	14.81
Gezira	GZ/A13	10.883^{b}	3.303^{a}	17.77^{n}	16.91 ^{i}	13.62^{b}	37.51^{ce}
Gezira	GZ/A14	8.597^{gh}	2.253^{eg}	25.97^{f}	21.83^{d}	11.44^{df}	29.91^{g}
Gezira	GZ/A15	8.707^{fh}	2.780^{cd}	20.30^{kl}	18.52^{gh}	12.51^{bd}	37.18^{ce}
Gezira	GZ/A16	8.737^{fh}	2.343^{eg}	27.22^{ef}	23.02^{bc}	10.47^{f}	28.21^{g}
Mean (GZ)		9.231	2.66975	22.815	20.07	12.01	33.2025
Gedarif	GF/A17	6.727^{j}	2.320^{eg}	18.25^{mn}	17.80^{hi}	8.93^{g}	45.98^{a}
Gedarif	GF/A18	9.527^{cd}	2.417^{ef}	23.66^{gh}	20.80^{ef}	8.03^{gi}	35.56^{df}
Gedarif	GF/A19	10.917^{b}	3.147^{ab}	19.94^{kl}	19.08^{g}	6.95^{i}j	39.97^{b}
Mean (GF)		9.057	2.628	20.61667	19.22667	7.97	40.50
W. Nile	WN/A20	9.060^{cg}	2.647^{c}	22.16^{i}j	18.68^{gh}	11.29^{df}	36.16^{de}
W. Nile	WN/A21	8.520^{gh}	1.840^{hj}	35.52^{d}	21.82^{d}	8.53^{gh}	23.77^{h}
Mean (WN)		8.79	2.2435	28.84	20.25	9.91	29.965
Blue Nile	BN/A22	8.357^{h}	2.377^{ef}	20.96^{jk}	18.25^{gh}	11.94^{ce}	38.91^{bc}
Blue Nile	BN/A23	9.600^{c}	2.413^{df}	19.51^{lm}	18.46^{gh}	12.58^{bd}	36.53^{ce}
Mean (BN)		8.9785	2.395	20.235	18.355	12.26	37.72
Overall mean		8.93	2.37	28.22	20.9	10.56	29.44
± SE		0.16	0.12	0.45	0.32	0.43	0.76
C.V%		3.6	8.9	2.8	2.6	7.1	4.5

*Means followed by the same letter/s are not significantly different using DMRT

A10) had the lowest value, while the GZ landrace (PR/A17) had the highest value. These values were within the range of 19 % reported by Ardabili et al. (2011) for pumpkin seeds and within the range of the same value obtained by Elinge et al. (2012), which was 28.03 %, and the value of 25 % reported by Qamar et al. (2019).

Fatty acid compositions of seeds belonging to different landraces and locations are shown in Table 2. According to obtained results, dominant fatty acids of landraces pumpkin seed oils were linoleic, oleic, palmitic, and stearic acids. Other fatty acids (palmitoleic acid, myristic acid, arachidic acid and behenic acid) were determined in small quantities. The major and most representative fatty acid in all analyzed landraces is linoleic which was exhibited in high percentage 41.58 % obtained by White Nile landrace (WN/A20) and low percentage 33.39 % obtained by Blue Nile landrace (BN/A23) and followed by oleic acid which was ranged from 11.03 % to 33.59 obtained by W. Nile landrace (WN/A20) and N. Kordofan landrace (NK/A11), palmitic which was ranged from 16.71 % to 27.56 % obtained by N. Kordofan landrace (NK/A11) and Gazira landrace (GZ/A13), and stearic acid which was ranged from 2.24 % to 15.69 % obtained by Gazira landrace (GZ/A13) and Gedarif landrace (GF/A19), respectively.

In the present study the content of most the fatty acids in the analyzed landraces pumpkin seed oils mainly affected by soil type in which the plants are grown, climate and state of ripeness; interaction between these factors was found to be significant for the content of polyunsaturated fatty acid (PUFA) percentage (37.02 %) and monounsaturated fatty acid percentages (27.50 %) ;and the total of unsaturated fatty acid (TUFA) which was ranged from 55.2 % to 70.2 %; while the total of saturated fatty acids (TSFA) content amounted in all landraces was ranged from 29.45 % to 45.27 %, that indicate the landraces pumpkin seeds oil in almost all samples collected from different locations in Sudan is highly unsaturated oil. It worth mention that the Sudanese landraces pumpkin seeds oil had a significant amount of squalene content in the all investigated landraces, which was ranged from 0.34 % to 1.29 % these values highly lower than values registered by Gorjanović et al. (2011), this non-glyceride component has been proposed to be an important part of the diet as it may be a chemo-preventative substance that protects people from cancer (Smith, 2000); that makes it contributes to the significant nutritive and medicinal value of the of the Sudanese landraces pumpkin seeds oil. With regard to location no significant difference in the percentages of fatty acids among the samples from same area but slightly differ from area to another, oil extracted from Gedarif samples (GF/A17, GF/A18 and GF/A19) had a lower average of linoleic acid content (34.07 %) and oil extracted from White Nile samples (WN/A20 and WN/A21) had a higher average of linoleic acid content (39.90 %). White Nile area had the lowest average on the oleic acid (14.51 %), while North Kordofan area had the highest average (29.05 %) in the investigated races. Palmitic acid average was lowest in the investigated races from South Kordofan (18.94%), while highest in the Gedarif races (24.93 %). Stearic acid was lowest on the average of investigated races from Gazira which had (10.855 %), while was highest in investigated races from Gedarif (14.50 %). The variability in the fatty acid composition is very high resulting from a broad genetic diversity; Study of the average fatty acid compounds studied by Soltani, (2016) on different accessions of *Cucurbita pepo* L. in Iran showed that oleic acid (39.24 %) was higher than linoleic (38.76 %), palmitic (11.09 %), and stearic (5.37 %), other study reported by Aktaş et al. (2018) on two types of pumpkin seeds belonging to *C. pepo* species, revealed that linoleic acid is an essential fatty acid followed by oleic acid which were ranged from (40.04 %-43.19 %) and (37.48 % -39.66 %) respectively, which were conforms with the present study. Linoleic acid is an essential fatty acid for humans as it is required for the formation of cellular membranes, vitamin D, and various hormones (Fruhwirth & Hermetter, 2007).

Table 2: Means (%) of fatty acids composition of 23 pumpkin landraces collected from different areas of Sudan

Location	Race	Myristic C14:0	Pentadecanoic C15:0	Palmitoleic C16:1n-7	Palmitic C16:0	Margaric C17:0	Linoleic C18:2	Oleic C18:1	Others*
South Kordfan	SK/A1	0.25	0.02	0.16	20.41	0.22	36.16	21.10	21.68
South Kordfan	SK/A2	0.17	0.03	0.26	17.70	0.37	33.62	33.20	14.65
South Kordfan	SK/A3	0.12	0.02	0.18	16.87	0.36	39.98	28.84	13.63
South Kordfan	SK/A4	0.17	0.02	0.12	21.11	0.20	38.38	23.85	15.70
South Kordfan	SK/A5	0.17	0.02	0.12	20.68	0.19	36.11	26.89	15.82
South Kordfan	SK/A6	0.17	0.01	0.13	21.29	0.23	34.95	28.32	14.90
South Kordfan	SK/A7	0.14	0.02	0.23	17.56	0.30	37.77	29.74	14.24
South Kordfan	SK/A8	0.13	0.01	0.16	19.08	0.21	35.96	30.62	13.83
South Kordfan	SK/A9	0.21	0.02	0.19	17.68	0.36	35.95	30.70	14.89
South Kordfan	SK/A10	0.13	0.02	0.22	17.04	0.31	34.87	32.50	14.91
Mean (SK)		0.17	0.02	0.18	18.87	0.28	36.38	28.58	15.52
North Kordfan	NK/A11	0.24	0.03	0.18	16.71	0.29	34.39	33.59	14.57
North Kordfan	NK/A12	0.31	0.03	0.21	22.44	0.34	34.14	24.52	18.01
Mean (NK)		0.28	0.03	0.20	19.58	0.32	34.27	29.10	16.22
Gezira	GZ/A13	0.35	0.03	0.25	27.56	0.41	36.03	22.7	12.67
Gezira	GZ/A14	0.40	0.03	0.26	24.57	0.43	35.31	18.02	20.98.
Gezira	GZ/A15	0.45	0.04	0.30	22.16	0.43	34.06	23.7	18.86
Gezira	GZ/A16	0.44	0.04	0.27	24.45	0.32	36.81	16.28	21.39
Mean (GZ)		0.41	0.07	0.27	24.69	0.39	35.55	20.18	18.44
Gedarif	GF/A17	0.49	0.03	0.29	22.97	0.4	33.62	23.38	18.82
Gedarif	GF/A18	0.39	0.04	0.22	25.74	0.28	34.9	17.07	21.36
Gedarif	GF/A19	0.37	0.03	0.22	26.08	0.32	33.69	16.67	22.62
Mean (GF)		0.41	0.03	0.24	24.93	0.33	34.07	19.04	20.95
White Nile	WN/A20	0.42	0.04	0.3	24.3	0.38	41.58	11.03	21.95
White Nile	WN/A21	0.36	0.05	0.53	21.28	0.91	38.23	18.00	20.64
Mean (WN)		0.39	0.05	0.42	22.64	0.65	39.91	14.51	21.43
Blue Nile	BN/A22	0.59	0.05	0.4	24.25	0.44	38.62	13.37	22.28
Blue Nile	BN/A23	0.65	0.05	0.43	23.32	0.54	33.39	22.09	19.53
Mean (BN)		0.62	0.05	0.42	23.79	0.49	36.00	17.73	20.90
Overall mean		0.31	0.03	0.25	21.53	0.36	36.02	23.75	17.75
SD		0.15	0.01	0.10	3.25	0.15	2.19	6.57	01.77

*Other fatty acids include Stearic (C18:0), Linolenic (C18:3), and Gadoleic (C20:1)

Table 2: Means (%) of fatty acids composition of 23 pumpkin landraces seed collected from different areas of Sudan

Location	Race	Stearic	Arachidic	Behenic	Squalene	PUFA	MUSAF	SFA
South Kordfan	SK/A1	12.44	1.30	0.50	1.25	37.22	22.00	36.53
South Kordfan	SK/A2	11.38	1.07	0.30	0.46	34.21	34.06	31.21
South Kordfan	SK/A3	10.94	0.76	0.24	0.35	40.73	29.47	29.45
South Kordfan	SK/A4	12.80	0.99	0.26	0.49	39.52	24.32	35.67
South Kordfan	SK/A5	12.34	0.97	0.24	0.41	37.56	27.42	34.61
South Kordfan	SK/A6	12.20	0.99	0.23	0.38	35.69	26.81	36.60
South Kordfan	SK/A7	12.03	1.01	0.23	0.34	37.77	30.37	31.52
South Kordfan	SK/A8	11.43	0.86	0.22	0.39	36.43	31.09	32.09
South Kordfan	SK/A9	12.77	0.95	0.18	0.49	35.95	31.30	32.26
South Kordfan	SK/A10	11.95	1.05	0.33	0.46	35.21	34.21	30.06
Mean (SK)		12.028	0.939	2730.	0.513	37.02	29.10	33.00
North Kordfan	NK/A11	12.01	0.83	0.32	0.39	34.73	34.18	30.62
North Kordfan	NK/A12	12.85	1.72	0.34	0.52	35.92	25.35	38.21
Mean (NK)		12.43	1.275	0.33	0.455	35.325	29.765	34.415
Gezira	GZ/A13	2.24	1.94	0.57	0.71	37.14	23.66	38.39
Gezira	GZ/A14	14.41	1.78	0.48	0.68	36.44	20.42	42.46
Gezira	GZ/A15	12.65	2.09	0.54	0.89	35.58	24.81	36.44
Gezira	GZ/A16	14.12	2.05	0.50	0.88	38.16	18.81	42.15
Mean(GZ)		10.855	1.965	0.522	0.790	36.83	21.92	39.86
Gedarif	GF/A17	12.81	2.27	0.56	0.96	34.96	24.34	39.74
Gedarif	GF/A18	15.04	1.76	0.47	0.80	35.9	19.3	44.00
Gedarif	GF/A19	15.67	1.95	0.48	0.81	34.53	19.39	45.27
Mean (GF)		14.50	1.99	0.50	0.85	35.13	21.01	43.00
White Nile	WN/A20	13.62	1.96	0.47	0.93	43.34	14.4	41.59
White Nile	WN/A21	12.90	1.91	0.37	0.97	39.65	21.29	38.09
Mean(WN)		13.26	1.93	0.42	0.95	41.49	17.84	39.84
Blue Nile	BN/A22	13.43	2.52	0.6	1.18	40.33	16.34	42.15
Blue Nile	BN/A23	12.12	2.79	0.83	1.29	34.43	23.49	40.79
Mean (BN)		12.27	2.65	0.715	1.24	37.38	19.92	41.47
Overall mean		12.35	1.544	0.403	0.697	37.02	25.08	36.95
SD		2.487	0.0	0.162	0.304	2.352	5.738	4.824

Table 3 shows the dimensions of whole landrace pumpkin seeds. The length of seeds ranged from 11.00 mm to 20.00 mm, which was observed between the GZ (GZ/A13) and the SK (PR/A10). The width of the seeds ranged from 4.30 cm, which was obtained by the GZ (GZ/A15) and the GF (GF/A17), to 9.30 cm, which was obtained by the GF (GF/A19). The thickness of the seeds ranged from 1.333 mm, which was obtained by the BN (PR/A23), to 4.667 mm, which was obtained by the SK (SK/A9) and the NK (NK/A12). Milani et al. (2007) reported that seeds had lengths ranging from 12 to 14 mm, widths between 7 and 13 mm, and thicknesses ranging from 2.0 to 4.0 mm, which is moderately lower than current results.

As shown in Table 3, sample from Blue Nile (BN/A23) recorded a minimum 100 seed mass of 12.55 g. The maximum 100-seed mass was obtained from SK (SK/A7) and SK (SK/A2), with 32.2 g and 27.52 g, respectively. These results were higher than the range of 1000-seed mass recorded for different genotypes reported by Türkmen et al. (2017), which ranged from 168.9 to 196.6 g. Can-Cauich et al. (2021) reported that two pumpkin species had values of 12.00 and 25.01 g per 100 seeds, which is in accordance with the current study. Furthermore, the minimum and maximum mass of whole seed, kernel, and hull of landraces were determined by BN (BN/A22) and SK (SK/A7), which ranged from 0.125 g WN (WN/A22) to 0.322 g for SK (SK/A7), 0.0802 g WN (WN/A22) to 0.2471 g for SK (SK/A7), and 0.0342 g SK (SK/A7) to This difference is attributed to the seeds' dimensions and to embryo quality. According to the calculation of the hull to kernel ratio, the lowest ratio was observed in BN BN/A23 (1.9:1), while the highest ratio was observed in BN BN/A22 (4.02 : 1). The variability in seed index parameters between the studied samples can be attributed to the variability of production area, soil type, and location genotype.

The content of kernel and hull differed significantly ($p < 0.001$), as shown in Table (3): the highest percent of kernel with WN WN/A20 (78.45 %) and the lowest percent with BN BN/A23 (63.92 %), as it had a lower percent of kernel; BN BN/A23 had the highest percent of hull (32.36 %), while WN landrace WN/A22 had the lowest percent (18.68 %).The variation in physical characteristics of landrace seeds may be due to location, type of soil, and climate. Manda et al. (2018) discovered that the husk content of pumpkin seeds was 26.75 %.

According to Table 4, the seeds from 23 landrace pumpkin seeds were a significant source of minerals. The most dominant minerals were potassium (K), phosphorus (P), calcium (Ca), sodium (Na), iron (Fe), zinc (Zn), magnesium (Mg), and manganese (Mn), respectively ($p < 0.05\%$). Potassium is the most abundant element found in the seed. The highest level of K was determined in the GF (GF/A17) at 1100 mg 100 g^{-1}, and the lowest level was found in the GZ (GZ/A14) at 946 mg 100 g^{-1}. These values are higher than those reported by Rezig et al. (2012), which were 886.56 g 100 g^{-1}. Phosphorus (P) and calcium (Ca) were the most abundant minerals, with values varying from 209 mg 100 g^{-1} for the GF (GF/A17) to 374 g 100 g^{-1} for the SK (SK/A5) and from 130 g 100 g^{-1} for the BN (BN/A23) to 147 g 100 g^{-1} for the SK (SK/A1), respectively. Phosphates play key roles as buffers that prevent changes in the acidity of body fluids. Calcium has an important role in preventing rickets, osteoporosis, and tachycardia (Mergedus et al., 2015). The values of phosphorus and calcium obtained from landrace pumpkin seeds were higher than those reported by Elinge et al. (2012), which were 47.680.04 mg 100 g^{-1} and 9.78 mg 100 g^{-1}, respectively, and within the range of values reported by Amoo et al. (2004) for P (224.14 mg 100 g^{-1}) and Ca (29.47 mg 100 g^{-1}). The concentration of sodium (Na) in landrace samples ranged from 26.99 mg 100 g^{-1} to 38.6 mg 100 g^{-1} determined by GZ (GZ/A13) and SK (SK/A4), respectively; this element is needed by the body to regulate blood pressure and blood volume.

The values obtained were within the range of the result reported by Amoo et al. (2004), 29.69 mg 100 g^{-1}, and lower than that reported by Rezig et al. (2012), which was 356.75 mg 100 g^{-1}.

Iron contents (Fe) were found to range from 11.2 mg 100 g^{-1} to 17.3 mg 100 g^{-1} for the NK (NK/A12) and the GZ (GZ/A16). Iron has important functions in the body. It carries oxygen through the blood (as a part of the red blood cell) to muscles and the brain, making it crucial for both mental and physical health and performance (Abbaspour et al., 2014). According to a joint FAO/WHO report (2005), iron deficiency is the most common nutritional disorder in the world. The daily value (DV) of iron is 18 mg, and one ounce (28 g) of pumpkin seeds contains 2.5 mg of iron, which is 14 % of the DV according to the FDC (2019). As a result, the iron content values obtained from this study corresponded to the DV. The values obtained in this study were higher than those reported by Elinge et al. (2012), which were 3.75 mg 100 g^{-1}, and Amoo et al. (2004), which were 4.27 mg 100 g^{-1}, and within the ranges of 13.66 mg 100 g^{-1} and 15.37 mg 100 g^{-1} reported by Alfawaz (2004) and Rezig et al. (2012), respectively.

Landraces showed respectable amounts of magnesium (Mg), which had a lower value in the SK (SK/A8), which was 3.9 mg 100 g^{-1}, and a higher value in the WN (WN/A22), which was 16.9 mg 100 g^{-1}. According to the Office of Dietary Supplements (ODS, 2022), seeds are rich sources of magnesium. Mg has a role in the regulation of blood sugar levels and is involved in energy metabolism and protein synthesis (Mir-Marqures et al., 2015). The values obtained were lower than those reported by Elinge et al. (2012) and Rezig et al. (2012), which were 67.41 mg 100 g^{-1} and 146.13 mg 100 g^{-1}, respectively.

This study determined the zinc (Zn) content of landrace seeds ranged from 8.4 mg 100 g^{-1} to 16.2 mg 100 g^{-1}, which were obtained from the GF (GF/A19) and the SK (SK/A6), respectively. Zinc is a vital component of white blood cells (WBC), which fight infections and prevent susceptibility to flu, colds, and other viral infections such as COVID-19. Several clinical trials are currently investigating the use of zinc supplementation alone or in combination with hydroxychloroquine for the prevention and treatment of COVID-19 (Neha et al., 2020). The Recommended Dietary Allowance (RDA) for elemental zinc is 11 mg daily for men and 8.0 mg for non-pregnant women, according to the Office of Dietary Supplements (OSD, 2022). Therefore, Sudanese landrace pumpkin seeds are a rich source of zinc. These results were higher than those reported by Alfwaz et al. (2004) (1.09 g 100 g^{-1}) and Amoo et al. (2004) (3.98 mg 100 g^{-1}), but lower than the 25.19 mg 100 g^{-1} reported by Rezig et al. (2012).

Manganese (Mn) was found to be the least abundant among all the minerals studied in these samples, which ranged from 3.3 mg 100 g^{-1} to 6.8 mg 100 g^{-1} obtained by the GZ (GZ/A15) and the SK (SK/A10) seeds, respectively. The values obtained were higher than those obtained by Elinge et al. (2015) (0.060.01 mg 100 g^{-1}) and Amoo et al. (2004) (1.79 mg 100 g^{-1}), and within the range of the value (3.42 mg 100 g^{-1}) obtained by Rezig et al. (2012). The differences in mineral composition could be due to the climate, species, or soil type.

Table 3: Means of seed characters of 23 pumpkin landraces collected from different areas of Sudan

Location	Pumpkin Race pear Area	Length mm	Thickness mm	Width mm	Mass 100 Seed (g)	Mass Whole Seed (g)	Mass Kernel (g)	Mass Hull (g)	% Kernel	% hull	Hull: Kernel
S.Kordfan	SK/A1	13.00^{eh}	2. 667^{d}	5. 70^{d}	17.40^{jk}	0.1740^{jk}	0.1314	0.0372	75.57^{ad}	21.39^{gh}	3.5:1
S.Kordfan	SK/A2	17.00^{b}	3. 667^{ad}	6. 00^{d}	27.52^{b}	0.2752^{b}	0.1794	0.0828	65.19^{h}	30.09^{b}	2.1:1
S.Kordfan	SK/A3	15.00^{be}	2. 667^{d}	5. 00^{e}	25.19^{d}	0.2519^{d}	0.1905	0.0626	75.60^{ad}	24.85^{d}	3.1:1
S.Kordfan	SK/A4	13.67^{dg}	2. 667^{d}	4. 70^{e}	15.7l^{l}	0.1571 ^{l}	0.1191	0.0365	75.83^{ad}	23.24^{e}	3.2:1
S.Kordfan	SK/A5	12.33^{gh}	2. 667^{cd}	5. 70^{d}	18.25^{hi}	0.1825^{hi}	0.1300	0.0449	71.31^{fg}	24.63^{d}	2.8:1
S.Kordfan	SK/A6	15.67^{bd}	3. 333 ^{bd}	5. 00^{e}	20.21^{g}	0.2020^{g}	0.1475	0.0463	73.06^{cf}	22.93^{ef}	3.1:1
S.Kordfan	SK/A7	19.67^{a}	4. 333^{ab}	8. 30^{b}	32.21^{a}	0.3222^{a}	0.2471	0.0751	76.69^{ab}	23.31^{e}	3.2:1
S.Kordfan	SK/A8	16.33^{bc}	4. 333^{ab}	7. 30^{c}	15.45^{i}	0.1544 ^{l}	0.1175	0.0362	76.10^{ac}	23.44^{e}	3.2:1
S.Kordfan	SK/A9	19.67^{a}	4. 667^{a}	9. 30^{a}	26.03^{c}	0.2605^{c}	0.1873	0.0736	71.90^{eg}	28.27^{c}	2.5:1
S.Kordfan	SK/A10	20.00^{a}	3. 333^{bd}	9. 00^{a}	24.40^{e}	0.2440^{e}	0.1841	0.0621	75.93^{ac}	25.45^{d}	2.9:1
Mean (SK)		16.234	3.433	6. 60	22.237	0.22238	0.163	0.055	73.718	24.76	
N.Kordfan	NK/A11	12.67^{fh}	3. 667^{ad}	5. 00^{e}	22.92^{f}	0.2291^{f}			74.10b^{f}	22.92^{ef}	3.2:1
N.Kordfan	NK/A12	12.67^{fh}	4. 667^{a}	5. 30^{e}	23.42^{f}	0.2342^{f}	0.1697	0.0525	74.84^{be}	22.08^{fg}	3.3:1
Mean(NK)		12.67	4.167	5. 15	23.17	0.23165	0.1752	0.0517	74.47	22.5	
Gezira	GZ/A13	11.00 ^{h}	4. 00^{abc}	4. 70f	20.22^{g}	0.2022^{g}			73.00^{cf}	20.47 ^{i}	3.5:1
Gezira	GZ/A14	11.33 ^{h}	3. 33^{bd}	5. 00^{e}	17.58^{ik}	0.1757^{ik}	0.1467	0.0414	72.74^{df}	23.69^{e}	3.07:1
Gezira	GZ/A15	11.00^{h}	3. 00^{cd}	4. 30^{f}	16.12 ^{l}	0.1612 ^{l}	0.1277	0.0407	71.49^{fg}	22.30^{f}	3.2:1
Gezira	GZ/A16	12.33^{gh}	3. 00^{cd}	5. 00^{e}	16.89^{k}	0.1689^{k}	0.1151	0.0359	69.23^{g}	22.27^{f}	3.1:1
Mean(GZ)		11.4	3.33	4. 75	17.70	0.177	0.1169	0.0376	71.615	22.18	
Gedarif	GF/A17	12.00^{gh}	3. 667^{ad}	4. 30^{f}	15.71 ^{l}	0.1575 ^{l}			74.75^{be}	23.29^{e}	3.2:1
Gedarif	GF/A18	11.67^{gh}	3. 000^{cd}	4. 70^{f}	15.84 ^{l}	0.1584 ^{l}			76.77^{ab}	22.10^{fg}	3.4:1
Gedarif	GF/A19	12.67^{fh}	3.000^{cd}	5. 00^{e}	14.02^{m}	0.1402^{m}	0.1177	0.0375	74.13^{bf}	25.06^{d}	2.9:1
Mean (GF)		12.1	3. 22	4. 66^{f}	15.19	0.15	0.1216	0.035	75.21	23.48	
W. Nile	WN/A20	12.33^{gh}	3. 000^{cd}	6.00^{d}	17.98^{hj}	0.1797^{hj}	0.1039	0.0349	78.45^{a}	20.64 ^{hi}	3.8:1
W. Nile	WN/A21	14.67^{cf}	3. 333^{bd}	5. 70^{d}	27.30^{b}	0.2729^{b}			75.75^{ad}	23.23^{e}	3.2:1
Mean(WN)		13.5	3. 16	5. 85^{d}	22.64	0.2263			77.1	21.94	
Blue Nile	BN/A22	12.00^{gh}	3. 000^{cd}	4. 70^{d}	18.38^{h}	0.1838^{h}	0.141	0.0371	75.24^{bd}	18.68^{j}	4.02:1
Blue Nile	BN/A23	12.00^{gh}	1. 333^{e}	4. 70^{d}	12.55 ^{n}	0.1255 ^{n}	0.2067	0.0634	63.92^{h}	32.36^{a}	1.9:1
Mean(BN)		12.00	2.16	4. 70^{d}	15.46	0.1546			69.58	25.52	
Overall mean		13.94	3. 32	0.5681	20.05	0.2006			73.55	23.77	
SE±		0.0656	0.039	0.044	0.233	0.002	0.1382	0.0342	0.92	0.28	
C.V%		8.1	20.2	13.5	2.0	2.0	0.0802	0.0406	2.2	2.1	

*Means followed by the same letter/s are not significantly different using DMRT

Table 4: Means of minerals of 23 pumpkin landraces seeds* collected from different areas of Sudan (mg 100 g^{-1})

Location	Pumpkin	Ca	Fe	K	Mg	Mn	Na	P	Zn
South Kordfan	SK/A1	147 ^{a}	13.9^{be}	992^{b}	7.3^{cd}	4.8^{bf}	35.9^{ae}	370^{a}	12.5 ^{bg}
South Kordfan	SK/A2	143^{ab}	15.4^{ad}	989^{b}	7.1^{de}	6.2^{ac}	37.7^{ac}	373^{a}	13.4 ^{ae}
South Kordfan	SK/A3	143^{ab}	15.4^{ad}	989^{b}	7.1^{de}	5.3^{ae}	37.7^{ac}	373^{a}	15.5 ^{ab}
South Kordfan	SK/A4	138^{ch}	14.2^{ae}	959^{b}	5.5^{df}	4.9^{bf}	38.6^{a}	367^{a}	13.6 ^{ae}
South Kordfan	SK/A5	141^{bc}	14.7^{ae}	981^{b}	4.6^{df}	4.9^{bf}	36.4^{ad}	374^{a}	14.6 ^{ad}
South Kordfan	SK/A6	139^{be}	15.4^{ad}	954^{b}	6.0^{df}	6.2^{ac}	35.3^{ae}	264^{g}	16.21 ^{a}
South Kordfan	SK/A7	140^{bd}	12.6^{df}	955^{b}	5.6^{df}	5.9^{ac}	36.2^{ad}	276^{fg}	14.8 ^{ac}
South Kordfan	SK/A8	137^{ch}	15.1^{ad}	978^{b}	3.9 ^{f}	4.8^{bf}	38^{ab}	282^{eg}	12.8 ^{bf}
South Kordfan	SK/A9	136^{dh}	12.8^{df}	982^{b}	5.7^{df}	6.4^{ab}	33.2^{af}	325^{bc}	12.0 ^{ch}
South Kordfan	SK/A10	139^{be}	9.85^{f}	977^{b}	5.6^{df}	6.8 ^{a}	30.3^{eg}	304^{ce}	12.4 ^{bg}
Mean (SK)		140.3	13.935	975.6	5.84	5.62	35.93	330.8	13.781
North Kordfan	NK/A11	134^{gi}	12.8^{df}	1042^{a}	4.5^{ef}	5.6^{ad}	32.8^{bf}	314^{cd}	12.2^{ch}
North Kordfan	NK/A12	135^{fi}	11.2^{ef}	978b	5.7^{df}	4.4^{cf}	28.5^{fg}	272^{fg}	9.8^{fi}
Mean (NK)		134.5	12	1010	5.1	5	30.65	293	11
Gezira	GZ/A13	137^{ch}	12.1^{df}	987^{b}	5.6^{df}	3.6^{ef}	26.99^{g}	285^{eg}	9.9^{fi}
Gezira	GZ/A14	137^{ch}	12.7^{df}	946^{b}	4.5^{ef}	5.2^{af}	31.2^{dg}	320 ^{c}	10.6^{ei}
Gezira	GZ/A15	139^{be}	16.9^{ab}	983^{b}	5.9^{df}	3.3^{f}	34.8^{ae}	265^{fg}	9.9^{fi}
Gezira	GZ/A16	136d^{h}	17.3^{a}	977^{b}	4.8^{df}	3.4^{f}	32.5^{cf}	271^{fg}	9.4^{gi}
Mean (GZ)		137.25	14.75	973.25	5.2	3.875	31.3725	165.1857	9.95
Gedarif	GF/A17	133^{hi}	16.4^{ac}	1100^{a}	6.2^{df}	3.4^{f}	33.98^{af}	209 ^{h}	16.22 ^{a}
Gedarif	GF/A18	133^{h}i	14.7^{ae}	987^{b}	5.8^{df}	3.9^{df}	34.4^{ae}	319 ^{c}	8.99^{hi}
Gedarif	GF/A19	134^{gi}	14.5^{ae}	982^{b}	6.2^{df}	4.4^{cf}	35.5^{ae}	303^{ce}	8.4 ^{i}
Mean (GF)		133.3	15.2	1023	6.06	3.9	34.62	277	11.203
White Nile	WN/A20	138ch	14.9^{ad}	987^{b}	9.5^{c}	5.1^{af}	30.9^{dg}	348^{ab}	11.11^{ei}
White Nile	WN/A21	135ei	12.2^{df}	992^{b}	13.9^{b}	3.6^{ef}	32.95^{bf}	352 ^{a}	11.5 ^{di}
Mean(WN)		136.5	13.55	989.5	11.7	4.35	31.9	350	11.305
Blue Nile	BN/A22	134^{gi}	12.0^{df}	961^{b}	16.9^{a}	3.4^{f}	30.8^{dg}	291^{df}	8.67 ^{i}
Blue Nile	BN/A23	130^{i}	13.0^{cf}	1015^{a}	13.0^{b}	3.6^{ef}	34.4^{ae}	350^{a}	9.53 ^{fi}
Mean (BN)		132	12.5	988	14.95	3.5	32.6	320.5	9.1
Overall mean		137.4	13.91	986.8	7.00	4.75	33.88	313.3	11.90
SE±		1.51	1.01	31.17	0.78	0.545	1.65	8.26	0.97
C.V%		1.9	12.6	5.5	19.4	19.9	8.5	4.6	14.2

*Means followed by the same letter/s are not significantly different using DMRT

4 CONCLUSİONS

The results of this study show that Sudanese landrace pumpkin seeds are rich in oil, protein, and minerals. The data revealed that there were significant differences in seed characteristics and mineral content among landraces from six states; the results of the oil content determination revealed that pumpkin seeds can be used as a promising source of edible oil and protein for food fortification. More studies are recommended on pumpkin seeds, and more attention and care should be taken for pumpkin cultivation to produce seeds for oil production.

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Chemical profiling of male date palm (*Phoenix dactylifera* L.) leaflets in El M'Ghair region, Algeria: Insights into total phenols, flavonoids, proteins, and total sugars

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Chemical profiling of male date palm (*Phoenix dactylifera* L.) leaflets in El M'Ghair region, Algeria: Insights into total phenols, flavonoids, proteins, and total sugars

Abstract: This study extensively investigates the biochemical composition of male date palm leaflets (*Phoenix dactylifera* L.) from the El M'Ghair region in Algeria, focusing on the cultivars Deglet Nour, Degla Beida, and Ghars. The chemical analysis centers on proteins, total sugars, total phenols, and flavonoids, revealing significant differences in these compounds among the three cultivars. 'Degla Beida' exhibits the highest protein content (39.09 ± 4.58 %), contrasting with 'Ghars', which records the lowest (18.33 ± 5.35 %). Total sugars exhibit variability among cultivars, with 'Ghars' displaying the highest levels (74.54 ± 6.92 %) and 'Degla Beida' the lowest (46.28 ± 9.11 %). The production ratios of crude methanolic aqueous extracts show consistent extraction efficiencies across cultivars. Abundant phenolic compounds, notably in 'Degla Beida', and significant variations in flavonoid content are observed. Specifically, 'Degla Beida' and 'Deglet Nour' boast the highest flavonoid levels (36.02 ± 3.79 and 30.18 ± 3.88 µg QE. mg^{-1}, respectively), whereas 'Ghars' demonstrates the lowest (22.02 ± 2.31 µg QE. mg^{-1}). The correlation matrix reveals positive associations among proteins, total phenols, and flavonoids, contrasting with a strong negative association with total sugar content.

Key words: *Phoenix dactylifera* L., leaflets, biochemical composition, El M'Ghair region

Kemijski profil lističev moških datljevcev (Phoenix *dactylifera* L.) na območju El M'Ghair v Alžiriji: Vpogled v vsebnost celokupnih fenolov, flavonoidov, proteinov in celokupnih sladkorjev

Izvleček: V raziskavi je bila preučevana biokemična sestava lističev moških dateljevih palm (*Phoenix dactylifera* L.) z območja El M'Ghair v Alžiriji, s poudarkom na sortah Deglet Nour, Degla Beida in Ghars. Kemijske analize so bile osredotočene na vsebnosti beljakovin, celokupnih sladkorjev, celokupnih fenolov in flavonoidov. Analiza je odkrila značilne razlike v vsebnostih teh spojin med tremi sortami. 'Degla Beida' je imela največjo vsebnost beljakovin (39,09 ± 4,58 %), v nasprotju s 'Ghars', ki jih je vsebovala najman (18,33 ± 5,35 %). Vsebnost celokupnih sladkorjev se je med sortami razlikovala, pri čemer je imela 'Ghars' največjo vsebnost (74,54 ± 6,92 %) in 'Degla Beida' najmanjšo (46,28 ± 9,11 %). Ekstrakcije preučevanih sestavin z metanolom in vodo se je izkazala za učinkovito pri vseh sortah. Pri 'Degla Beida'je bila ugotovljena velika vsebnost celokupnih fenolov, vsebnost flavonoidov je med sortami značilno variirala. 'Degla Beida' in 'Deglet Nour' sta imeli največjo vsebnost flavonoidov (36,02 ± 3,79 in 30,18 ± 3,88 µg QE. mg^{-1}), 'Ghars' je imela najmanjšo (22,02 ± 2,31 µg QE. mg^{-1}). Korelacija je odkrila pozitivne povezave v vsebnosti beljakovin, celokupnih fenolov in flavonoidov, a zelo negativno povezavo z vsebnostjo celokupnih sladkorjev.

Ključne besede: *Phoenix dactylifera* L., lističi, biokemična sestava, območje El M'Ghair

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1 INTRODUCTION

Phoenix dactylifera L., commonly known as the date palm, is of enormous importance both globally and specifically in Algeria. Its cultivation plays an important role in supporting the Algerian Sahara population, significantly contributing to food security and rural livelihoods (Mansouri et al., 2005). Moreover, the palm has a rich cultural and historical significance, deeply rooted in the traditions of different societies. This woody perennial plant, which belongs to the Arecaceae family, is recognized as one of the oldest and most widely cultivated fruit trees in arid and semi-arid regions, particularly in the Gulf countries of the Middle East (Al Harthi et al., 2015). Globally, Algeria ranks fourth among the largest palm producers and ninth in palm fruit exports (Benouamane et al., 2022). The date palm is also considered one of the important plants that can tolerate salinity in its final stages. Dates are considered one of the most important crops and non-traditional commodities that can be exported and consumed locally with good financial returns. The area of date palm cultivation in the world is increasing daily, especially in desert areas with sandy soil (Shareef et al., 2023).

Date palm fruits are known for their numerous health and nutritional benefits attributed to their rich phenolic content and strong antioxidant capacity (Daoud et al., 2019). Many studies are available on the phenolic composition of certain cultivars of date palm seeds and fruits (Abu-Reidah et al., 2017; Bentrad et al., 2017; Habib et al., 2014; Hilary et al., 2020; Jenny & Fereidoon, 2019; Ma et al., 2019; Mansouri et al., 2005; Messaoudi et al., 2013), and these by-products have shown promising results in pharmaceutical formulations, as they exhibit antifungal, antioxidant, and anti-inflammatory activities and anti-diabetic. The nutritional value of these fruits has been explored, leading to the identification of their various benefits when incorporated into animal diets (Al-Shahib & Marshall, 2003). However, it enjoys scarce studies on the leaves of *Phoenix dactylifera* L., specifically in the phytochemical and nutritional fields.

Determining the content of total phenolic compounds and flavonoids in phytochemical studies provides valuable insights into plant extracts' antioxidant potential and bioactive properties.

Phenolic compounds, among them flavonoids are known for their significant antioxidant activity, which can help neutralize harmful free radicals in the body and reduce oxidative stress, potentially leading to various health benefits. These compounds have been associated with anti-inflammatory, anti-cancer, and cardiovascular protective effects, making them essential components in assessing the health-promoting properties of plant materials (Djeridane et al., 2006; Martínez et al., 2022; Tungmunnithum et al., 2018).

On the other hand, determining the protein and total sugars content in proximate composition provides crucial information about the nutritional value of the analyzed samples. Proteins are vital for building and repairing tissues in the body, supporting the immune system, and serving as enzymes and hormones. Assessing protein content is essential for evaluating the nutritional quality of food and feed materials. Similarly, the determination of total sugars, which includes both simple sugars like glucose and fructose and polysacharide like starch, is significant in understanding the energy content and sweetness of the analyzed samples. This information is critical for evaluating the nutritional value and potential applications of plant-based products in both human and animal diets (Akintimehin et al., 2022; Radha et al., 2021; Zhou et al., 2022).

The biological dimension of this study aims to explore the chemical content of male date palm leaves from three cultivars (Deglet Nour, Degla Beida, and Ghars) grown in the El M'Ghair region of Algeria. The study focuses on analyzing the levels of total protein, total sugars, totalphenolic, and flavonoids in these leaves. The objective is to comprehend the changes in these substances among the various cultivars, which may impact their therapeutic and nutritional qualities.

2 MATERIALS AND METHODS

2.1 CHEMICALS

Sigma-Aldrich (St. Louis, USA) provided the following chemicals for the study: trichloroacetic acid, chloroform, methanol, sodium hydroxide, phenol, sulfuric acid, vanillin, orthophosphoric acid, Coomassie brilliant blue, hexane, Folin-Ciocalteu, sodium carbonate, and aluminum trichloride.

2.2 PLANT MATERIAL

In January 2020, leaflets from three male cultivars of the El M'Ghair region were collected, situated at coordinates 33°57'02.4"N 5°55'27.3"E (Figure 1). The leaflets were taken from the middle crown of date palms. Following collection, the samples were washed with distilled water and dried in an oven at 40 °C for 24 hours. Subsequently, an electric blender was used to grind the dried leaflets.

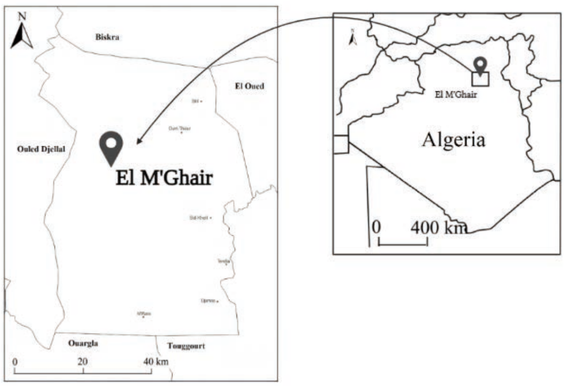

Figure 1: Sketch map of collection sites for study samples (El M'Ghair)

2.3 CLIMATIC DATA

To analyze the climatic conditions of El M'Ghair region over the period from 2008 to 2019, monthly averages of temperature and precipitation were computed. The x-axis of the graph corresponds to the months of the year, while the y-axis denotes the temperature (°C) on the left side and precipitation (mm) on the right side. The data for the climate map was sourced from both the World Weather and Local Weather Forecast (Tutiempo Network, 2023) for the specified time frame (2008-2019).

2.4 EXTRACTION AND ESTIMATION OF SUGARS

10 g of fresh leaflets (ground) were peeled in 50 ml of distilled water then complete the volume up to 100 ml, agitate for 15 min, left in contact for 24 hours, and agitate for 15 min. We obtain the extract. In each test tube, place 2 ml of the carbohydrate extract diluted 1/100, previously prepared. Then, 2 drops of phenol at (5 %) and 3 ml of concentrated sulfuric acid are added; then, the tubes are allowed to cool for 3 min in the dark. Place the tubes in a water bath at (30 °C) for 20 minutes (appearance of the yellow-red color), and stop the reaction with a stream of cold water. The absorbances were read at a wavelength of 490 nm by a UV-visible spectrophotometer Dubois et al. (1951). The concentration of total sugars was determined using a calibration curve constructed using glucose ($y = 0.0216x + 0.1471$, $R^2 = 0.9881$) and expressed in percentage (%).

2.5 EXTRACTION AND ESTIMATION OF PROTEIN

The proteins are extracted by primary hydrolysis (Snyder & Desborough, 1978). We took 0.15 g of leaflets into a beaker. Add 5 ml of (5 %) NaOH solution, stir the mixture with a vortex, then let stand for 30 hours. Then, the mixture was put into a cold centrifuge at a speed of 5000 rpm. Finally, the supernatant was obtained and stored at (-20 °C) until used for the assay.

To determine the total proteins, the method of Bradford (1976) was used. 100 µl of the supernatant was taken and 4 ml of BBC was added, which was prepared by dissolving 30 mg of BBC in 15 ml of ethanol (95 %), to which 30 ml of phosphoric acid (85 %) was added. Then complete the volume up to 300 ml with distilled water. The absorbance was read at a wavelength of 595 nm by a UV-vis spectrophotometer. Protein concentration was performed using a calibration curve generated with BSA ($y = 0.0096x + 0.0114$, $R^2 = 0.9967$) and expressed as percentage (%).

2.6 PREPARATION OF EXTRACTS

The ground leaflets (10 g) were soaked with 100 ml of methanol/water (80/20 %) and left to rest with stirring at a cold temperature for 48 hours. The mixture was filtered using a clean cloth and then filter paper to obtain the first filtrate, which was kept at (4 °C). Then the extraction was refined, and the two obtained filtrates were evaporated at (40 °C) using a rotating steam (Heidolphe) to remove most of the solvent. Then, the crude extract was hexane-extracted in a separating funnel to remove lipids and chlorophyll. The aqueous phase was evaporated, oven-dried at (40 °C) and scraped with a scalpel to obtain a powder. The powder was stored at (4 °C), and the following equation determined the extraction yield:

$$R\ (\%) = (M_2 - M_1) / M_0 \times 100$$

R (%): Yield expressed in (%).
M_0: Mass in grams of the sample.
M_1: Mass in grams of empty petrie dish.
M_2: Mass in grams of petrie dish full by the extract.

2.7 ESTIMATION OF TOTAL PHENOLIC CONTENT

The determination of total polyphenols by the Folin-Ciocalteu reagent was described by Singleton and Rossi (1965). The protocol consisted of incubating a mixture of 200 µl of sample and 1 ml of Folin-Ciocalteu reagent (10 %) for 4 min, then 800 µl of sodium carbonate (7.5 %) was added. After incubation in the dark and at room temperature, the absorbance was read at a wavelength of

765 nm. Gallic acid was used as a standard, and a calibration curve ($y = 0.0049x + 0.1279$, $R^2 = 0.9762$) was prepared under the same conditions. The phenolic content was expressed in µg GAE. mg^{-1} of extract.

2.8 ESTIMATION OF FLAVONOID CONTENT

The aluminum trichloride method (Bahorun et al., 1996) is used to quantify flavonoids in various extracts. 1 ml of each sample or standard (Quercetin), diluted in methanol (1 mg of extract in 1 ml of methanol), was added to 1 ml of an $AlCl_3$ solution (2 % in methanol). After 10 minutes of incubation, a UV-vis spectrophotometer read the absorbance at a wavelength of 430 nm.

The flavonoid concentrations of the different extracts are derived from a calibration range ($y = 0.0216x + 0.1471$, $R^2 = 0.9881$), which was established with quercetin, and the flavonoid content is expressed as: (µg QE. mg^{-1} of extract).

2.9 STATISTICAL ANALYSIS

All estimates were performed in triplicate, and the results were expressed as the mean ± standard deviation. One-way ANOVA (Analysis of Variance) and Duncan's test in SPSS 15.0 were used to determine the significance of the results, and the correlation matrix was also determined.

3 RESULTS AND DISCUSSION

3.1 CLIMATIC DATA OF EL M'GHAIR

The ombrothermic diagram for the El M'Ghair region from 2008 to 2019 (Figure 2) shows the monthly precipitation (P in mm) and temperature (T in °C) values. It reveals the variations in weather conditions throughout the year, with different levels of precipitation and temperature for each month. In the El M'Ghair region from 2008 to 2019, the hottest months were June and July, with temperatures reaching 31.58 °C and 34.94 °C, respectively. January is the coldest month, with a temperature of 11.46 °C. The wettest month is April, with a precipitation value of 7.31 mm, while June is the driest month, with no recorded precipitation.

Based on the information provided in the ombrothermic diagram for the El M'Ghair region, it is reasonable to classify the area's climate as either desert or arid. The hot temperatures during June and July, coupled with the dry conditions in June (no recorded precipitation), suggest characteristics commonly associated with desert climates. Additionally, the low precipitation levels throughout the year, with the wettest month being April (7.31 mm), further indicate arid conditions.

3.2 BIOCHEMICAL CONTENT

Figures 3, 4, 5, and 7 show the results of the chemical content analysis of the leaflets of three male palm cultivars (Deglet Nour, Ghars, and Degla Bieda) cultivated in El M'Ghair region, Algeria. The analysis focused on determining the total protein content and total sugars from the dry biomass, as well as total phenols and flavonoids from the water-methanolic extract. Data are presented in units of µg. mg^{-1} or percentages (%). The results of analyzing the chemical content of the leaflets of three male palm cultivars, namely Deglet Nour, Ghars, and Degla Beida, showed significant differences in the levels of the different compounds.

Figure 3 shows the protein content in palm leaflets of the three cultivars, Deglet Nour, Deglet Beida, and Ghars, and the statistical study ($p < 0.05$) shows dis-

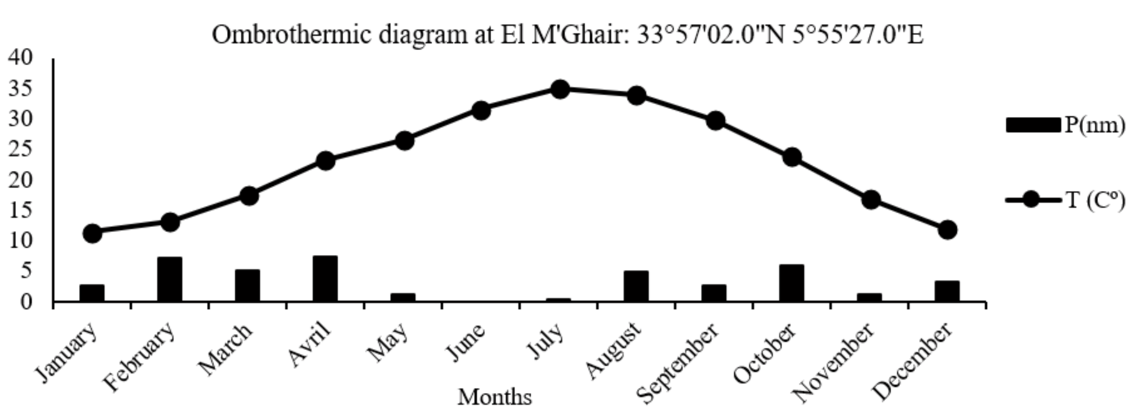

Figure 2: Ombrothermic diagram at El M'Ghair region from 2008 to 2019

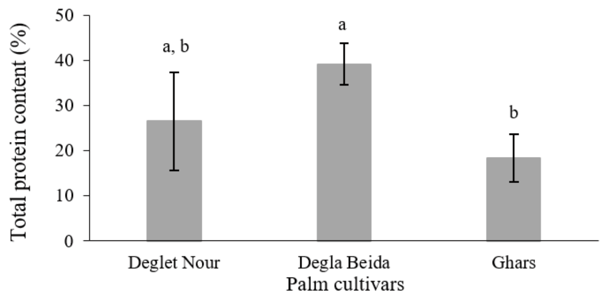

Figure 3: Protein content of date palm leaflets extracts (*Phoenix dactylifera* L.) for three Algerian cultivars (Deglet Nour, Degla Beida and Ghars). Different letters indicate significant differences between cultivars (p < 0.05)

tinct differences between them. Among the three cultivars, Degla Beida showed the highest protein content (39.09 ± 4.58%). It was followed by Deglet Nour (26.53 ± 10.84%). On the other hand, Ghars had the lowest protein content among the three cultivars, reaching 18.33 ± 5.35%.

Compared to the study of Harrak and Hamouda (2005), the protein content in the studied cultivars is high, as the percentage of protein in Moroccan dates ranged between 1.99 to 4.22 % (Langyan et al., 2021).

According to the results obtained from determining the content of total sugars in palm leaflets (Figure 4), it is clear that there are differences between the three cultivars, namely Deglet Nour, Degla Beida, and Ghars. The highest sugar content was observed in Ghars leaflets with a value of 74.54 ± 6.92 %. Deglet Nour leaflets showed the highest total sugar content with a value of 61.41 ± 10.68 %. On the other hand, Degla Beida leaflets showed the lowest total sugar content among the three cultivars, with a value of 74.54 ± 6.92 %.

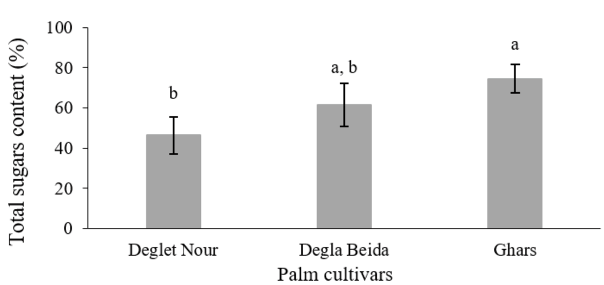

Figure 4: Total sugars content of date palm leaflets extracts (*Phoenix dactylifera* L.) for three Algerian cultivars (Deglet Nour, Degla Beida and Ghars). Values in the same column followed by different letters indicate significant differences (*p* < 0.05)

The results of this study are nearly identical to those found by Taouda et al. (2014), who reported that the content of five Moroccan date cultivars (Medjool, Khalas, Sukkari, Barhi, and Zaghloul) in total sugars ranged from 58 ± 2.76 % to 83 ± 0.26 %. It is higher than that Mehaoua (2006) obtained from date palm leaflets infected with *Parlatoria blanchardi* (Targioni Tozzetti, 1892) in the Biskra region, with content ranging between 0.02 %-0.04 %. This is because diseases affecting palm trees affect the levels of primary metabolites (Zaynab et al., 2019).

Naser et al. (2016) also reasoned that the high rate of highly efficient photosynthesis in the vast photosynthetic zone and the high amount of photosynthetic pigments may be directly responsible for the high content of total sugars and some bioactive components. Elevated glucose and sucrose levels available under stress are physiological characteristics associated with stress tolerance. Furthermore, accumulation of carbohydrates and amino acids is required to control osmotic activities and protect the cellular structure from stresses by maintaining membrane stability and water balance in the cell.

3.3 CONTENT OF PHYTOCHEMICALS

Figure 5 shows the yield percentages of the crude hydro-methanolic extracts obtained from leaflets of male date palms cultivars (Deglet Nour 14.67 %, Degla Beida 16 %, and Ghars 14.33 %). The results indicate no significant differences among the cultivars (p > 0.05), suggesting similar extraction efficiency for all three cultivars. Further exploration of the extracted compounds' chemical composition and potential applications may be warranted.

The statistical study results, expressed with a p > 0.05, indicate no statistically significant differences in yield percentage between the three date palm cultivars. Similar results were reported by Benouamane et al. (2022) in their study on five cultivars of date palms in Biskra, where no significant difference was observed in the yield of extraction with different solvents.

According to the results of the quantitative assessment of total phenols (Figure 6), it is clear that the three water-methanolic extracts had an abundant content of phenolic compounds, whereas the leaflets of 'Degla Beida' had a higher content (206.03 ± 30.55 µg GAE. mg^{-1} D), followed by the leaflets of 'Deglet Nour' (179.42 ± 17.27 µg GAE. mg^{-1} D), then the leaflets of 'Ghars', which had the lowest content among the samples (135.81 ± 11.38 µg GAE. mg^{-1} D).

Laouini et al. (2014) found that the hydroethano-

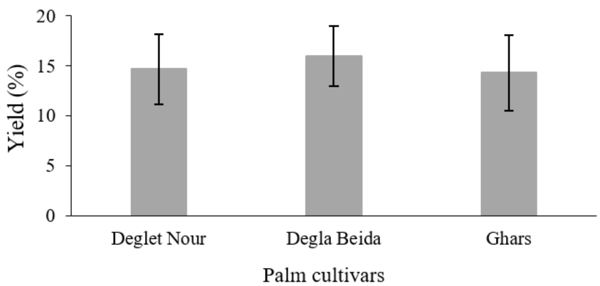

Figure 5: Yield (%) of crude hydro-methanolic extracts of leaflets of male date palms. (n = 3, One-factor ANOVA test, p > 0.05)

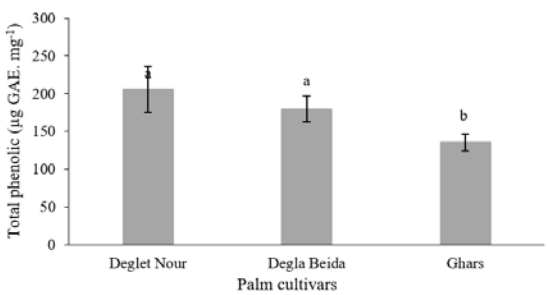

Figure 6: Total phenolic content of date palm leaflets extract (*Phoenix dactylifera* L.) for three Algerian cultivars (Deglet Nour, Degla Beida and Ghars). Different letters indicate significant differences between cultivars ($p < 0.05$)

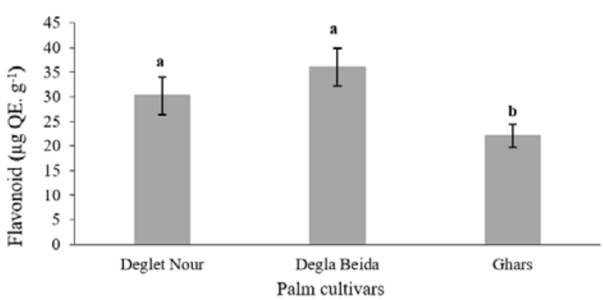

Figure 7: Flavonoid content of date palm leaflets extract (*Phoenix dactylifera* L.) for three Algerian cultivars (Deglet Nour, Deglet Beida and Ghars). Different letters indicate significant differences between cultivars ($p < 0.05$)

lic extract of 'Ghars' leaflets contained 342.45 ± 12.5 mg GAE. g^{-1} D of phenolic content. 'Deglet Nour' and 'Hemraya' leaflets contained phenolic compounds with a concentration of 221.75 ± 9.59 and 190.27 ± 6.55 mg GAE. g^{1}.

These agricultural varieties are all feminine and growing in the valley region. In a study by Benouamane et al. (2022), the phenolic content did not exceed the value of 180 mg GAE. g^{-1} in the methanolic, ethanolic, and acetone extracts of the female palm leaflets of five cultivars: Hamray, Safray, Ghars, Horra, and Deglet Nour, grown in the Biskra region.

While the results of this study are higher than those obtained by Kriaa et al. (2012), as the content ranged from 69.06 ± 0.41 to 146.46 ± 2.61 mg GAE. g^{-1} of the methanolic extract of three Tunisian cultivars (Deglet Nour, Medjool, and Barhi).

The difference in total' phenolic content among the *Phoenix dactylifera* L. cultivars studied in the four studies can be attributed to the different geographical locations in which they were grown, which resulted in a difference in climate and soil conditions. Geographical location plays an important role in shaping the environment in which palm trees thrive. Factors such as temperature, humidity, sun exposure, precipitation patterns, and soil composition can significantly influence plants' metabolic processes, including synthesizing phenolic compounds (Zakraoui et al., 2023).

According to the results shown in Figure 7 and the statistical study, it is clear that the three methanolic extracts of the leaflets of cultivars Deglet Nour, Deglet Beida and Ghars have a highly significant difference ($p < 0.01$) at the level of flavonoid content. 'Deglet Beida' and 'Deglet Nour' leaflets had the highest content of 36.02 ± 3.79 and 30.18 ± 3.88 µg QE. mg^{-1}. 'Ghars' leaflets had the lowest content of 22.02 ± 2.31 µg QE. mg^{-1}.

According to the results, it is concluded that there is a difference in the content of flavonoids according to the different cultivars of palm trees, as shown in the results of the estimation of total phenols (Figure 6), and this is what was obtained in the previous studies mentioned above (Benouamane et al., 2022; Kriaa et al., 2012).

Table 1: Correlation matrix between the biochemical parameters studied

Variables	Total phenolic	Flavonoid	Protein	Total sugars
Total phenolic	1	0.829	0.781	-0.768
Flavonoid	0.829	1	0.530	-0.965
Protein	0.781	0.530	1	-0.437
Total sugars	-0.768	-0.965	-0.437	1

*Correlation is significant at the 0.05 level (2-tailed)

3.4 CORRELATION RELATIONSHIP AMONGELEMENTS CONTENT

Table 1 presents the correlation matrix of the studied biochemical contents (total phenolic content, flavonoids, protein, total sugars) of palm leaflets in the three cultivars: Deglet Nour, Degla Beida, and Ghars. The values in the table represent correlation coefficients, which indicate the strength and direction of relationships between variables. Where it is clear that among the three variables; proteins, total phenols, and flavonoids had a positive correlation among them, with values ranging from 0.53 to 0.829. Specifically, there is a weak positive relationship between proteins and flavonoids and between them and total phenols, while total phenols and flavonoids have a strong positive relationship. This indicates that when one of these biochemical contents increases, the others also tend to increase. This positive correlation indicates these compounds' possible co-occurrence or association within the studied samples. On the other hand, there is a strong negative correlation between these three biochemical contents (proteins, total phenols, flavonoids) and total sugar content, with correlation coefficient values ranging from -0.965 to -0.437. This indicates that as the levels of proteins, total phenols, and flavonoids increase in the sample, the total sugar content tends to decrease.

4 CONCLUSIONS

This analytical study of the leaflets of different cultivars of date palm shows significant differences in their chemical content, including proteins, total sugars, total phenols, and flavonoids. Among the cultivars, Degla Beida showed the highest protein, total phenolic and flavonoids content, making it the class with the highest levels of these compounds.

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Rumen gas kinetics: a comparative analysis of two *in vitro* assessment methods for forage evaluation

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Rumen gas kinetics: a comparative analysis of two *in vitro* assessment methods for forage evaluation

Abstract: Gas production from thirty samples of feedstuffs (10 samples of corn silage, grass silage, and grass hay, respectively) was assessed *in vitro* using two methods: the Hohenheim gas test (HGT) and the ANKOM RF Gas Production System (ANKOM). Samples were incubated in buffered rumen fluid. Gas kinetic parameters were calculated using the Gompertz model. Results revealed significantly lower gas production with the ANKOM compared to the HGT. Significant differences were observed between the HGT and ANKOM in the specific gas production rate (parameter C), maximum fermentation rate (MFR) and gas produced after 24 h of incubation (Gas24) for each feed group. High coefficients of determination (R^2) were calculated between the methods for the gas kinetic parameters MFR, Gas24, total potential gas production (parameter B), decrease in the specific gas production rate (parameter A), moderate R^2 for C, and low R^2 for time of maximum fermentation rate (TMFR). Despite the lower quantities of gas generated with the ANKOM, there are strong correlations in the parameters of gas kinetics that promise the possibility of developing correction models. Future development of such models could position the ANKOM as a viable alternative to HGT, particularly for calculating metabolizable energy and net energy for lactation in feedstuffs.

Key words: animal nutrition, rumen, gas production, gas kinetic parameters, Ankom RF Gas Production System, Hohenheim Gas Test

Kinetika plinov v vampu: primerjalna analiza dveh *in vitro* metod za ocenjevanje krme

Izvleček: Tvorba plina iz tridesetih vzorcev krme (po 10 vzorcev koruzne silaže, travne silaže in sena) je bila ocenjena *in vitro* z dvema metodama: Hohenheimskim plinskim testom (HGT) in ANKOM RF Gas Production System (ANKOM). Vzorci so bili inkubirani v puferiranem vampovem soku. Parametri kinetike produkcije plinov so bili izračunani z uporabo Gompertzovega modela. Rezultati so pokazali statistično značilno manjšo tvorbo plina pri metodi ANKOM v primerjavi s HGT. Med metodama HGT in ANKOM smo opazili razlike v specifični hitrosti fermentacije (parameter C), največji hitrosti fermentacije (MFR) in plinu, proizvedenem po 24 urah inkubacije (Gas24), za vsako skupino krmil. Visoki koeficienti determinacije (R^2) med metodama so bili izračunani za MFR, Gas24, skupno potencialno tvorbo plina (parameter B) in faktor mikrobne (ne)učinkovitosti (parameter A), zmeren R^2 za parameter C in nizek R^2 za čas, v katerem je bila dosežena največja hitrost fermentacije (TMFR). Kljub manjšim količinam plina, ki je nastal z uporabo metode ANKOM, obstajajo močne korelacije v parametrih kinetike tvorbe plinov, ki kažejo na možnost razvoja korekcijskih modelov. S prihodnjim razvojem takih modelov bi bila metoda ANKOM lahko uporabljena kot zadovoljiva alternativa HGT, zlasti za izračun presnovljive energije in neto energije za laktacijo v krmi.

Ključne besede: prehrana živali, vamp, produkcija plinov, kinetika produkcije plinov, parametri, metoda ANKOM, Hohenheimski plinski test

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1 INTRODUCTION

In vitro measurements of gas production in the rumen are an important scientific method for estimating metabolizable energy (ME) and net energy for lactation (NEL) in conjunction with chemical analyses of feedstuffs. They are also used to determine the suitability of feed additives or feed rations for ruminants, the activity and biomass of microorganisms in the rumen and the quantification of volatile fatty acids produced during the incubation of substrata (Menke and Steingass, 1988; Aiple et al., 1995). The standard method for measuring *in vitro* gas production in the rumen is the Hohenheim gas test (HGT; Menke and Steingass, 1988). In this method, substrata are incubated in glass syringes and gas production is measured manually at fixed times. The method is therefore very labour-intensive. Since the development of the HGT, new methods have been developed that aim to automate and simplify the measurement process (Davies et al., 2000). One of the first such systems was developed by Theodorou et al. (1994), which measured gas production using a pressure transducer. The incubations were carried out in gas-tight culture flasks in which gases could accumulate during fermentation. The pressure in each flask was displayed on a digital indicator and then recorded manually. Excess gas that accumulated in each flask was removed manually with a syringe needle. Davies et al. (2000) then developed an automatic system where the gas that accumulates in the headspace of the flask during fermentation is automatically recorded and released when the pressure in the flask reaches a certain level. This was a direct improvement over the classic HGT system, as the researcher did not have to be present at certain times to manually record the amount of gas produced. For the accurate calculation of the gas kinetic parameters, it is important to record the gas volumes more frequently. This makes manual recording very labour intensive. Therefore, an automatic system such as the ANKOM^{RF} gas production system, which is able to automatically record the gas pressure produced at more frequent times during the incubation (e.g. every 30 minutes), should improve the accuracy and reliability of the measurements. The disadvantages of automatic systems are that they are prone to electronic and mechanical errors. Such a system is also more expensive than the manual HGT. For the correct estimation of rumen degradability of feedstuffs and the effects of feed additives, it is very important that the different systems are as accurate as possible when measuring rumen *in vitro* gas production.

A comparison of the manual (HGT) and automatic (ANKOM) methods of measuring rumen *in vitro* gas production was carried out to determine whether the automatic method is a valid replacement for the standard HGT. The aim of the study was to compare the HGT with the ANKOM based on the gas production measured with both methods. The hypothesis was that the ANKOM does not differ from the HGT in terms of gas production measurements and therefore kinetic parameters of *in vitro* gas production.

2 MATERIALS AND METHODS

2.1 SUBSTRATES AND CHEMICAL COMPOSITION

Thirty samples of forages, ten samples of varying quality of grass silage (GS), 10 of corn silage (CS) and 10 of grass hays (H), were used as substrates. The substrata were dried at 50 °C to the constant mass and ground through a 1 mm sieve. Samples were analysed for dry matter (DM), crude protein (CP), ash and ether extract (EE). Neutral detergent fibre (NDF) content was determined using the Van Soest method (Goering and Van Soest, 1970). Chemical compositions of each feed group are presented as means ± standard deviation in Table 1. All values are presented as g kg^{-1} DM unless specified otherwise.

2.2 EXPERIMENTAL DESIGN AND *IN VITRO* GAS PRODUCTION

For the evaluation of *in vitro* gas production, two measurement techniques were used: manual (HGT) with the measurement of the volume of gas in the glass syringes as described by Menke and Steingass (1988),

Table 1: Chemical composition of corn silages, grass hays and grass silages (arithmetic means ± standard deviations; n = 10 for each feed group)

Indices	Corn silages	Grass hays	Grass silages
DM (g kg^{-1})	940 ± 10.6	937 ± 11.8	928 ± 15.9
Ash	33 ± 3.5	70 ± 15.6	108 ± 38.1
EE	27 ± 3.1	15 ± 3.7	26 ± 6.0
CP	67 ± 11.9	95 ± 18.6	136 ± 33.2
NDF	465 ± 96.7	630 ± 45.3	533 ± 89.3
NFC	407 ± 101.0	190 ± 62.5	197 ± 110.2

DM – dry matter; EE – ether extract; CP – crude protein; NDF – neutral detergent fibre; NFC – non-fibre carbohydrates (1000 – (CP + EE + Ash + NDF)) and automatic with the measurements of gas pressure within the 100 ml glass flasks (ANKOM).

Rumen fluid was taken from two mature castrated Jezersko Solčavska × Romanovska rams (*Ovis aries*), with an average weight of 70 kg, fitted with permanent rumen cannula. A daily ration consisting of average quality hay (Ash = 38 g kg^{-1} DM, CP = 172 g kg^{-1} DM, NDF = 579 g kg^{-1} DM) was offered to them ad libitum (approx. 1.5 kg consumed) with the addition of 0.25 kg pelleted commercial compound feed (160 g CP kg^{-1}), and mineral and vitamin mix (0.025 kg) once per day. The diet composition was calculated according to the German metabolizable energy (ME) and utilisable protein requirements (nXP; DLG, 1997) with which the protein and energy requirements for maintenance were met and the energy-to-protein ratio of the rumen was balanced. Animals were kept in compliance with animal welfare regulations (U33401-12/2019/9 dated 16.7.2019, issued by the Food Safety, Veterinary and Phytosanitary Inspectorate, Ministry of Agriculture, Forestry and Food, Republic of Slovenia Food Safety, Veterinary and Plant Protection Administration, Ljubljana, Slovenia).

The study was conducted from October 2022 to March 2023 in 10 total consecutive runs (1 week = 1 run). All samples were incubated in rumen fluid using the HGT (runs = 3) and ANKOM (runs = 7). The number of consecutive runs for the ANKOM was higher due to the smaller number of ANKOM modules available. With both methods, the buffer medium was prepared as described by Menke and Steingass (1988) using the rumen fluid to buffer solution ratio of 1:2. Both methods included two blank samples (only inoculum without substrate) and two samples of Italian ryegrass (*Lolium multiflorum*) 2nd cut in flowering period as hay standard. Gas production after 24 h for the hay standards was known (HGT: 196 ml 1 g^{-1} DM^{-1}; ANKOM: 140 ml g^{-1} DM^{-1}). Hay standard factors (measured/known gas production) were then calculated between runs and ranged from 0.92 to 1.12. The mean standard factor for this trial was 1.004, hence gas production measurements for each run were not corrected.

Sheep rumen fluid was taken before morning feeding, and was transported to the laboratory immediately inside a thermo flask heated to 39 °C, and strained through two and then four layers of cheesecloth. Using the manual method, gas production kinetics were evaluated by anaerobically incubating each feed sample (250 ± 5 mg/syringe) in four 100 ml glass syringes filled with 30 ml of buffered rumen fluid. Syringes were kept in a water bath at 39 °C. Gas production was measured manually after 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, and 72 h. The syringes were manually shaken at each measurement. If gas production exceeded 80 ml in the first 36 h, the volume was recorded and the gas was released. In each run, two blank samples and two hay standard samples were included.

Using the automatic method, developed by Ankom Technology® (Macedon, NY, USA; ANKOM^{RF} gas production system), each feed sample (250 ± 5 mg/flask), was anaerobically incubated in the 30 ml of buffered rumen fluid. Each unit consisted of a 100 ml glass flask (actual volume: 137 ml; headspace volume: 107 ml) and an ANKOM pressure sensor module, equipped with a microchip and a radio sender. The system automatically measures gas pressure inside the unit and automatically releases the pressure when it reaches a set threshold of 7.5 kPa. The decision to set the pressure threshold to 7.5 kPa was based on methodologies of studies using the ANKOM method for *in vitro* gas production measurements (Tagliapietra et al. 2010; Cornou et al. 2013; Bachmann et al. 2020). The gas pressure was recorded every 30 minutes for 72 h. After the start of the incubation, the flasks were manually shaken daily. After incubation, the gas pressure was converted into amount units (moles) of gas using the "ideal" gas law (Equation 1) and then converted to millilitres (ml) of gas produced by Avogadro's law (Equation 2):

$$n = p\left(\frac{V}{RT}\right) \qquad (1)$$

where n is gas produced in moles (mol), p is cumulative pressure in kilopascals (kPa), V is the headspace volume in the glass flask in litres (l), T is the temperature in Kelvin (K) and R is gas constant (8.314472 J $(K \times mol)^{-1}$). The gas production is then calculated as:

$$GP\ (ml) = n \times 22.4\ \frac{l}{mol} \times 1000\ \frac{ml}{l} \qquad (2)$$

where GP is the volume of gas produced.

2.3 CALCULATIONS AND STATISTIC ANALYSES

Gas production kinetic parameters were calculated as described by Lavrenčič et al. (1997). The net volume of gas produced at each incubation time was calculated as the difference between the total volume of gas produced and the volume of gas produced from the blank sample at each time of incubation. Net volumes at each time of incubation were adjusted afterwards to 1 g of substrate DM. The obtained *in vitro* gas production data were then fitted with the Gompertz model (Lavrenčič et al., 1997):

$$Y_t = B \times e^{-C \times e^{-At}} \quad (3)$$

Where Y_t is gas produced at the time "t" (ml g^{-1} DM), B is the total potential gas production (ml g^{-1} DM), C is the specific gas production rate, A is the decrease in specific gas production rate and t is the time in hours (h).

The parameters were calculated in SAS 9.4. (SAS Software ver. 9.4; SAS Institute, Cary, NC, USA), using the PROC NLIN procedure for a nonlinear regression method with the Marquardt compromise to estimate the kinetic parameters and fit the curve for each syringe within a substrate. By inserting a fixed time of 24 h in the equation of the Gompertz model with known parameters, the amount of gas produced in 24 h was calculated. By setting the second derivative of the Gompertz model to zero (0) and solving for "t", times of maximum fermentation rates (TMFR) were calculated:

$$\frac{d^2y}{dt^2} = A \times B^2 \times C^2 \times (e^{-At})^2 \times e^{-C \times e^{-At}} \\ -A \times B \times C^2 \times e^{-C \times e^{-At}} = 0 \ \rightarrow TMFR \quad (4)$$

Using the corresponding value of TMFR in the first derivative equation, maximum fermentation rates (MFR) were calculated:

$$MFR = B \times C \times A^{-A \times TMFR \times e^{-C \times e^{-A \times TMFR}}} \quad (5)$$

With a one-way analysis of variance (ANOVA), using the general linear model (PROC GLM), the effect of the feed group, sample, method and interaction between the feed group and method on the estimated and calculated kinetic parameters of gas production, was compared. The results are presented as least square means (Table 1):

$$Y_{ijkl} = \mu + F_i + S_j + M_k + FM_{ik} + e_{ijkl} \quad (6)$$

Where Y_{ijkl} are the estimated and calculated kinetic parameters of gas production, F_i is the effect of the feed group (i = corn silage, grass silage, grass hay), S_j is the effect of the sample (j = 1, 2… 30), M_k is the effect of the method (k = ANKOM, HGT) and FM_{ik} is the interaction between feed groups and methods.

3 RESULTS AND DISCUSSION

Within each feed group, significant differences ($p < 0.05$) were found between the ANKOM and HGT for the parameters C, MFR and Gas24, but not for B, A and TMFR (Table 2). Parameter C measured with ANKOM was 25 % lower for maize silage, 27 % lower for grass silage and 38.5 % lower for grass hay compared to the HGT. Using the ANKOM, the MFR parameter was lower in every of the feed groups compared to the HGT. The differences were –41.3 % for corn silages, –50 % for grass silages and –62.8 % for grass hays, respectively. The rate at which gas is produced with the ANKOM

Table 2: Effect of method, individual sample, and feed group on estimated and calculated kinetic parameters of *in vitro* gas production

Feed group	Method	B (ml g^{-1} DM)	C	A	MFR (ml h^{-1})	TMFR (h)	Gas24 (ml g^{-1} DM)
Corn silage	ANKOM	240	2.11	0.096	8.4	7.9	191
	HGT	259	2.99	0.166	14.3	6.6	242
Grass silage	ANKOM	160	1.97	0.087	5.3	8.6	120
	HGT	182	2.69	0.156	10.6	6.3	168
Grass hays	ANKOM	177	1.63	0.049	2.9	12.5	97
	HGT	206	2.56	0.102	7.8	9.5	162
		P – values					
F		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
S		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
M		<.0001	<.0001	<.0001	<.0001	0.0005	<.0001
F × M		0.6470	0.0168	0.0625	<.0001	0.5486	0.0082

F – feed group; S – sample; M – method; F × M – interaction between feed group and method; B – total potential gas production; C – specific gas production rate; A – decrease in specific gas production rate; MFR – maximum fermentation rate; TMFR – time of maximum fermentation rate; Gas24 – gas produced after 24 h of incubation could be affected by the amount of gas dissolved in the medium. With the ANKOM, the gas produced is released out of the headspace of the flask only when the pressure inside reaches a certain threshold. According to Henry's law, a certain proportion of these gases are dissolved in the medium and are released into the headspace of the flask only after the excess gasses are already released (Theodorou et al., 1994). It has also been pointed out that microbial activity could be disturbed, if the pressure exceeds 48 kPa (Theodorou et al., 1994). Lowman (1998) implied that continuous venting of flasks at 4.5 kPa should prevent the saturation of the solution. They also found out that, if the flasks were not shaken at all, their gas production was higher than in flasks shaken recurrently at pre-set times or continuously on an orbital shaker. Supersaturation of CO_2 in the medium may also occur at high gas pressures in closed systems, which can lead to biased measurements of gas production (Tagliapietra et al. 2010; Cattani et al. 2014). In our study, the threshold for venting the ANKOM flasks was set at 7.5 kPa, which was similar to Cornou et al. (2013), and the flasks were shaken regularly. With the HGT, the plungers of the syringes were greased to avoid high pressures by allowing the accumulated gases to expand, and they were shaken at fixed times.

Gas produced after 24 h for feeds measured with the HGT (Table 2) was similar to the findings of Getachew et al. (2004). Their corn silage produced 232 ml g^{-1} DM gas, while their wheat silage, which was similar to our grass silages, produced 172 ml g^{-1} DM gas. Compared to HGT, the ANKOM resulted in significantly lower ($p < 0.05$) gas production in the first 24 h

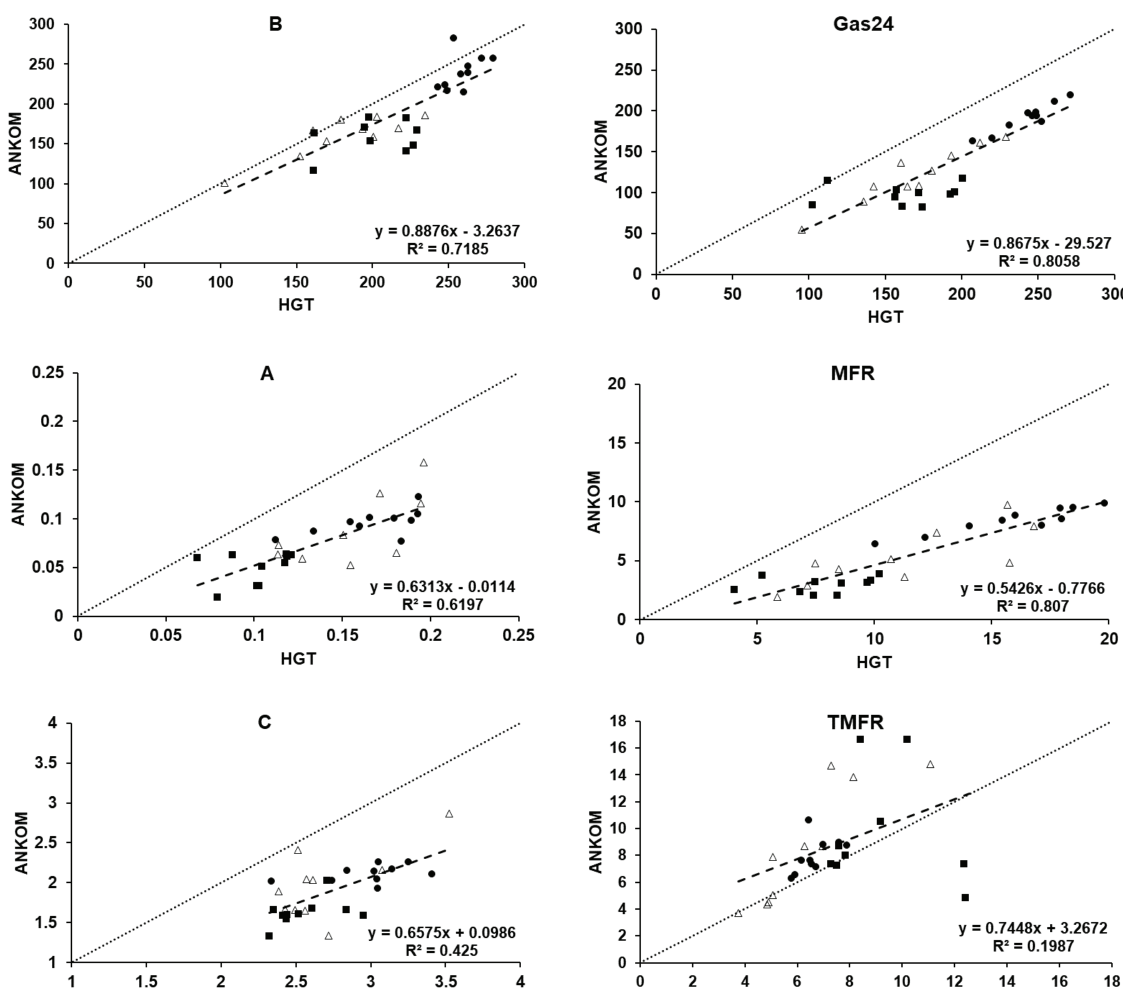

Figure 1: Relationship between parameters B, Gas24, A, MFR, C, and TMFR using the HGT (Hohenheim gas test) versus the ANKOM RF gas system (dashed line = regression line; dotted line = constant diagonal) for corn silage (●), grass hays (■) and grass silage (Δ)

of incubation (Gas24) for all feed groups. The differences were –21.0 % for corn silages, –28.5 % for grass silages and –40.1 % for grass hays. Bachmann et al. (2020) reported lower gas production measured in general with the ANKOM compared to the HGT. They also reported that the rankings of substrates remained the same regardless of the system used. These results were similar to our findings, as corn silages produced the most gas, grass silages were ranked second and grass hay produced the least gas, in both the HGT and ANKOM system. Our results also partly agree with Gierus et al. (2008), who have shown that their automated pressure evaluation system produced significantly less gas after 24 h compared to HGT for grass silages but not for grass hay, while Elberg et al. (2018) have shown, that gas production in 24 h of incubation in the automated system compared to HGT, was significantly lower for corn silages and for grass hays, but not for grass silages. Gas production can be affected by the ratio between rumen fluid and buffer, the diet of donor animals, and the general variability of rumen fluid on collection day (Rymer et al., 2005). In this study, the incubation parameters were standardised across both methods, except in times of rumen fluid collection. Due to the smaller number of ANKOM modules available, we performed the *in vitro* incubation in a higher number of runs than with the HGT. Considering all these factors, we cannot fully explain the differences between the compared systems.

Figure 1 shows the distribution of parameters for each sample comparing the ANKOM to the HGT. All the single values for each parameter were plotted. The coefficients of determination (R^2) show a strong correlation between the methods across all samples in the parameters B, A, MFR, and Gas24, a moderate correlation for parameter C, and a very weak correlation for parameter TMFR. Bachmann et al. (2020) reported an R^2 of 0.57 for gas produced after 24 h (Gas24) between the two methods from substrates used, however, they used a smaller number of substrates (n = 6), compared to our study (n = 30).

4 CONCLUSIONS

The gas kinetic parameters C, MFR and Gas24 obtained with the ANKOM differed significantly from the HGT. Despite these differences, the rankings for each feed group in gas produced and gas kinetic parameters were equal between both methods and showed strong correlations between the methods in all parameters except TMFR. In this regard, the methods are comparable in terms of ranking, but not in terms of absolute values. For the calculation of ME and NEL, with the results obtained from the ANKOM, caution should be taken as there are significant differences between the methods in the Gas24 parameter. It is also necessary to be aware of the difference in gas production when using the ANKOM and to reference the method used when listing the results in the study. Despite the lower quantities of gas generated *in vitro* with the ANKOM compared to the HGT, there are strong correlations in the parameters of gas kinetics that show the possibility of developing correction models. With the development of correction models in the future, the ANKOM could become a valid replacement for the HGT for calculations of gas kinetic parameters and more importantly, for calculating ME and NEL of feedstuffs.

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Interactions between aphids and aphidophages in citrus orchards in the Chlef region (North West of Algeria)

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Interactions between aphids and aphidophages in citrus orchards in the Chlef region (North West of Algeria)

Abstract: The objective of this study is to inventory and identify the different species of aphids and aphidophages associated with them in citrus orchards in the Chlef region (Algeria) in order to promote predation and parasitism interactions for the ultimate purpose of biological control of these formidable pests. Surveys are conducted twice a month for an entire year. For sampling, we used yellow sticky traps, yellow pans, and visual determination. This study allowed us to identify seven species of aphids and 34 species of aphidophages, including 30 predator species and 4 parasitoid species. The most abundant aphids are *Aphis spiraecola* (Patch, 1914) and *Aphis gossypii* (Glover, 1877), while the most common aphidophages are *Episyrphus balteatus* (De Geer, 1776), *Chrysoperla carnea* (Stephens, 1836), *Coccinella septempunctata* (Linné, 1758), *Aphidoletes aphidimyza* (Rondani, 1847) and *Lysiphlebus fabarum* (Marshall, 1896). In terms of frequency, aphidophages are dominated by ladybugs, followed by lacewings, then syrphids, then bugs, and aphid midges in last place. The diversity of the aphidophages fauna is not very important, but the highest values are observed towards the end of April. Predation activities in the study area extend from the end of March to November. Aphidophages associated with aphids are divided into generalists and specialists. Specialist aphidophages show preferences for certain prey over others, in the case of aphid diversity according to both intrinsic and extrinsic factors.

Key words: aphids, aphidophages, citrus, natural enemies, Chlef region

Interakcije med listnimi ušmi in afidofagi v nasadih citrusov v regiji Chlef (severozahod Alžirije)

Izvleček: Cilj te raziskave je bil popisati in identificirati različne vrste listnih uši in z njimi povezane afidofage v nasadih citrusov v regiji Chlef (Alžirija), da bi spodbudili interakcije plenjenja in parazitizma za končni namen biotičnega zatiranja teh nevarnih škodljivcev. Raziskave so potekale dvakrat mesečno skozi celo leto.Za vzorčenje smo uporabili rumene lepljive pasti, rumene posode in vizualno določanje. Ta študija nam je omogočila identifikacijo sedmih vrst listnih uši in 34 koristnih vrst, vključno s 30 vrstami plenilcev in 4 vrstami parazitoidov. Najštevilčnejši vrsti listnih uši sta *Aphis spiraecola* (Patch, 1914) in *Aphis gossypii* (Glover, 1877) medtem, ko so najpogostejši plenilci *Episyrphus balteatus* (De Geer, 1776), *Chrysoperla carnea* (Stephens, 1836), *Coccinella septempunctata* (Linnaeus, 1758), *Aphidoletes aphidimyza* (Rondani, 1847) in *Lysiphlebus fabarum* (Marshall, 1896). Glede na pogostnost prevladujejo med plenilci polonice, sledijo jim čipkarke, nato trepetavke, nato plenilski hrošči, na zadnjem mestu so plenilske hržice. Raznolikost afidofagne favne ni zelo pomembna, vendar so največje vrednosti opažene proti koncu aprila. Dejavnosti plenjenja na območju študije trajajo od konca marca do novembra. Afidofagi, povezani z listnimi ušmi, se delijo na generaliste in specialiste. Afidofagi specialisti kažejo preferenco za določen plen, v primeru raznolikosti listnih uši glede na notranje in zunanje dejavnike.

Ključne besede: listne uši, afidofagi, agrumi, naravni sovražniki, Chlef regija

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1 INTRODUCTION

Citrus fruits are one of the most important fruit tree crop in the world. They are cultivated in 168 countries on an area of 12.7 million hectares (FAOSTAT, 2022).

Algeria, due to its geographical location, is one of the world's leading producers of citrus fruits. The country has a total area of 77,895 ha with a production of 2 million tons (MADR, 2021).

Chlef is one of the most productive regions in the country, unfortunately, this crop hosts several pests and diseases. Aphids are considered to be not only among the most formidable pests of citrus (Ait-Amar et al., 2022), but also among the main vectors of phytopathogenic viruses (De Moya-Ruiz et al., 2023). They are phytophagous and all piercing-sucking. This mode of nutrition can lead to various reactions in the plant, both to the bite and to the toxicity of the saliva (Herrbach, 2022). Their honeydew allows the development of fungi that hinder the photosynthesis of the plant and its chlorophyll state (Hullé et al., 1999).In turn, aphids provide food for a variety of predatory species. This natural chain helps to maintain biological balance. This balance can be disrupted by the decline in the diversity of entomophages. It is in this approach that Straub & Snyder (2006) decline the importance of the relationship between the biodiversity of predators and biological control of bio-aggressors, as studies have shown that predators can complement or interfere with each other (Snyder & Ives, 2003; Finke & Denno, 2004).

In our study region, this auxiliary fauna is unfortunately poorly studied and still poorly known. In this context, the present study consisted of a survey of the predators of aphids on citrus as well as the aphidophages associated with them in one of the largest citrus-growing regions of Algeria. This opens the way for other studies on the impact of the predation of each of these entomophages on citrus pest aphids and facilitates the control of these pest populations by the implementation of sustainable biological pest management strategies.

2 MATERIALS AND METHODS

2.1 DESCRIPTION OF THE STUDY SITES

Three sites were selected for this study, the first orchard is a clementine orchard of the Montreal variety, 15 years old and located in Ouled Fares (Latitude: 36.2328, Longitude: 1.24028 36° 13′ 58″ North 1° 14′ 25″ East). It is located at an altitude of 136 meters and covers an area of 7 hectares. The second orchard is a Thomson Navel orange orchard, 21 years old and located in Ouled Abbes (Latitude: 36.2167, Longitude: 1.48333 36° 13′ 0″ North, 1° 28′ 60″ East). It is located at an altitude of 151 meters and covers an area of 3 hectares. The third orchard is a Washington Navel orange orchard, 19 years old and located in Labiodh Medjadja (Latitude: 36.25, Longitude: 1.4 36° 15′ 0″ North, 1° 24′ 0″ East) (Fig 1). It is located at an altitude of 196 meters and covers an area of 5 hectares. Chlef's climate is warm and temperate, of the Mediterranean type (Köppen classification: Csa). All three orchards are irrigated by a drip irrigation system that also provides fertilizer and pesticide applications. The soils in the study area (Chlef) are generally characterized by a high degree of homogeneity and agricultural aptitude, and are predominantly clay-loam (ABH; Chelliff Zahrez, 2003).

2.2 SAMPLING METHOD

This study was conducted between September 2021 and August 2022 in 3 citrus orchards in the Chlef region. This study consists of identifying and classifying the different types of aphids and their natural enemies present in citrus orchards in the study region. For this, we used three sampling methods, which are described below and we spread the prospections over the whole year in order to offer ourselves the chance to find more insects and other auxiliary arthropods regardless of their biological characteristics.

2.2.1 Sticky yellow traps

The installation of traps allows to follow the flight activity of the different species and to know precisely which periods of the year this activity will take place. The

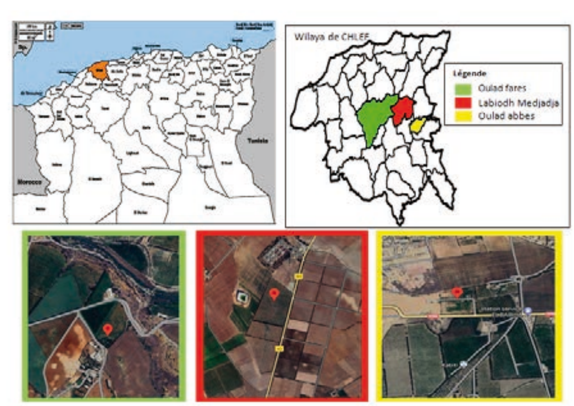

Figure 1: Geographic location of the experimental sites

flight phase of aphids plays an important role in the dispersal of species, in the search for host plants, and in the transmission of viral diseases.

In order to control all of these phenomena, it was necessary to carry out an air sampling by capturing winged aphids with freely moving yellow sticky traps (Hullé, 2010). Five yellow traps per orchard were placed at the four cardinal corners and in the center for a representative sampling of the orchard. Every 15 days (2 weeks), the previously installed traps are retrieved at the same time that new traps are installed in other parts of the orchard, so that the sampling is spread over the entire study area. Each retrieved trap is wrapped in a transparent plastic film to preserve all the trapped insects. In the laboratory, aphids and aphidophagous insects are collected and placed in test tubes filled with 70 °C ethyl alcohol for later identification.

2.2.2 Hand gathering of shoots

To count and identify the different species of aphids, their developmental stages, and their natural enemies, we carried out hand-gathering of shoots. To do this, at each sampling (2 per month), 10 trees are randomly selected and distributed across the different orientations of the orchard. From each tree, 5 shoots are randomly collected along the entire diagonal of the orchard (east, west, north, south, and center) and transported in transparent bags to the laboratory, where immediate identification is undertaken before the plants dry out. Individuals whose identification is difficult or doubtful are preserved for later identification or confirmation.

2.2.3 The yellow basins

These are circular plastic basins 20 cm in diameter that were placed at the level of the trees between the leaves and branches at an average height of 1 m above the ground. The basins were filled to ¾ with soapy water, which helped to fix the insects inside the basins. This type of trap captures not only winged aphids, but also their natural enemies, notably parasitoid Hymenoptera and other predatory insects. The trapped insects were collected every month in small plastic tubes containing 75 % alcohol, and counting and identification were carried out in the laboratory.

2.3 METHOD OF DATA ANALYSIS

2.3.1 Ecological diversity indices

To interpret the results, we based our analysis on the calculation of ecological indices of composition, such as total richness (S), centile frequency, and constancy, as well as ecological indices of structure, such as the Shannon-Weiner index H', evenness, and Simpson's index.

2.3.1.1 Ecological indices of composition

- Total richness:

According to Guillaum et al. (2009), richness tells us about the elements present in a given space. It is expressed by the number of species of the population considered in a given ecosystem (Ramade, 1984).

- Relative abundance of centile frequency (%):

According to Dajoz (1985), it is the percentage of individuals of a species compared to the total number of individuals. It is calculated by the following formula:

$$F.C.\% = \frac{ni}{N} \times 100$$

With: ni: Number of individuals of a species and N: Total number of individuals.

- Coefficient of abundance-dominance or frequency of occurrence

It is expressed as a percentage of the number of statements containing species i taken into consideration, divided by the total number of statements (Dajoz, 2003).

$$C = \frac{i}{N} \times 100$$

C: is the number of statements containing the species studied and N: is the total number of statements carried out.

Depending on the value of C, the following categories are distinguished:

- Very frequent or omnipresent species if C = 100 %.

- Constant species if 75 % < C < 100 %.
- Regular species if 50 % < C < 75 %.
- Accessory species if 25 % < C < 50 %.
- Accidental species if 5 % < C < 25 %.
- Rare species if C < 5 %.

2.3.1.2 Ecological structure indices

- The Shannon-Weaver diversity index

The Shannon-Weaver diversity index is considered to be the best index of diversity; it is calculated as follows (Blondel, 1979; Barbault, 1993):

$$H' = \Sigma p_i log_2(p_i)$$

H' is the diversity index expressed in bits, and pi is the proportional abundance or percentage abundance of a present species (pi = ni/N). Thus, a community will be more diversified the larger the H' index is. The maximum diversity (H'max) corresponds to the highest possible value of the population and translates to a heterogeneous population for which all individuals of all species are distributed equally.

It is calculated by the following formula:

$$H'max = \log 2\,(S)$$

S: is the total richness or the total number of species present.

- The Pielou evenness index

The Shannon index is often accompanied by the Pielou evenness index (J), or equipartition index (E) (Blondel, 1979). It is expressed as the ratio between the observed diversity and the theoretical maximum diversity and is calculated as follows:

$$E = H'/Hmax$$

E being the evenness, H' is the observed diversity index and H'max is the maximum diversity index expressed in bits.

The value of E varies from 0 to 1. It tends towards 0 when the population is composed of a few species and many individuals. When this value tends towards 1, it translates to a population represented by many species with approximately the same number of individuals. The high diversity values reflect the presence of a large number of aphidophages in the agrosystems studied, so biological regulation of aphid pests by their natural enemies would be of great benefit.

- The Simpson index

The Simpson index measures the probability that two individuals selected at random belong to the same species and is defined by the formula:

$$L = \Sigma\, ni(ni-1)/N(N-1)$$

Where Ni is the number of individuals of the given species and N is the total number of individuals.

This index is inversely proportional to diversity. As a result, another formulation has been proposed to establish an index directly representative of heterogeneity by subtracting the Simpson index from its maximum value. This new formulation constitutes the Simpson diversity index, which is expressed by the formula D = 1 - L. Therefore, this index varies from 0 (minimum diversity) to 1 (maximum diversity) (Ramade, 1984).

2.3.2 Statistical analysis (AFC)

The results of the presence-absence of the different species of aphids and those of the entomophages during the surveys carried out in the study environments were the subject of a correspondence factor analysis (AFC) using a trial version of XlSTAT. This latter allows the structure of the data to emerge, the way in which the modalities of each variable are situated in relation to each other, in a differential and relational way. According to Escoffier and Pages (2008), correspondence factor analysis can, on different types of data, describe the dependence or correspondence between two sets of characters. For the present study, this analysis allows us to investigate the affinities between aphid species and the aphidophagous insects that are associated with them in the agrosystems studied during the survey period. In other words, we can identify the aphids that are most preferred by each predator, the impact of predation by a given natural enemy at a given time or period, or the interactions between aphidophagous species (competition, association, etc.). This information is necessary for the selection of effective auxiliaries for use in one of the desired biological control methods (conservation, introduction, or augmentation) or for their use in an integrated pest management process.

3 RESULTS

3.1 THE APHIDS

3.1.1 The aphid fauna found in the study environments

Through our surveys conducted over a full year, seven different species of aphids distributed over three different genera were identified. The genus *Aphis* is represented by *Aphis spiraecola*, *Aphis gossypii*, *Aphis faba* and *Aphis nerii* (Boyer De Fonscolombe, 1841).

The genus *Toxoptera* is represented by *Toxoptera citricida* (Kirkaldy 1907) and *Toxoptera aurantii* (Boyer de Fonscolombe, 1841)**.** Finally, the genus *Myzus* is represented by *Myzus persicae* (Sulzer, 1776) (Table 1).

3.1.2 The total richness, and the relative abundance of aphid populations during the surveys conducted

The results of Table 3 on the specific richness, dominance, and centile frequency of citrus-damaging aphids in the Chlef region showed that the greatest infestations are noted during the autumn and spring periods. In fact, at the end of October, we recorded a very large aphid population reaching 5074 individuals (17.37 %). This population declines significantly before starting to increase again to reach very high levels exceeding 8000 individuals (28.45 %) by the end of May, then they disappear again from the end of June. It should be noted that during the periods from December to February and from the end of June to the end of September, aphids were completely absent in the study areas.

3.1.3 Total richness, relative abundance, and occurrence frequency of aphid species in the Chlef region

The results of Table 2 show that *Aphis gossypii* and

Table 2: Specific richness and centile frequency of aphids during the surveys

Date	Taxa_S	Effective	frequency %
10 /9	0	0	0
25 /9	2	8	0,03
10 /10	5	666	2,28
25 /10	6	5074	17,37
10 /11	2	928	3,18
25 /11	1	65	0,22
10 & 25 /12	0	0	0
10 & 25 /1	0	0	0
10 & 25 /2	0	0	0
10 /3	0	0	0
25 /3	2	776	2,66
10 /4	3	386	1,32
25 /4	8	1215	4,16
10 /5	7	7609	26,05
25 /5	8	8311	28,45
10 /6	6	4171	14,28
25 /6	0	0	0
10 & 25 /7	0	0	0
10 & 25 /8	0	0	0
Total	6	29209	100

Table 1: The aphid fauna recorded in the three citrus orchards surveyed

Families	Subfamilies	Tribe	genus	Species
Aphididae	Aphidinae	Aphidini	*Aphis*	*Aphis spiraecola* (Patch, 1914) *Aphis gossypii* (Glover,1877) *Aphis nerii* (Boyer De Fonscolombe, 1841) *Aphis fabae* (Scopoli, 1763)
			Toxoptera	*Toxoptera aurantii* (Boyer de Fonscolombe, 1841) *Toxoptera citricida* (Kirkaldy 1907)
		Macrosiphini	*Myzus*	*Myzus persicae* (Sulzer, 1776)

Aphis spireacola are the two most abundant species in citrus orchards in the Chlef region, with a richness of 12,933 and an abundance of (50.16 %) for the first species, and a richness of 12,585 corresponding to an abundance of (48.81 %) for the second. The other species are much less abundant, with a richness not exceeding 147 individuals and rates below 1 %.

In terms of occurrence, *Aphis. gossypii* and *Aphis. spireacola* were found to be regular species, *T. aurantii* was found to be an accidental species, while the other species are rare in our study areas.

3.2 APHIDOPHAGES

3.2.1 Aphidophages associated with aphids recorded in the study orchards

Thirty-four species of aphidophages that accompany aphids in their emergence and are involved in the biological regulation of their populations were also identified (Table 5). They are divided into 30 predators and 4 parasitoids, composed mainly of insects and dominated by beetles (13 species), hymenopterans (6 species), and

Table 3: Richness, abundance, and dominance of different aphids collected in citrus orchards in the Chlef region

Settings	*A. spiraecola*	*A. gossypii*	*T. aurantii*	*M. persicae*	*A. fabae*	*T. citricida*	*A. nerii*
Richness	14257	14651	167	102	17	4	11
Abundance (%)	48,81	50,16	0,57	0,35	0,06	0,01	0,04
Constancy	Accessory	Accessory	Accessory	Accidental	Accidental	Rare	Accidental

Table 4: Abundance of aphid species during the different surveys

	Species							
Date	*A. spiraecola*	*A. gossypii*	*T. aurantii*	*M. persicae*	*A. fabae*	*T. citricida*	*A. nerii*	Total
10/9	0	0	0	0	0	0	0	0
25/9	2	6	0	0	0	0	0	8
10/10	274	380	12	0	0	0	0	666
25/10	2799	2231	26	18	0	0	0	5074
10/11	491	405	21	11	0	0	0	928
25/11	27	38	0	0	0	0	0	65
10 &25/12	0	0	0	0	0	0	0	0
10 & 25/1	0	0	0	0	0	0	0	0
10 & 25/2.	0	0	0	0	0	0	0	0
10/3	0	0	0	0	0	0	0	0
25/3	277	496	3	0	0	0	0	776
10/4	127	245	6	8	0	0	0	386
25/4	299	884	17	15	0	0	0	1215
10/5	3783	3760	31	22	9	0	4	7609
25/5	4313	3909	42	28	8	4	7	8311
10/6	1865	2297	9	0	0	0	0	4171
25/6	0	0	0	0	0	0	0	0
10 & 25/7.	0	0	0	0	0	0	0	0
10 & 25/8	0	0	0	0	0	0	0	0
Total	14257	14651	167	102	17	4	11	29209

hemipterans (5 species). The most widespread are the hoverfly *Episyrphus balteatus* (De Geer, 1776), the green lacewing *Chrysoperla carnea* (Stephens, 1836), the ladybugs *Coccinella septempunctata* (Linné, 1758) and *Coccinella algerica (*Kovàr 1977)**, t**he gall midge Aphidoletes aphidimyza (Rondani, 1847), and a parasitic hymenopteran, *Lysiphlebus fabarum* (Marshall, 1896).

3.2.2 Richness, abundance, and dominance of the main aphidophages predators present in the citrus-growing environments surveyed

To calculate the indices of composition related to predators, we found it useful to limit ourselves to the most abundant and efficient species. In terms of richness

Table 5: List of aphidophages associated with aphids in the study environments

Class	Order	Family	Species	Status
Arachnida	Araneae	Araneidae	*Araneus diadematus* (Clerck,1757)	Predator
			Araneidae sp	Predator
Insecta	Mantodea	Mantidae	*Mantis religiosa* (Linné, 1758)	Predator
			Sphodromantis sp. (Stal, 1871)	Predator
			Iris oratoria (Linné, 1758)	Predator
	Dermaptera	Forficulidae	*Forficula auricularia* (Linné, 1758)	Predator
		Anisolabidae	*Anisolabis* sp. (Fieber, 1853)	Predator
	Hemiptera	Lygaeidae	*Lygaeus* sp. (Fabricius, 1794)	Predator
		Anthocoridae	*Anthocoris* sp. (Fallen, 1814)	Predator
			Orius sp. (Wolff, 1811)	Predator
			Cardiastethus sp. (Fieber, 1860)	Predator
		Geocoridae	*Geocoris* sp. (Fallén, 1814)	Predator
	Coleoptera	Carabidae	Carabidae sp	Predator
			Brachinus sp. (Weber, 1801)	Predator
			Chlaenius sp.1 (Bonelli, 1810)	Predator
			Harpalus attenuatus (Steph, 1828)	Predator
			Ophonus pubescens (Mull, 1776)	Predator
			Acinopus sp. (Dejean, 1821)	Predator
			Agonum sp. (Bonelli, 1810)	Predator
			Zabrus distinctus (Lucas, 1842)	Predator
		Staphylinidae	*Ocypus olens* (Muller,1764)	Predator
			Anthophagus sp. (Grav, 1802)	Predator
		Coccinellidae	*Coccinella septempunctata* (Linné, 1758)	Predator
			*Coccinella algerica (*Kovàr 1977)	Predator
			Scymnus sp. (Kugelann, 1794)	Predator
	Diptera	Syrphidae	*Episyrphis balteatus* (De Geer, 1776)	Predator
		Cecidomyiidae	*Aphidoletes aphidimyza* (Rondani, 1847)	Predator
	Neuroptera	Chrysopidae	Chrysoperla carnea (Stephens, 1836)	Predator
	Hymenoptera	Vespidae	*Vespula germanica* (Fabrice, 1793)	Predator
			Vespidae sp. (Latreille, 1802)	Predator
		Braconidae	*Aphidius colemani* (Viereck, 1912)	Parasitoid
			Lysiphlebus fabarum (Marshall, 1896)	Parasitoid
		Ichneumonidae	Ichneumonidae sp.1 (Latreille, 1802)	Parasitoid
			Ichneumonidae sp. 2	Parasitoid

Table 6: Richness, abundance, and dominance of the main predators collected in citrus orchards in the Chlef region

	Predators						
Settings	Hoverflies	Lacewings	Ladybugs	Midges	Bugs	Others	Total
Richness	1	1	3	1	5	19	30
Abundance	65	75	100	4	45	30	319
Frequency%	20.37	23.51	31.35	1.25	14.11	9.4	100
Constancy	regular	regular	regular	Accidental	Accessory		

and frequency, ladybugs are the most numerous with 100 individuals (31.35 %), followed by lacewings with 75 individuals (23.51 %), then hoverflies with 65 individuals (20.37 %), then bugs with 45 individuals (14.11 %), and finally gall midges with only 4 individuals, or 1.25 %. Other generalist predators that can have an impact on aphid control are also present, with a total of 30 individuals (9.4 %), distributed over 19 different species (Table 6).As for the monthly frequency of these auxiliaries, it appears that ladybugs and hoverflies appear first in March and disappear last at the end of October, with high numbers in April and May. As for lacewings, they only appear from the end of April and disappear late in mid-November. As for bugs, gall midges, and other predators, their presence is limited only to spring and a little less in summer (Table 7).

Table 7: Temporal evolution of the numbers of aphidophages during the prospection period

Date	Hoverflies	Lacewings	Ladybugs	Midges	Bugs	Others	Total
10/9	2	3	3	0	0	0	8
25/9	1	2	2	0	0	0	5
10/10	0	1	5	0	0	0	6
25/10	1	0	4	0	0	0	5
10/11	0	2	0	0	0	0	2
25/11	0	0	0	0	0	0	0
10 &25/12	0	0	0	0	0	0	0
10 & 25/1	0	0	0	0	0	0	0
10/2	0	0	0	0	0	0	0
25/2	0	0	0	0	0	0	0
10/3	2	0	0	0	0	0	2
25/3	4	0	8	1	0	0	13
10/4	8	0	10	2	11	0	31
25/4	7	11	12	1	10	7	48
10/5	8	15	9	0	7	5	44
25/5	10	12	8	0	7	5	42
10/6	5	6	7	0	5	7	30
25/6	6	8	12	0	4	2	32
10/7	4	5	7	0	1	3	20
25/7	3	4	4	0	0	1	12
10/8	2	2	5	0	0	0	9
25/8	3	3	4	0	0	0	10
Total	65	75	100	4	45	30	319

3.2.3 Diversity of aphidophages in the study environments

The diversity of aphidophage was translated by calculating the Simpson, Shannon-Wiever, and Equitability indices (Table 8).The values of these indices show that the highest diversity of aphidophage is observed at the end of April, with D = 0.59, H = 2.42, and E = 0.49. This diversity then regresses until it becomes zero in November, before increasing again from the beginning of March.

3.3 TEMPORAL DISTRIBUTION OF WINGLESS APHIDS AND THEIR NATURAL ENEMIES THROUGH AN AFC

For the study of the temporal evolution of the different species of aphids as well as their natural enemies, on the one hand, and the interactions that could exist between them, on the other hand, we carried out an AFC, from which we retained the results of the first two axes, which explain 85.72 % of variability. The positive side of axis 1 shows a correlation between syrphids, ladybugs, and lacewings, which are in turn correlated with *Myzus persicae* and the end of March. The second axis shows, on the positive side, a correlation between *Aphis spiraecola, Aphis fabae, Toxoptera citricida*, and *Aphis nerii,* which are in turn correlated with the March-April period. On the negative side of the same axis, the interaction of the aphids *Toxoptera aurantii* and *Aphis gossypii* with

Table 8: Values of diversity indices for aphidophagous populations in citrus orchards

Date	Simpson_1-L	Shannon_H	Evenness Index
10/9	0,35	1,07	0,19
25/9	0,31	1,05	0,17
10/10	0,33	1,01	0,11
25/10	0,30	0,9	0,1
10/11	0,21	0,7	0,08
25/11	0	0	0
10 &25/12	0	0	0
10 & 25/1	0	0	0
10/2	0	0	0
25/2	0	0	0
10/3	0,21	0,7	0,08
25/3	0,39	1,13	0,24
10/4	0,42	1,21	0,31
25/4	0,59	2,42	0, 49
10/5	0,54	2,38	0,41
25/5	0,52	2,37	0,4
10/6	0,43	1,24	0,35
25/6	0,45	1,25	0,36
10/7	0,41	1,21	0,31
25/7	0,37	1,12	0,23
10/8	0,35	1,09	0,2
25/8	0,36	1,11	0,22

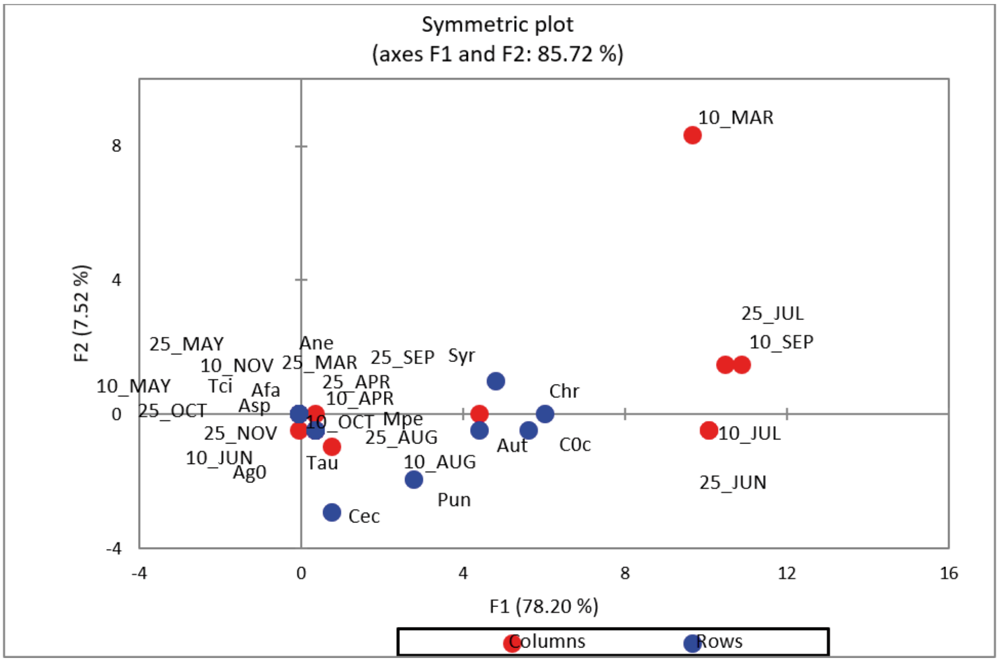

Figure 2: AFC applied to the populations of wingless aphids and their predators during the periods of prospections

gall midges and bugs during the period from August to November (Fig. 2) stands out.

4 DISCUSSION

In this study, the aphid fauna recorded is represented by seven species, the most widespread of which are *Aphis gossypii* and *Aphis spiraecola* , unlike *Toxoptera aurntii, Aphis fabae , Aphis nerii, Myzus persicae,* and *Toxoptera citricida* which were found in limited colonies. Aphids that are specialized in citrus are numerous. Barbagallo and Patti (1986) cited 17 species, but few of these species can have an economic impact on citrus production.

The abundance of *A. gossypii* and *A. spiraecola* reflects their cosmopolitanism and their polyphagy. The first is one of the main pests of citrus in many Mediterranean countries (Kavallieratos et al., 2002; Satar et al., 2014).In addition to these direct damages by feeding on tender shoots and flowers, it is responsible for the transmission of citrus tristeza virus. (Marroquín et al., 2004; Compra et al., 2000). As for the latter, it can, in addition to citrus, infest *Prunus* fruit trees in many Mediterranean countries (Ben Halima-Kamel and Ben-Hamouda, 2005). It is a key pet of *Citrus* x.*clementina* Tanaka in Spain, Algeria, France and Italy (Gomez-Marco, 2015). According to Mostefaoui et al. (2014), its abundance on the Clementine variety could be explained by a better tolerance to high levels of proline in the foliage.

In terms of species, predators are more numerous than parasitoids among natural enemies. However, the parasitism rate observed in aphid populations reflects the abundance of parasites in terms of numbers.

It is known that a parasitoid can only control a single host individual, unlike predators, of which a single individual can ingest a large number of pests. In fact, it has been proven that *Coccinella septempunctata* **c**an consume 469 to 725 individuals of *Myzus persicae* **i**n 17 to 19 days (Aroun, 2015), a syrphid larva can consume 400 to 700 aphids during its lifespan of 8 to 15 days (Deguine and Leclant, 1997) and a *Chrysoperla carnea* larva consumes 300 to 450 individuals **of** *Aphis craccivora* **(**Paulian, 1999).

The parasitoids encountered are four in number. It is worth noting that 29 species of aphid parasitoids are known in Algeria to date (Laamari et al., 2011).In our study areas, the most abundant parasitoid is *Lysiphlebus fabarum*, although Laamari et al. (2011) noted that *Aphidius matricariae* is the most frequent in aphid mummies in Algeria. *L. fabarum* was first reported in Algeria in 1993 in Mostaganem, (Guenaoui and Mahiout, 1993). It is associated with a wide range of host aphids worldwide (Stary, 1988). In Algeria, the sexual strain was found on 9 species of aphids associated with 18 species of host plants (Laamari et al., 2011). In Iran, 47 species of aphids have been reported as being parasitized by this species (Rakhshani et al., 2013).

Predators are mainly composed of insects, most of which are beetles. They even dominate the entomofauna associated with citrus fruits in the study region (Mohammedi et al., 2019).In terms of headcount; they are dominated by ladybugs, followed by lacewings and then hoverflies, although their abundance fluctuates according to the species' life cycle and the rate of prey presence. In Algeria, the fauna of ladybugs includes 48 species, of which 46 are biological control agents that can play a role in plant protection against certain pests (Sahraoui, 2017). However, 21 species that prey on citrus pests in a region of Algeria have been identified, of which Scymninae and Coccinellinae are quantitatively dominant (Sahraoui and Hemptinne, 2009).

Ladybugs are recognized as excellent predators of aphids at all stages of their life, they constitute the essential entomophagous group in the regulation of aphid populations. (Saharaoui et al., 2001).Their density increases with that of their prey (Sahraoui and Hemptinne, 2009). The presence of natural enemies is linked to climatic conditions, food availability (aphids) and species richness of the flora.

In addition to ladybugs, hoverflies are also known for their predation on aphids. The most widespread species is *Episyrphus balteatus,* but other species such as *Sphaerophoria scripta, Syritta pipiens* and *Eristalis tenax* are also abundant in a region of northeastern Algeria (Djellab et al., 2013). The larvae of hoverflies, especially those of *Episyrphus balteatus*, are also important predators for the control of aphids. Some predators show a preference for certain prey over others. Indeed, it has been shown that the effect of different prey species on the feeding capacity of *E. balteatus* larvae is higher on *Aphis gossypii and Myzus persicae* than on *A. craccivora* (Hong and Hung, 2010).

The diversity of aphidophages varies from season to season according to the life cycle of each species involved, as well as their reaction to variations in environmental conditions and prey availability. In fact, this diversity becomes important in the spring, but it regresses in the summer and autumn and becomes zero in the winter before appearing with low values at the beginning of spring. This translates to the life cycle of insects in general, which depends heavily on climatic conditions. Therefore, most insects die before the arrival of winter, and few of them hibernate in different shelters (Mohammedi, 2015). In addition, in temperate regions, adaptation to winter conditions is an important trait of the biological cycle that can influence their ecological and evolutionary success (Raymond et al, 2013). Some species of ladybugs, such as

C. algerica Kovár, 1977 **,** *Hippodamia variegata* (Goeze, 1777), and *P. subvillosus* Sturm, 1837, emerge from hibernation a little earlier and start laying eggs in early spring, and even earlier if climatic conditions become favorable. This is in contrast to the small-sized species **(***Scymnini, Platynaspini, Hyperaspini***)**, which begin their reproductive activities late and last until summer (Ben Halima-Kamel et al., 2011). Some authors think that the diversity of predator species has no effect on the strength of aphid suppression. Thus, for the biological control of aphids, conservation strategies that target the main predator species will be more effective than those that target the diversity of predators (Straub and Snider, 2006).In addition, the nature of prey can even influence the biological evolution of some predators, since it has been shown that females of *C. septempunctata* fed with *A. pisum* and *S. avenae* laid twice as many eggs as those fed with *A. fabae* and *A. craccivora* (Kalushkov and Hodek, 2004). Therefore, to succeed in biological control by conservation, it is necessary to know the effective entomopathogen and then act on the parameters that are favourable to it.

The AFC has identified affinities between aphid species and their potential predators. A large diversity of natural enemies coexist and share the same food (Sahraoui and Hemptinne, 2009; Sahraoui et al., 2015).In addition, it should be noted that the behaviour, abundance, and distribution of predators can be influenced by the physical characteristics of the habitat (Ben Halima Kamel et al, 2011), but also by the nature of the prey, regardless of its density. In fact, correlations between aphids and aphidophages, translating predation activities, are noted during the period from the end of March to November. The present analysis (AFC) also revealed a strong correlation between the ladybugs present, the hoverfly **(***E. balteatus***)** and the lacewing **(***Chrysoperla carnea***)** with *Myzus persicae*, unlike the bugs and the aphid midge **(***Aphidoletes aphidimyza***)** which showed a correlation with *Toxoptera aurantii* and *Aphis gossypii*. The choice of prey by the predator, in the case of aphid diversity, depends on both intrinsic and extrinsic factors. Thus, it has been shown that *C. septempunctata* showed higher predation efficiency for *Aphis craccivora, A. fabae* and *A. gossypii* than for other species (Sarker et al., 2019). On the other hand, *Acyrthosiphon pisum* Harris, 1766 and *Megoura viciae* Buckton, 1876 were more attractive to *E. balteatus***,** while *Aphis fabae* and all other aphids were less attractive. Similarly, the consumption of these two aphids increases the fecundity of the predator (Almohamad et al., 2007). It was also mentioned that the type of adjacent habitat and the identity of the predator affect the direction of predator movement. Thus, information on predator movement can be used to design the distribution of crops and natural habitats in agricultural landscapes that maximize pest control services (Samaranayake and Costamagna, 2019)**.** Even crop-associated plants are of great effect in the biological control of certain pests, as it has been shown that the sugar content of Mediterranean flowering plants, especially the trehalose content of pollen and nectar as a food resource for adult *Chrysoperla carnea*, has a positive impact on the fecundity and longevity of this insect predator (Gonzales et al., 2016).

The preservation and conservation of insect predators in general and aphidophages in particular allow for the biological and sustainable protection of agrosystems in general and citrus cultivation in particular. However, the success of this process requires the mastery of the interactions that occur between aphids, aphidophages, and the surrounding environment.

5 CONCLUSION

The aphid fauna recorded from the three citrus orchards surveyed is represented by seven species, the most widespread of which are *Aphis gossypii and Aphis spiraecola***.** The colonies of aphids are only present during the autumn and spring periods. These are associated with an aphidophages fauna consisting of 34 species, of which 30 are predatory and 4 are Parasitoid**.** However, the most widespread aphidophages are *Episyrphus balteatus***,** *Chrysoperla carnea, Coccinella septempunctata, Aphidoletes aphidimyza* (predators), and *Lysiphlebus fabarum* (parasitoid).

In terms of richness and frequency, ladybugs are the most numerous with 100 individuals (31.35 %), followed by lacewings with 75 individuals (23.51 %), then by hoverflies with 65 individuals (20.37 %), then by bugs with 45 individuals (14.11 %), and finally by gall midges with only 4 individuals, or 1.25 %. Other generalist predators that can have an impact on aphid control are also present, with a total of 30 individuals (9.4 %) distributed over 19 different species.

The highest diversity of aphidophages is noted towards the end of April with D = 0.59, H = 2.42, E = 0.49. This diversity gradually regresses until it becomes zero from November onwards, before manifesting itself again from the beginning of March.

The study revealed affinities between aphid species and their potential predators. Indeed, correlations between aphids and aphidophages, translating predation activities, were noted during the period from the end of March to November. The choice of prey by the predator, in the case of aphid diversity, depends on both intrinsic and extrinsic factors.

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Bio-efficacy, persistence and residual toxicity of greener insecticides against predominant flea beetles on cabbage crop in Kashmir

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Bio-efficacy, persistence and residual toxicity of greener insecticides against predominant flea beetles on cabbage crop in Kashmir

Abstract: The flea beetles, *Phyllotreta striolata* (Fabricius, 1803) and *Altica himensis* (Shukla, 1960) (Coleoptera: Chrysomelidae: Alticinae) are one of the most serious pests of *Brassica oleracea* L. in Kashmir. In order to find eco-friendly control against these pests, bio-efficacy, persistence and residual toxicity of some newer insecticides *viz*., Emamectin benzoate 5SG @ 0.002 and 0.004 per cent, Spinosad 45SC @ 0.0035 and 0.007 per cent was evaluated against *P. striolata* and *A. himensis* infesting cabbage crop. Mortality caused by these insecticides was recorded in all the treatments. The result revealed that spinosad 45SC @ 0.007 per cent exhibited significantly lowest pest population and the highest efficacy against cabbage flea beetles. The persistence and residual toxicity of these insecticides was worked and it was found that spinosad 45SC @ at 0.007 per cent revealed the highest PT value of (346.11 & 321.43) for *P. striolata* and (299.57 & 322.38) for *A. himensis* compared to other insecticides. LT_{50} values of (4.13 & 3.38) for *P. striolata* and (3.08 & 3.84) for *A. himensis* were the highest for Spinosad 45SC @ 0.007 per cent. It was concluded that spinosad 45SC @ 0.007 per cent offers a feasible choice for the management of *P. striolata* and *A. himensis.*

Key words: bio-efficacy, persistence, residual toxicity, *Phyllotreta striolata, Altica himensis*, Spinosad 45SC, emamectin benzoate 5SG

Biološka učinkovitost, obstojnost in rezidualna toksičnost bolj zelenih insekticidov pri zatiranju prevladujočih hroščev bolhačev na zelju v Kašmirju

Izvleček: Hrošči bolhači, kot sta vrsti *Phyllotreta striolata* (Fabricius, 1803) in *Altica himensis* (Shukla, 1960) (Coleoptera: Chrysomelidae: Alticinae), so najnevarnejši škodljivci na zelju v Kašmirju. Z namenom najti okolju prijazni nadzor, biološko učinkovitost, obstojnost in rezidualno toksičnost nekaterih novih incekticidov sta bila pri zatiranju teh *škodljivcev* na zelju preiskušena emamktin benzoat 5SG @ 0,002 in 0,004 % in spinosad 45SC @ 0,0035 in 0,007 %. Smrtnost, ki sta jo povzročila ta dva insekticida je bila zabeležena pri vseh obravnavanjih. Rezultati so pokazali, da je spinosad 45SC @ 0,007 % povzročil značilno manjšo populacijo škodljivca in je imel večjo učinkovitost pri zatiranju hroščev bolhačev na zelju. Preučeni sta bili obstojnost in rezidulana toksičnost teh dveh insekticidov, pri čemer je bilo ugotovljeno, da je imel spinosad 45SC @ pri 0,007 % največjo PT vrednost (346,11 & 321,43) za vrsto *P. striolata* in (299,57 & 322,38) in za vrsto *A. himensis* v primerjavi z drugimi insekticidi. LT_{50} vrednosti (4,13 & 3,38) za vrsto *P. striolata* in (3,08 & 3,84) in za vrsto *A. himensis* so bile največje za spinosad 45SC @ 0,007 %. Zaključeno je bilo, da ponuja spinosad 45SC @ 0,007 % primerno izbiro za upravljanje s škodljivcema *P. striolata* in *A. himensis.*

Ključne besede: bio-učinkovitost, obstojnost, rezidulana toksičnost, *Phyllotreta striolata, Altica himensis,* spinosad 45SC, emamektin benzoat 5SG

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1 INTRODUCTION

Vegetables are an important component of the human diet and cruciferous crops are among the important vegetables grown in many parts of the world. Vegetables have a vital role in feeding the world population and have economic and commercial worth due to their nutritional, crop rotation, bio-industrial and biocontrol properties (Ahuja et al., 2010). Cabbage (*Brassica oleracea* var. *capitatata* L.) commonly known as Pata Kopi or Bhadha Kopi are important vegetables produced in Kashmir. Cabbage is a highly exported vegetable in Kashmir and offers a source of living to all those who are intricated in cabbage production from its cultivation till it goes to the final user. Cabbage is well-known as the king of curries and is used in salads. Several biotic and abiotic factors reduce the yield of cabbage crops. Among biotic factors, insect pests are one of the major constraints and impose severe limitations on profitable cabbage production in India. Flea beetles are one of the key insect pests that attack vegetables specifically brassicaceous crops (Feeny et al., 1970; Palinswamy and Lamb, 1992). Alticine beetles attack agricultural, horticultural, floricultural and ornamental crops. They represent an important group, as they cause enormous injury to seedlings and nibble small round holes into leaves slowing the growth of a plant and decreasing assimilation. Flea beetles in enormous number can even kill the plant in an early phase of their development and also affect the yield of a plant (Anderson & Walker, 1934; Feeny et al., 1970). The adult beetle produces massive economic damage as they feed on different plant parts including leaves and non-woody stems (Konstantinov & Tishechkin, 2004). Present crop production is reliant heavily on insecticidal application against pests in the field condition (Way and Van Embden, 1999). Management by insecticides in the world of agricultural practices has led to chemical resistance, residue problem (Mandal et al*.,* 2006), pest resurgence (Metcalf, 1994) and are harmful to natural enemies (Desneux et al*.,* 2011). At the ecosystem level, they cause loss of biodiversity (Tilman et al., 2002). On the other hand, there is an increase in the demand for residue-free food. Thus, there is a need for switching from chemical insecticides to greener insecticides. Greener insecticides are a safer choice than chemical insecticides and are eco-friendly as they remain viable in the environment for short time compared to conventional insecticides. Unlike chemical insecticides, they prevent the development of resistance (Copping & Menn, 2000). Many greener insecticides have been used for the management of pests as a substitute to conventional insecticides (Shrestha & Reddy, 2017) including flea beetles (Antwi & Reddy, 2016). The present study was aimed to evaluate the effect of some greener insecticides against flea beetles on cabbage crop both in the field and the laboratory.

2 MATERIALS AND METHODS

2.1 FIELD ASSAY

The effectiveness of greener insecticides against flea beetles on cabbage was evaluated during the year 2019-20 and 2020-21 at Mirgund, Baramulla Kashmir with latitude $34^0 13'79''$ and longitude of $74^0 65'66''$. Cabbage seedlings were sown on the 29th of February 2019 and the 9th of March 2020 in well-raised nursery beds. The nursery beds were irrigated regularly till transplantation. Beds were made as per the design and dimensions to start transplantation. Cabbage seedlings (F1 hybrid) were transplanted after 30 days of sowing. There were five treatments including an untreated check with four replications *viz.*, emamectin benzoate 5SG @ 0.002 and 0.004 per cent, spinosad 45SC @ 0.0035 and 0.007 per cent. In control, only water was used for investigation. The plot size for each treatment was 4 x 3 m per replication. A buffer zone of 1.25 m was maintained between different treatments. In each plot crop spacing of 50 x 50 cm was maintained. The crop was raised as per recommended packages of practices. When the flea beetle population exceeded ETL of 3 beetles/leaf, the crop was sprayed with insecticides having a spray volume of 500 l ha^{-1} using the foot-pump sprayer. For control plots, only water was used. A buffer area of 1.5 m was kept around each experimental plot to protect against possible drift during the spraying operation. The pre-count was taken one day prior to application and mortality of pests was recorded at 1, 3, 7, 14 and 21 days. Data obtained on mean per cent mortality of adult flea beetles in different treatments were subjected to statistical analysis using Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT).

2.2 LABORATORY BIOASSAY

2.2.1 Leaf dip method

For determining laboratory bio-assay, untreated leaves were collected from the tagged plants. Test liquid was prepared followed by agitation. Individual leaves were dipped for 5 seconds in the insecticidal solution. The surface liquid was dried from leaves in ambient condition before placing them in Petri-plates. The stalk of leaves was covered with paraffin wax to maintain their turgidity for 24 hours of exposure. Leaf discs were placed upside down over moist filter paper in Petri-plates in a laboratory. Adult beetles were collected using an aspirator. Ten numbers of flea beetles of the same size were released on each leaf disc by using a sable brush (Fig. 1a). These Petri-plates were kept in B.O.D at 28 ± 1 ^{0}C and 70 ± 2 % relative humidity (Fig. 1b). Observations on mortality was recorded after 24 hours and continued till mortality reached less than 10 %. Mortality was assessed by tapping the insect and looking for beetle movement. The insects not able to make coordinated movements were counted as dead. Each treatment was replicated thrice. Control mortality of test insects was also observed by releasing insects on untreated leaves.

2.3 STATISTICAL ANALYSIS

2.3.1 Corrected mortality

The per cent mortality was worked out using Abbott's formula (Abbott, 1925).

$$Corrected\ \%\ mortality = \left(1 - \frac{n\ in\ T\ after\ treatment}{n\ in\ Co\ after\ treatment}\right) \times 100$$

Where n = no of insects
T = Treated
Co = control

2.3.2 Calculation of persistent residual toxicity

Persistent residual toxicity (PT) was determined as per the method given by Pradhan (1967) and Sarup et al. (1970). The average persistent toxicity (T) was determined by adding the values of corrected per cent mortalities of each observation and dividing the total by the total number of observations.

Persistent toxicity (PT) = Average residual toxicity x *period for which toxicity was observed*

Based on PT values the order of efficacy of each treatment was determined. Relative persistence of each insecticide was determined as per Bharti et al. (2015), by taking PT values of least toxic insecticide as unity

$$Relative\ persistence\ (RP) = \frac{PT\ \text{value of insecticides}}{\text{Insecticide with lowest}\ PT\ \text{value}}$$

2.3.3 Relative efficacy of insecticides based on PT and LT_{50} values

The relative efficacy of each insecticide based on LT_{50} and persistent toxicity (PT) values were determined. The data were subjected to probit regression analysis (Finney, 1971) for determining LT_{50} values.

3 RESULTS

3.1 BIO-EFFICACY

3.1.1 Bio-efficacy of insecticides against *Phyllotreta striolata* during 2019-20 and 2020-21

All insecticidal treatments were found significantly superior over untreated control in minimizing the pest

Figure 1a: Release of flea beetles on cabbage leaves

Figure 1b: Maintaining insects in a B.O.D incubator

population. Statistically, a non-significant difference was noted in the pre-treatment count of the flea beetle population. Perusal of the data (Table 1) revealed that the highest cumulative mean per cent mortality of 78.20 ± 18.59 was recorded in Spinosad 45SC @ 0.007 per cent, followed by 65.56 ± 24.89 @ 0.0035 per cent. Cumulative mean per cent mortality of 65.24 ± 25.17 was recorded in emamectin benzoate @ 0.004 per cent followed by 52.46 ± 17.28 @ 0.002 per cent. Untreated check (water spray) recorded the lowest cumulative mean per cent mortality of 8.36 ±3.58 per cent. All the treatments were statistically different from untreated control, whereas the treatments of emamectin benzoate 5SG @ 0.004 and spinosad 45 SC @ 0.0035 per cent were statistically at par with each other and statistically different with rest of the concentrations when tested at p = 0.05 at 21 days after application of treatment. However, the treatments of emamectin benzoate 5SG @ 0.002 and 0.004 per cent, spinosad 45SC @ 0.0035 and 0.007 per cent were statistically different from each other. Among the treatments, emamectin benzoate 5SG @ 0.002 and spinosad 45SC @ 0.0035 per cent were also statistically different from each other when tested at p = 0.05 at 21 days after application of treatment. The order of efficacy was observed as spinosad 45SC @ 0.007 per cent > spinosad 45SC @ 0.0035 per cent > emamectin benzoate 5SG @ 0.004 per cent and emamectin benzoate 5SG @ 0.002 per cent respectively.

Data recorded in (Table 2) revealed that the highest cumulative mean per cent mortality of 78.16 ± 18.29 was recorded in spinosad 45SC @ 0.007 per cent followed by 65.60 ± 18.29 @ 0.0035 per cent. Cumulative mean per cent mortality of 63.95 ± 26.29 was recorded in emamectin benzoate @ 0.004 per cent followed by 51.95 ± 17.70 @ 0.002 per cent. Untreated check (water spray) recorded the lowest pooled mean per cent mortality of 8.43 ±3.20. All the treatments were statistically different from untreated control, whereas the treatments of emamectin benzoate 5SG @ 0.004 and spinosad 45 SC @ 0.0035 per cent were statistically at par with each other and statistically different with rest of the concentrations when tested at p = 0.05 at 21 days after application of treatment. However, the treatments of emamectin benzoate 5SG @ 0.002 and 0.004 per cent, spinosad 45SC @ 0.0035 and 0.007 per cent were statistically different from each other. Among the treatments, emamectin benzoate 5SG @ 0.002 and spinosad 45SC @ 0.0035 per cent were also statistically different from each other when tested at p = 0.05 at 21 days after application of treatment. The order of efficacy was observed as spinosad 45SC @ 0.007 per cent > spinosad 45SC @ 0.0035 per cent > emamectin benzoate 5SG @ 0.004 per cent and emamectin benzoate 5SG @ 0.002 per cent respectively.

3.1.2 Bio-efficacy of insecticides against *Altica himensis* during 2019-20 and 2020-21

All insecticidal treatments were found significantly superior over untreated control in minimizing the pest population. Statistically, a non-significant difference was noted in the pre-treatment count of the flea beetle population. Perusal of the data (Table 3) revealed that the highest cumulative mean per cent mortality of 77.30 ± 19.64 was recorded in spinosad 45SC @ 0.007 per cent, followed by 69.18 ± 18.93 @ 0.0035 per cent. Cumulative mean per cent mortality of 64.12 ± 23.17 was recorded in emamectin benzoate @ 0.004 per cent followed by 54.26 ± 28.0 @ 0.002 per cent. Untreated check (water spray) recorded the lowest cumulative mean per cent mortality of 12.92 ± 5.93. All the treatments were statistically different from untreated control, whereas the treatments of emamectin benzoate 5SG @ 0.004 and spinosad 45 SC @ 0.0035 per cent were statistically at par with each other and statistically different with rest of the concentrations when tested at p = 0.05 at 21 days after application of treatment. However, the treatments of emamectin benzoate 5SG @ 0.002 and 0.004 per cent, spinosad 45SC @ 0.0035 and 0.007 per cent were statistically different from each other. Among the treatments, emamectin benzoate 5SG @ 0.002 and spinosad 45SC @ 0.0035 per cent were also statistically different from each other when tested at p = 0.05 at 21 days after application of treatment. The order of efficacy was observed as spinosad 45SC @ 0.007 per cent > spinosad 45SC @ 0.0035 per cent > emamectin benzoate 5SG @ 0.004 per cent and emamectin benzoate 5SG @ 0.002 per cent respectively.

Data recorded from (Table 4) revealed that the highest cumulative mean per cent mortality of 75.11 ± 18.43 was recorded in spinosad 45SC @ 0.007 per cent followed by 69.69 ± 18.42 @ 0.0035 per cent. Cumulative mean per cent mortality of 62.34 ± 24.54 was recorded in emamectin benzoate @ 0.004 per cent followed by 54.24 ± 26.54 @ 0.002 per cent. Untreated check (water spray) recorded the lowest pooled mean mortality of 11.42 ± 5.39. All the treatments were statistically different from untreated control, whereas the treatments of emamectin benzoate 5SG @ 0.004 and spinosad 45 SC @ 0.0035 per cent were statistically at par with each other and sta-

Table 1: Effect of different insecticides on the population of *Phyllotreta striolata* on cabbage during 2019-20

Treatment	Concentration (%)	Pretreatment count/10 leaves	Percent mortality of *Phyllotreta striolata* (Days after treatment)					Cumulative mean mortality (%) ± SD
			1	3	7	14	21	
Emamectin benzoate 5 SG	0.002	43.57 $ (6.60)	*24.88 **(29.92)	53.01 (46.72)	52.60 (46.49)	59.92 (50.72)	71.91 (57.99)	52.46 (± 17.28)
	0.004	44.77 (6.69)	31.95 (34.41)	48.17 (43.95)	68.58 (55.90)	85.85 (67.90)	91.66 (73.21)	65.24 (± 25.17)
Spinosad 45 SC	0.0035	45.20 (6.72)	32.63 (34.83)	48.68 (44.24)	68.82 (56.05)	86.22 (68.20)	91.46 (73.01)	65.56 (± 24.89)
	0.007	42.87 (6.55)	52.00 (46.14)	66.87 (54.85)	83.27 (65.85)	91.76 (73.32)	97.12 (80.23)	78.20 (± 18.59)
Untreated control	0	44.33 (6.66)	4.04 (11.59)	6.96 (15.29)	8.16 (16.59)	8.76 (17.21)	13.88 (21.87)	8.36 (± 3.58)
C.D ($p \leq 0.05$)		1.03	1.11	0.89	1.08	1.24	1.32	

*mean of three replications
**Figures in parenthesis indicate Arc transformed value
$ Figures in parenthesis indicate SQRT transformed value

Table 2: Effect of different insecticides on the population of *Phyllotreta striolata* on cabbage during 2020- 21

Treatment	Concentration (%)	Pretreatment count/10 leaves	Percent mortality of *Phyllotreta striolata* (Days after treatment)					Cumulative mean mortality (%) ± SD
			1	3	7	14	21	
Emamectin benzoate 5 SG	0.002	43.23 $ (6.58)	*23.67 **(29.24)	51.39 (45.79)	53.64 (47.08)	59.13 (50.26)	71.93 (58.00)	51.95 (± 17.70)
	0.004	44.07 (6.61)	29.50 (32.89)	45.54 (42.44)	67.70 (55.36)	85.40 (67.53)	91.60 (73.15)	63.95 (± 26.29)
Spinosad 45 SC	0.0035	45.53 $ (6.75)	32.50 (34.75)	48.98 (44.41)	69.04 (56.19)	86.24 (68.22)	91.22 (72.76)	65.60 (± 18.29)
	0.007	42.93 (6.55)	52.70 (46.55)	66.68 (54.74)	82.83 (65.52)	91.54 (73.08)	97.05 (80.11)	78.16 (± 18.29)
Untreated control	0	44.33 (6.66)	4.12 (11.71)	6.54 (14.81)	8.93 (17.38)	10.14 (18.56)	12.40 (20.61)	8.43 (± 3.20)
C.D ($p \leq 0.05$)		2.62	2.31	1.24	1.54	1.46	2.62	

*mean of three replications
**Figures in parenthesis indicate Arc transformed value
$ Figures in parenthesis indicate SQRT transformed value

tistically different with rest of the concentrations when tested at $p = 0.05$ at 21 days after application of treatment. However, the treatments of emamectin benzoate 5SG @ 0.002 and 0.004 per cent, spinosad 45SC @ 0.0035 and 0.007 per cent were statistically different from each other. Among the treatments, emamectin benzoate 5SG @ 0.002 and spinosad 45SC @ 0.0035 per cent were also statistically different from each other when tested at $p = 0.05$ at 21 days after application of treatment. The order of efficacy was observed as spinosad 45SC @ 0.007 per cent > spinosad 45SC @ 0.0035 per cent > emamectin benzoate 5SG @ 0.004 per cent and emamectin benzoate 5SG @ 0.002 per cent respectively.

Table 3: Effect of different insecticides on the population of *Altica himensis* on cabbage during 2019-20

Treatment	Concentration (%)	Pretreatment count/10 leaves	Percent mortality of *Altica himensis* (Days after treatment) 1	3	7	14	21	Cumulative mean mortality (%)± SD
Emamectin benzoate 5 SG	0.002	30.47 $ (5.52)	*25.82 **(30.53)	28.38 (32.19)	51.49 (45.85)	79.62 (63.16)	85.98 (68.00)	54.26 (± 28.0)
	0.004	29.87 (5.47)	35.03 (36.28)	48.52 (44.15)	64.17 (53.22)	80.90 (64.08)	91.97 (73.53)	64.12 (± 23.17)
Spinosad 45 SC	0.0035	34.33 (5.86)	48.64 (44.22)	54.65 (47.66)	65.34 (53.93)	84.85 (67.09)	92.43 (74.02)	69.18 (± 18.93)
	0.007	44.50 (6.67)	48.74 (44.27)	67.18 (55.05)	81.35 (64.41)	92.36 (73.95)	96.85 (79.78)	77.30 (±19.64)
Untreated control	0	30.67 (5.54)	4.97 (12.88)	9.19 (17.64)	13.76 (21.77)	16.94 (24.30)	19.76 (26.39)	12.92 (± 5.93)
C.D ($p \leq 0.05$)			2.23	2.61	1.95	1.85	1.74	

*mean of three replications
**Figures in parenthesis indicate Arc transformed value
$ Figures in parenthesis indicate SQRT transformed value

Table 4: Effect of different insecticides on the population of *Altica himensis* on cabbage during 2020- 2021

Treatment	Concentration (%)	Pretreatment count/10 leaves	Percent mortality of *Altica himensis* (Days after treatment) 1	3	7	14	21	Cumulative mean mortality (%) ± SD
Emamectin benzoate 5 SG	0.002	30.53 $ (5.52)	*25.99 **(30.65)	28.55 (32.29)	51.18 (45.67)	79.67 (63.19)	85.80 (67.86)	54.24 (± 26.54)
	0.004	33.87 (5.82)	27.34 (31.52)	50.20 (45.11)	64.47 (53.41)	81.79 (64.73)	87.89 (69.63)	62.34 (± 24.54)
Spinosad 45 SC	0.0035	34.40 (5.87)	49.23 (44.55)	55.72 (48.28)	66.48 (54.62)	84.97 (67.19)	92.05 (73.62)	69.69 (± 18.42)
	0.007	32.23 (5.68)	50.43 (45.24)	63.61 (52.89)	76.83 (61.22)	89.01 (70.63)	95.64 (77.95)	75.11 (± 18.43)
Untreated control	0	31.00 (5.54)	3.66 (11.02)	8.92 (17.37)	12.37 (20.59)	14.41 (22.30)	17.74 (24.90)	11.42 (±5.39)
C.D ($p \leq 0.05$)			1.06	2.22	2.67	1.48	1.37	

*mean of three replications
**Figures in parenthesis indicate Arc transformed value
$ Figures in parenthesis indicate SQRT transformed value

3.1.3 Persistent toxicity of various insecticides against *Phyllotreta striolata* and *Altica himensis* on cabbage

The persistent toxicity of different insecticides *viz*., emamectin benzoate @ 0.002, 0.004 per cent and spinosad @ 0.0035 and 0.007 per cent was computed revealing important data. Perusal of data (Table 5) revealed that mortality with emamectin benzoate 5SG was observed up to 8 days at both concentrations of 0.002 and 0.004 per cent. The average residual toxicity was found to be 24.74 and 30.90 for emamectin benzoate 5SG @ 0.002 and 0.004 with the persistent toxicity index of 197.94 and 247.2. The mortality with spinosad 45SC was observed up to 9 days at both concentrations of 0.0035 and 0.007 per cent. The average residual toxicity was found to be

28.56 and 38.45 for spinosad 45SC @ 0.0035 and 0.007 with the persistent toxicity index of 257.1 and 346.11. The order of relative efficacy of insecticides based on the index of persistent toxicity is 1, 2, 3 and 4 for spinosad @ 0.007, 0.0035 and emamectin benzoate @ 0.004, 0.002 per cent. Relative persistence values for emamectin benzoate @ 0.004, spinosad @ 0.0035 and 0.007 per cent were 1.24, 1.29 and 1.74 times more persistent than emamectin benzoate @ 0.002. Overall spinosad 45SC @ 0.007 was having maximum mortality against *Phyllotreta striolata* (79.32) after one day with higher persistence > spinosad 45SC @ 0.0035 (71.51) > emamectin benzoate 5SG @ 0.004 (69.20) > emamectin benzoate 5SG @ 0.002 (56.55) as depicted from (Fig. 2).

Perusal of data (Table 6) revealed that mortality with emamectin benzoate 5SG was observed up to 7 days at both concentrations of 0.002 and 0.004 per cent. The average residual toxicity was found to be 26.32 and 28.81 for emamectin benzoate 5SG @ 0.002 and 0.004 with the persistent toxicity index of 184.24 and 201.7. The mortality with spinosad 45SC was observed up to 8 and 9 days at concentrations of 0.0035 and 0.007 per cent. The average residual toxicity was found to be 29.9 and 35.71 for spinosad 45SC @ 0.0035 and 0.007 with the persistent toxicity index of 239.24 and 321.43. The order of relative efficacy of insecticides based on the index of persistent toxicity is 1, 2, 3 and 4 for spinosad @ 0.007, 0.0035 and emamectin benzoate @ 0.004, 0.002 per cent. Relative persistence values for emamectin benzoate @ 0.004, spinosad @ 0.0035 and 0.007 per cent were 1.09, 1.29 and 1.74 times more persistent than emamectin benzoate @ 0.002. Overall spinosad 45SC @ 0.007 was having maximum mortality against *P. striolata* (80.60) after one day with higher persistence > spinosad 45SC @ 0.0035 (70.60) > emamectin benzoate 5SG @ 0.004 (66.21) > emamectin benzoate 5SG @ 0.002 (60.79) as depicted from (Fig. 3).

3.1.4 Persistent toxicity of various insecticides against *Altica himensis* on cabbage during 2019-2020 and 2020-2021

Perusal of data (Table 7) revealed that mortality with emamectin benzoate 5SG was observed up to 8 days at both concentrations of 0.002 and 0.004 per cent. The average residual toxicity was found to be 20.84 and 24.86 for emamectin benzoate 5SG @ 0.002 and 0.004 with the persistent toxicity index of 166.77 and 198.89. The mortality with spinosad 45SC was observed up to 8 days at both concentrations of 0.0035 and 0.007 per cent. The average residual toxicity was found to be 29.92 and 37.44 for spinosad 45SC @ 0.0035 and 0.007 with the persistent toxicity index of 209.49 and 299.57. The order of relative efficacy of insecticides based on the index of persistent toxicity is 1, 2, 3 and 4 for spinosad @ 0.007, 0.0035 and emamectin benzoate @ 0.004, 0.002 per cent. Relative persistence values for emamectin benzoate @ 0.004, spinosad @ 0.0035 and 0.007 per cent were 1.19, 1.25 and 1.79 times more persistent than emamectin benzoate @ 0.002. Overall spinosad 45SC @ 0.007 was having maximum mortality against *A. himensis* (82.46) after one day with higher persistence > spinosad 45SC @ 0.0035 (71.32) > emamectin benzoate 5SG @ 0.004 (65.88) > emamectin benzoate 5SG @ 0.002 (56.49) as depicted from (Fig. 4).

Perusal of data (Table 8) revealed that mortality with emamectin benzoate 5SG was observed up to 8 and 7 days at a concentration of 0.002 and 0.004 per cent. The average residual toxicity was found to be 19.68 and 28.55 for emamectin benzoate 5SG @ 0.002 and 0.004 with the persistent toxicity index of 157.51 and 199.91. The mortality with spinosad 45SC was observed up to 9 days at both concentrations of 0.0035 and 0.007 per cent. The average residual toxicity was found to be 27.95 and 35.82 for spinosad 45SC @ 0.0035 and 0.007 with the persistent toxicity index of 251.55 and 322.38. The order of relative efficacy of insecticides based on the index of persistent toxicity is 1, 2, 3 and 4 for spinosad @ 0.007, 0.0035 and emamectin benzoate @ 0.004, 0.002 per cent. Relative persistence values for emamectin benzoate @ 0.004, spinosad @ 0.0035 and 0.007 per cent were 1.26, 1.59 and 2.04 times more persistent than emamectin benzoate @ 0.002. Overall spinosad 45SC @ 0.007 was having maximum mortality against *A. himensis* (80.11) after one day with higher persistence > spinosad 45SC @ 0.0035 (69.79) > emamectin benzoate 5SG @ 0.004 (68.11) > emamectin benzoate 5SG @ 0.002 (55.00) as depicted from (Fig. 5).

Table 5: Persistent residual toxicity of different insecticides against *P. striolata* on cabbage during 2019-20

Insecticide	Concentration %	Corrected percent mortality									P	ART	PT	ORE	RP
		(Days after treatment)													
		1	2	3	4	5	6	7	8	9					
Emamectin benzoate 5 SG	0.002	56.55	46.20	34.21	22.31	18.60	13.62	4.83	1.62	0	8	24.74	197.94	4	1
Emamectin benzoate 5 SG	0.004	69.20	58.79	42.10	35.94	20.01	14.76	5.46	1.01	0	8	30.90	247.27	3	1.24
Spinosad 45 SC	0.0035	71.51	53.65	46.42	32.41	22.21	17.79	8.02	3.3	1.79	9	28.56	257.10	2	1.29
Spinosad 45 SC	0.007	79.32	72.49	64.06	56.39	38.79	21.02	9.26	3.67	1.11	9	38.45	346.11	1	1.74

P = Period for which toxicity
ART = Average residual toxicity
PT = Index of persistent toxicity
ORE = Order of relative efficacy based on PT values

Table 6: Persistent residual toxicity of different insecticides against *P. striolata* on cabbage during 2020-21

Insecticide	Concentration %	Corrected percent mortality									P	ART	PT	ORE	RP
		(Days after treatment)													
		1	2	3	4	5	6	7	8	9					
Emamectin benzoate 5 SG	0.002	60.79	48.10	35.94	20.01	11.79	5.46	2.20	0	0	7	26.32	184.24	4	1
Emamectin benzoate 5 SG	0.004	66.21	50.11	39.78	21.75	14.26	7.11	2.49	0	0	7	28.81	201.71	3	1.09
Spinosad 45 SC	0.0035	70.60	59.79	42.79	30.01	20.48	11.20	3.36	1.01	0	8	29.90	239.24	2	1.29
Spinosad 45 SC	0.007	80.60	68.14	56.56	48.20	34.11	20.70	10	2.11	1.01	9	35.71	321.43	1	1.74

P = Period for which toxicity
ART = Average residual toxicity
PT = Index of persistent toxicity
ORE = Order of relative efficacy based on PT values

Table 7: Persistent residual toxicity of different insecticides against *A. himensis* on cabbage during 2019-20

Insecticide	Concentration %	Corrected percent mortality									P	ART	PT	ORE	RP
		(Days after treatment)													
		1	2	3	4	5	6	7	8	9					
Emamectin benzoate 5 SG	0.002	56.49	40.48	31.79	17.89	10.02	5.88	3.11	1.11	0	8	20.84	166.77	4	1
Emamectin benzoate 5 SG	0.004	65.88	56.23	36.49	22.49	12.11	3.46	1.23	1.00	0	8	24.86	198.89	3	1.19
Spinosad 45 SC	0.0035	71.32	51.23	42.32	23.82	12.11	6.23	1.42	1.01	0	8	26.18	209.49	2	1.25
Spinosad 45 SC	0.007	82.46	70.03	58.79	41.36	29.18	11.32	5.32	1.11	0	8	37.44	299.57	1	1.79

P = Period for which toxicity
ART = Average residual toxicity
PT = Index of persistent toxicity
ORE = Order of relative efficacy based on PT values

Table 8: Persistent residual toxicity of different insecticides against *A. himensis* on cabbage during 2020-21

Insecticide	Concentration %	Corrected percent mortality									P	ART	PT	ORE	RP
		(Days after treatment)													
		1	2	3	4	5	6	7	8	9					
Emamectin benzoate 5 SG	0.002	55.00	38.40	31.20	12.11	10.00	6.46	3.11	1.23	0	8	19.68	157.51	4	1
Emamectin benzoate 5 SG	0.004	68.11	50.03	39.63	24.11	11.11	5.83	1.09	0	0	7	28.55	199.91	3	1.26
Spinosad 45 SC	0.0035	69.79	58.11	52.41	32.76	17.11	14.23	4.5	1.63	1.01	9	27.95	251.55	2	1.59
Spinosad 45 SC	0.007	80.11	72.56	64.30	47.86	29.11	18.14	6.83	2.46	1.01	9	35.82	322.38	1	2.04

P = Period for which toxicity
ART = Average residual toxicity
PT = Index of persistent toxicity
ORE = Order of relative efficacy based on PT values

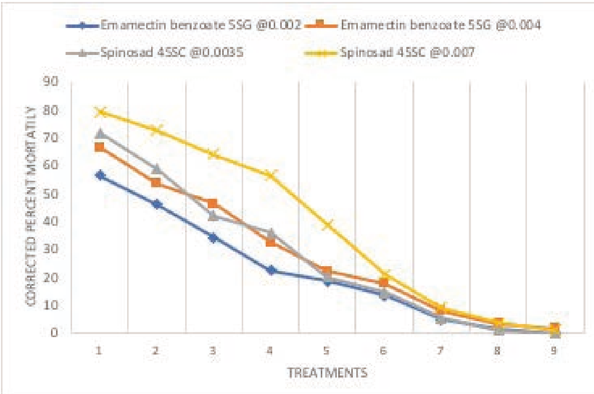

Figure 2: Persistence of toxicity of different insecticides in terms of corrected per cent mortality of P. striolata on cabbage during 2019-20

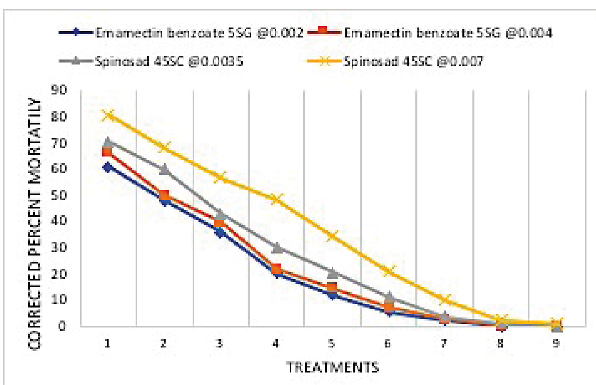

Figure 3: Persistence of toxicity of different insecticides in terms of corrected per cent mortality of P. striolata on cabbage during 2020-21

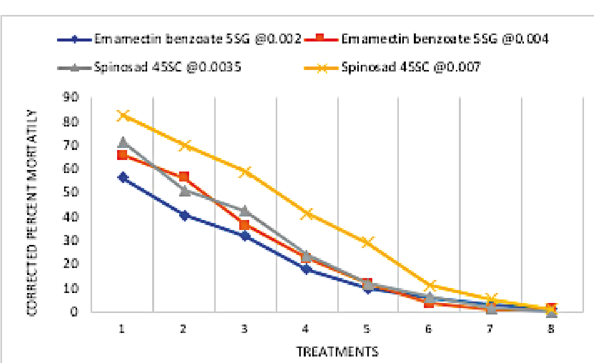

Figure 4: Persistence of toxicity of different insecticides in terms of corrected per cent mortality of A. himensis on cabbage during 2019-20

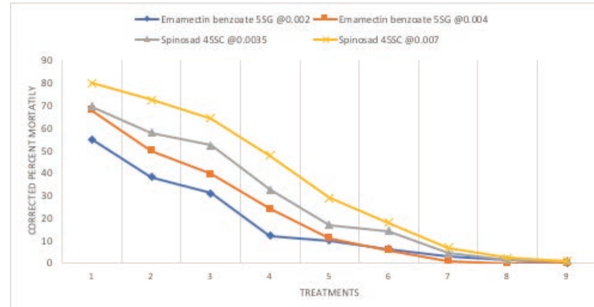

Figure 5: Persistence of toxicity of different insecticides in terms of corrected per cent mortality of A. himensis on cabbage during 2020-21

3.2 LT_{50} VALUES

3.2.1 LT_{50} of various insecticides against *P. striolata* on cabbage during 2019-2020 and 2020-2021

From Table 9 it is clear that during 2019-2020, residual toxicity in terms of LT_{50} values for *P. striolata* was found to be the highest for spinosad 45SC @ 0.007 per cent (4.13) > spinosad 45SC @ 0.0035 (2.26) > emamectin benzoate 5SG @ 0.002 (2.17) > emamectin benzoate 5SG @ 0.004 (1.88). From Table 10 it is clear that during 2020-2021 residual toxicity in terms of LT_{50} values for *P. striolata* was found to be the highest for spinosad 45SC @ 0.007 (3.38) > spinosad 45SC @ 0.0035 (2.40) > emamectin benzoate 5SG @ 0.002 (2.10) > emamectin benzoate 5SG @ 0.004 (1.99). Overall spinosad 45SC @ 0.007 per cent had maximum LT_{50} against *P. striolata.*

3.2.2 LT_{50} of various insecticides against *Altica himensis* on cabbage during 2019-2020 and 2020-2021

From Table 11 it is clear that during 2019-2020, residual toxicity in terms of LT_{50} values for *A. himensis* was found to be the highest for spinosad 45SC @ 0.007 (3.08) > spinosad 45SC @ 0.0035 (1.97) > emamectin benzoate 5SG @ 0.004 (1.88) > emamectin benzoate 5SG @ 0.002 (0.92). From Table 12 it is clear that during 2020-2021, residual toxicity in terms of LT_{50} values for *A. himensis* was found to be the highest for spinosad 45SC @ 0.007 (3.84) > spinosad 45SC @ 0.0035 (3.05) > emamectin benzoate 5SG @ 0.004 (1.99) > emamectin benzoate 5SG @ 0.002 (0.92). Overall spinosad 45SC@ 0.007 per cent had maximum LT_{50} against *A. himensis.*

Table 9: Residual toxicity in terms of LT_{50} value of different insecticides against *P. striolata* on cabbage during 2019-20

Insecticide	y = a + bx	LT_{50}	LT_{50}	95% of Confidential limit	R	R^2	x^2
	Regression equation	(in hours)	(in days)	(Lower-upper limit)	(Correlation coefficient)	(Coefficient of determination)	Chi-square
Emamectin benzoate 5 SG 0.002 %	y = 265-71.5x	52.31	2.17	39.41-59.75	-0.962	0.925	2.72
Emamectin benzoate 5 SG 0.004 %	y = 251-45.2x	45.21	1.88	35.21-52.3	-0.922	0.972	3.12
Spinosad 45 SC 0.0035 %	y = 296-71.3x	54.36	2..26	46.58-67.28	-0.922	0.886	2.56
Spinosad 45 SC 0.007 %	y = 238-42.6x	99.2	4.13	75.26-109.38	-0.961	0.971	2.99

*Significant at $p \leq 0.004$

Table 10: Residual toxicity in terms of LT_{50} value of different insecticides against *P. striolata* on cabbage during 2020-21

Insecticide	y = a + bx	LT_{50}	LT_{50}	95% of Confidential limit	R	R^2	x^2
	Regression equation	(in hours)	(in days)	(Lower-upper limit)	(Correlation coefficient)	(Coefficient of determination)	Chi-square
Emamectin benzoate 5 SG 0.002 %	y = 280-70.5x	50.61	2.10	39.26-62.76	-0.971	0.92	2.76
Emamectin benzoate 5 SG 0.004 %	y = 232-64.7x	47.98	1.99	40.12-58.36	-0.924	0.918	3.03
Spinosad 45 SC 0.0035 %	y = 298-73.6	57.68	2.40	45.23-69.27	-0.923	0.899	2.63
Spinosad 45 SC 0.0007 %	y = 286-73.2x	81.32	3.38	70.11-90.20	-0.992	0.926	2.83

*Significant at $p \leq 0.004$

Table 11: Residual toxicity in terms of LT_{50} value of different insecticides against *A. himensis* on cabbage during 2019-20

Insecticide	y = a + bx	LT_{50}	LT_{50}	95% of Confidential limit	R	R^2	x^2
	Regression equation	(in hours)	(in days)	(Lower-upper limit)	(Correlation coefficient)	(Coefficient of determination)	Chi-square
Emamectin benzoate 5 SG 0.002 %	y = 299-86x	22.16	0.92	16.46-33.26	-0.912	0.992	3.12*
Emamectin benzoate 5 SG 0.004 %	y = 246-94x	45.29	1.88	36.22-52.11	-0.991	0.926	2.93*
Spinosad 45 SC 0.0035 %	y = 301-76x	47.29	1.97	39.26-57.88	-0.988	0.992	3.03*
Spinosad 45 SC 0.007 %	y = 265-74.6x	74.12	3.08	60.22-86.99	-0.972	0.899	2.78*

*Significant at $p \leq 0.004$

Table 12: Residual toxicity in terms of LT_{50} value of different insecticides against *A. himensis* on cabbage during 2020-21

Insecticide	y = a + bx	LT_{50}	LT_{50}	95% of Confidential limit	R	R^2	x^2
	Regression equation	(in hours)	(in days)	(Lower-upper limit	(Correlation coefficent)	(Coefficent of determination)	Chi-square
Emamectin benzoate 5 SG 0.002 %	y = 269-70.2x	22.29	0.92	15.96-37.29	-0.932	0.901	3.00*
Emamectin benzoate 5 SG 0.004 %	y = 282-81x	47.99	1.99	33.26-56.76	-0.906	0.902	2.89
Spinosad 45 SC 0.0035 %	y = 301-85.29x	73.20	3.05	60.52-86.29	-0.907	0.932	3.12
Spinosad 45 SC 0.007 %	y = 236-41.3x	92.29	3.84	81.16-98.11	-0.988	0.977	2.97

*Significant at $p \leq 0.004$

4 DISCUSSION

In the present study, the greener insecticides employed for the control of flea beetles, *viz*., spinosad 45 SC and emamectin benzoate 5 SG were the promising treatments compared to control. Although greener insecticides have been used elsewhere for the control of flea beetles, nevertheless no work has been done for evaluation of these newer insecticides against *Phyllotreta striolata* and *Altica himensis* on cabbage crop in Kashmir. In the present investigation, it was found that spinosad 45SC @ 0.007 per cent caused the highest per cent mortalities of 78.20 and 78.16 per cent against *Phyllotreta striolata* and 77.30 and 75.11 per cent against *Altica himensis* during 2019-2020 & 2020-21 respectively. Spinosad 45SC @ 0.0035, emamectin benzoate 5SC @ 0.004 and emamectin benzoate 5SC @ 0.002 per cent were next effective treatments. This promising activity of spinosad 45 SC @ 0.007 per cent was supported by findings of Brickle et al. (2001), Ahmed et al. (2004) and Jat et al. (2017) who evaluated same insecticide at different concentrations against different insect pests and found that spinosad was an effective pest control agent. Earlier studies are in line with the present study as Meena & Raju (2014) investigated the efficacy of spinosad 45 SC, profenophos 5 EC, fipronil 5 SC and indoxacarb 14.5 SC against *H. armigera* in India and found that spinosad 45 SC was significantly superior to all used treatments and was best and most effective treatment. These results are in agreement with the findings of Kumar et al., (2008) who reported that spinosad was most toxic to *Erias vitelli* when compared with emamectin benzoate.

Persistent residual toxicity of spinosad 45SC @ 0.007 & 0.0035 per cent and emamectin benzoate @ 0.002 & 0.004 per cent was studied against *Phyllotreta striolata* and *Altica himensis*. The study revealed that the highest persistent toxicity of 346.11 & 321.43 and 299.57 & 322.38 was observed for spinosad 45SC @ 0.007 per cent against *P. striolata* and *A. himensis* during 2019-2020 and 2020-21, followed by spinosad 45SC @ 0.0035, emamectin benzoate 5SG @ 0.004 and emamectin benzoate 5SG @ 0.002 percent. However, the results are more or less in conformity with studies of Dake et al. (2017) and Deole et al. (2018) who reported similar results. Our findings showed that spinosad 45 SC @ 0.007 per cent persisted in cabbage crop from 8- 9 days at low and high doses and has the highest PT and LT_{50} values. Sharma et al., (2007) also reported that spinosad persisted in cabbage and cauliflower up to 7 and 10 days, respectively following spinosad application at lower and higher doses. Present studies are in conformity with Shinde et al. (2010) who concluded that on the basis of PT values, spinosad 0.005 % (1026.2) was effective. During the present investigation, spinosad 0.007 per cent was having the highest LT_{50} values of (4.13 & 3.38) against *P. striolata* and (3.08 & 3.84) against *A. himensis* during two years of study. Studies are in line with McLeod et al. (2002) who found that LT_{50} values for thiamethoxam, chlorfenapyr and spinosad against eggplant flea beetle were 1.8, 3.0 and 3.6 respectively. Our study revealed that spinosad 45SC @ 0.007 per cent resulted in maximum mortalities of 79.32, 80.60 against *Phyllotreta striolata* and 82.46, 80.11 against *Altica himensis* after one day of spray and persisted up to 9 days. This is in conformity with McLeod et al., (2011) who found that spinosad is toxic to eggplant flea beetle and found significant mortality of flea beetles after one day of spray. Mortality of beetles on treated foliage declined to 65 % one day after treatment and continued to drop to 63 % after 6 days indicating that activity of chemical had degraded within one week. These results suggested that spinosad 0.007 per cent has great potential for the management of *Phyllotreta striolata* and *Altica himensis* on cabbage crop and could be used as eco-friendly alternative to chemical control.

5 CONCLUSIONS

The use of chemical insecticides against insect pests is the main cause of environmental pollution and the death of natural enemies. The present study was thus aimed to evaluate the use of newer greener insecticides against predominant flea beetles on cabbage crops. There was a shift from chemical to greener insecticides. Control through spinosad 45 SC @ 0.007 per cent proved to be an effective approach against *P. striolata* and *A. himensis* on cabbage crop. The persistence and residual toxicity of greener insecticides was also studied, indicating an effective period over which greener insecticide could persist in the field. The present study would provide insight to make future investigations and also reduces the practice of using toxic chemicals for insect pest control of major crops.

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Investigation of fluctuation in infestation of citrus whitefly *Dialeurodes citri* (Ashmead, 1885) and its predators in organic citrus orchards under varying climatic conditions: A case study from north-west Algeria

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Investigation of fluctuation in infestation of citrus whitefly *Dialeurodes citri* (Ashmead, 1885) and its predators in organic citrus orchards under varying climatic conditions: A case study from north-west Algeria

Abstract: The citrus pest, *Dialeurodes citri* (Ashmead, 1885), poses a significant threat to citrus crops, impacting growth. Various control methods, including biological measures using natural predators like *Clitostethus arcuatus* (Rossi, 1794), *Chrysoperla carnea* (Stephens, 1836), and *Semidalis aleyrodiformis* (Stephens, 1836), were studied over a year in the Chlef region of northwest Algeria. Results indicated that a temperature range of 19.5 °C to 30.5 °C, with 65 % humidity and no precipitation, favored the emergence of *D. citri* adults. Throughout the study, *S. aleyrodiformis* and *C. arcuatus* emerged as the predominant predators. A strong negative correlation (r = -0.91) was observed between *D. citri* abundance and *C. arcuatus* caught on traps. The correlation between nymphal infestation rates and monthly average temperatures was negative (r = -0.72), with temperatures ranging from 13 °C to 27 °C and humidity between 47 % and 73 %, conducive to *D. citri* nymphal infestation. Precipitation negatively impacted *D. citri* and its predators, causing a decline in adult numbers during the rainy season. The overarching goal is to establish an integrated biological control system, bolstering the economic viability of citrus cultivation in the region.

Key words: *D. citri*, predators, citrus, climatic parameters, abundance, north-west Algeria

Preučevanje nihanja napada agrumovega ščitkarja (*Dialeurodes citri* [Ashmead, 1885]) in njegovih plenilcev v ekološki pridelavi pri različnih podnebnih razmerah: vzorčna raziskava iz severozahodne Alžirije

Izvleček: Škodljivec agrumov *Dialeurodes citri* (Ashmead, 1885) predstavlja pomembno grožnjo pridelavi agrumov zaradi negativnega vpliva na rast. Različne metode njegovega zatiranja, vključno z biotičnimi, ki vključujejo plenilce, kot so vrste *Clitosthutus arcuatus* (Rossi, 1794), *Chrysoperla carnea* (Stephens, 1836) in *Semidalis aleyrodiformis* (Stephens, 1836), so bile preučevane v eni rastni dobi na območju Chlef v severozahodni Alžiriji. Rezultati so pokazali, da je temperatura v območju od 19,5 do 30,5 °C, ob 65 % relativni zračni vlagi in brez padavin, pospeševala pojav odraslih osebkov škodljivca. Skozi celotno obdobje raziskave sta se vrsti *S. aleyrodiformis* in *C. arcuatus* pojavljali kot glavna plenilca. Ugotovljena je bila močna negativna korelacija (r = -0,91) med številčnostjo škodljivca (*D. citri*) in plenilca *C. arcuatus* na pasteh. Korelacija med številčnostjo nimf in poprečno mesečno temperaturo je bila negativna (r = -0,72), s temperaturo od 13 do 27 °C in relativno zračno vlago med 47 in 73 %, kar je pospeševalo napad nimf škodljivca. Padavine so negativno vplivale na škodljivca in njegove plenilce, kar je povzročilo upad števila odraslih osebkov v deževnem obdobju. Glavni namen raziskave je vzpostaviti sistem integriranega varstva, kar bi pospešilo bolj gospodarno gojenje agrumov na območju raziskave.

Ključne besede: *D. citri*, plenilci, citrus, podnebni parametri, številčnost, severozahodna Alžirija

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1 INTRODUCTION

Citrus is a major fruit crop in many parts of the world (Ahmed & Azmat, 2019, Anjos et al., 2021) including Africa (Oke et al., 2020). Algeria, for example, is the fifth largest citrus producer in Africa with more than 1.478.053 tons generated from 71.470 ha (FAO, 2020). A major problem is that citrus products around the world have been affected by numerous insect pests (Uygun & Satar,2008), such as the citrus whitefly *Dialeurodes citri* (Wang et al., 2021, Butter & Dhawan, 2021), which can cause high yield losses due to sucking of plant sap by nymphs (Zhang & Li, 2012; Yuan et al., 2017; Boulahia-Kheder, 2021). Recently, Zhang et al. (2019) reported that *D. citri* can be an effective vector for virus CYVCV to citrus. It is also one of the most important citrus pests in the Mediterranean region (Bellows & Meisenbacher, 2007; Martin et al., 2000; Boulahia-Kheder, 2021) including Algeria (Boukhalfa & Bonafonte, 1979).

Indeed, chemical treatment has been one of the best management strategies for controlling the citrus whitefly (Yuan et al., 2017). However, pesticides can have a negative effect on natural enemies and sustainability of crop systems (Corallo et al., 2021).Several environmentally friendly approaches have been developed to control citrus pest (Wuryantini et al., 2021). In the biological control of pests in citrus orchards, predatory insects can play a decisive role and can be a good alternative to chemical treatment (Rattanapun, 2017; Ahmed & Azmat, 2019; Oke et al., 2020; Smaili et al., 2020; Mansour et al., 2021). Their feeding habits are typically polyphagous, and thus help to prevent the buildup of populations of insects that feed on green plants. The identity and abundance of predatory insects has been studied in numerous citruses producing areas around the world. Indeed, Jacas et al. (2010) reported that biological control has been very effective in managing whiteflies. The lacewings and *Clitostethus arcuatus* (Rossi, 1794), for instance, are the main predators of whiteflies (Sagar et al., 2020).

The ladybird beetle, *C. arcuatus,* is one of the most effective predators of the whitefly since its feeds on all development stages of Aleyrodidae (Yazdani & Samih, 2012). A single beetle consumes more than 50 *D. citri* eggs per day (Onillon, 1975). Another predator is *Chrysoperla carnea* (Stephens, 1836), one of the species of common green lacewing insects in the Chrysopidae family. Although the adults feed on nectar, pollen and aphid honeydew, the larvae are active predators and feed on whiteflies and other small insects. This species has been used in the biological control of insect pests on crops (Villenave et al., 2005).

The study of insect-pest biology is very important for developing effective biological control strategies (Umeh & Adeyemi, 2011). However, Benmessaoud-Boukhalfa & Chebrou (2014) reported that the study of the dynamics of whitefly populations is complicated because of their polyvoltinism (*i.e.,* having several generations per year), and their intricate and variable interactions with climatic factors such as temperature, humidity, and precipitation, as well as their host plant.

In the current investigation, the relationship between the citrus whitefly pest *D. citri* and its predators *C. arcuatus, C. carnea* and *S. aleyrodiformis* (Stephens, 1836) was examined over a one-year period under varying climatic conditions. Organic citrus orchards of the Chlef region in north-west Algeria was utilized as a case study. The long-term aim is to develop an integrated biological control system to enhance the economics of citrus growth in the region.

2 MATERIALS AND METHODS

2.1 STUDY REGION AND EXPERIMENTAL SITES

The current investigation was conducted in the citrus region of Chlef located in the north-west of Algeria close to the coast of the Mediterranean Sea. According to meteorological data provided by the Chlef meteorological station for the period 2009-2017, the climate of the area is semi-arid, with hot summers and mild winters (Mahmoudi et al., 2018). The altitude varied from 83 to 156 m. Precipitation was irregular, and the wettest year was 2012 (525 mm) while the driest year was 2015 (256 mm).The mean annual temperature was 21 °C with an average maximum temperature of 30.8 °C in August. Furthermore, the selection of sampling sites was based on the choice of an orchard infested by citrus whitefly populations that had not been treated with pesticides. The experimental sites were in two localities, Boukadir

Table 1: Description of orchards studied

Study sites	Study sites key	Variety	Age (years)	Area (ha)	Geographic coordinates
Boukadir	BOK	*Citrus sinensis* 'Thomson Navel'	59	1.2	-36.04391 10.9496
Ouedfodda	ODF	*Citrus sinensis* 'Valencia'	38	3.2	-36.11569 13.4438

and Oued Fodda (Table 1). The Boukadir investigational site was located 39.6 km from the Oued Fodda citrus orchard.

2.2 METEOROLOGICAL DATA AND INSECT SAMPLING

Monthly maximum and minimum temperature, together with monthly humidity, were recorded from the Chlef meteorological station. The sampling design allows for the collection of data on insect infestation, with a focus on adult insects caught in sticky traps, along with the condition of leaves sampled from different trees. Sampling of insects was made under field conditions approximately every month during the winter periods and fortnightly during the entire season of insect activity (Soto, 1999; Rodrigues & Cassino, 2012) from the first week of July 2015 to the last week of June 2016. For the same sampling periods, the first emerged adults were reported by visual observations on infested young shoots. For each sampling visit, the experiment is replicated and each replication involves selecting 10 trees randomly from each orchard. From each selected tree, 5 leaves are chosen for sampling chosen from different orientations around the tree: North, South, East, West, and the center. With 10 trees sampled and 5 leaves per tree, a total of 50 leaves are sampled per replication and were placed separately in plastic bags to be observed on the laboratory under a binocular microscope. Living nymphs were counted on the leaves to estimate infestation rate of *D. citri* larvae. Nymphal infestation rate and adult abundance in each grove were correlated with climatic parameters. Larval infestation rate equals (number of live larvae/total of larvae)/100. However, adult abundance refers to the mean number of adults for each sampling.

D. citri adults and their predators were caught by suspending randomly three yellow sticky traps (25 x 10 cm) at a height of 1.5 m on the outside of the tree *canopy* (Calabuig et al., 2015; Eserkaya & Karaca, 2016; Hernández-Landa et al., 2018).The yellow sticky traps were placed diagonally, and spaced 50 meters apart from each other, and replaced at every visit and the insects captured were counted under a binocular microscope (x20, ×40, and ×80) (Ekbom & Rumei, 1990). This study employed a total of 120 yellow sticky traps.

2.3 INSECT IDENTIFICATION

Butter and Dhawan (2021) reported that the taxonomists use the characteristic features of the fourth instar pupa of whitefly for species identification.

Insect identification relying solely on external morphology indeed has its limitations, particularly when dealing with species that exhibit morphological variations. In such cases, genetic analysis through DNA barcoding can provide more accurate species identification, with complement morphological observations (Tahir et al., 2018).

Collaborating with taxonomic experts can aid in accurate species identification. In our case, Mr. Streito (INRA Montpellier) and Mr. Reynaud (ANSES Plant Health Laboratory) were actively involved in the identification of whitefly specie as well as their predators.

We utilized identification keys based not only on external morphological criteria but also on internal organs within insect bodies, such as the cement gland of *D. citri* female adult's. By incorporating both external and internal characteristics, we were able to enhance the accuracy and reliability of our identification methods. This comprehensive approach allowed us to effectively distinguish between different species.

Indeed, the fourth pupal stages of *D. citri* were collected from infested leaves and prepared for microscopic observation according to the method established by Martin et al. (2000) and Hodges and Evans (2005). Female adults were removed from sticky traps, their cement glands were extracted and observed under optical microscope to confirm the identification of the pupa (Guimarães, 1996). Martin (1987) reported that the identification of adult whiteflies based on external morphology is difficult when the populations of adults captured through the sticky traps are heterogeneous with more than one species. In the current study, only the *D.citri* were registered without other whiteflies species. The adults were 1.2–1.4 mm long, with body and wings covered with white wax powder (Alford, 2012). Identification of adult predators captured was based on external morphology, *C. carnea* adult's body was green and slender (*i.e.,* 15-20 mm long) with golden eyes (Dreistadt, 2012). However, the *C. arcuatus* body was yellow to reddish brown, with an arc-shaped design on the dorsal side (1.2-1.5 mm long) (Klausnitzer, 2011). Although often confused with whiteflies because their bodies and wings are covered with whitish waxy secretion (Lee et al., 2010), *S. aleyrodiformis* is larger and has four wings with front wings larger than the hind ones (Mcewen et al., 2001).

2.4 STATISTICAL ANALYSIS

The statistical analysis of the data involved the utilization of Past program version 3.24. This software was chosen for its capability to handle the specific analyses required for our study. In particular, Pearson correlation tests were employed to assess the relationship between insect abundance and various meteorological parameters. This choice of statistical tool was based on its suitability for examining the linear association between two continuous variables, which aligns with the nature of our research objectives.

Furthermore, significance levels were established at $p < 0.05$, adhering to conventional standards in statistical analysis to denote a statistically significant result. This threshold ensures that observed correlations are unlikely to have arisen by chance alone, thus lending credibility to our findings.

In interpreting the strength of correlations, we followed the guidelines outlined by Schober et al. (2018). According to their classification, correlations with an *r*-values ranging from 1.0 to 0.9 were considered very strong, indicating a robust and almost perfect linear relationship between variables. Correlations falling between 0.7 and 0.89 were deemed strong, suggesting a substantial linear association. Those ranging from 0.4 to 0.69 were classified as moderate, indicating a moderate degree of linear relationship. Finally, correlations between 0.10 and 0.39 were categorized as weak, signifying a minimal linear association.

By adhering to these established criteria and employing rigorous statistical methods, we aimed to ensure the reliability and validity of our data analysis, thereby enhancing the credibility of the results obtained.

3 RESULTS AND DISCUSSION

3.1 PERIODS OF *D. citri* EMERGENCE

Table 2 shows that the adults of the first generation were recorded in the autumnal period (2015) generally from the end of August to the beginning of September, coinciding with the citrus autumn growth flush, when the mean maximum daily temperature exceeded 37 °C with a mean minimum daily temperature of 24 °C. The mean daily humidity was estimated at 63 % with an absence of precipitation. Furthermore, the second emergence period was recorded in the spring period in mid-April (2016). In the spring citrus growth flush when the mean maximum daily temperature exceeded 26 °C with a mean minimum daily temperature of 13 °C. The mean daily humidity was estimated at 65 % without precipitation (Table 2).

3.2 NUMBER OF ADULTS OF *D. citri* AND ITS PREDATORS DETECTED IN THE CASE STUDY ORCHARDS

The *D. citri* adults were measured in the Oued fodda (ODF) and Boukadir (BOK) groves. The number of citrus whitefly *D. citri* adult pests was found to be the largest in the BOK orchard compared to the ODF grove. In contrast *C. carnea* adult predators were captured in just very small numbers in each orchard with only some adults being trapped from January through June 2016 (Table 3). For example, for the ODF grove the *C. carnea* predator count was only 0.3 ±0.39 from January to July 2016, while the *D. citri* adult pest count was 309.6 ± 421.75. The *C. arcuatus* and *S. aleyrodiformis* adults were more abundant than *C. carnea*. In the ODF grove *S. aleyrodiformis* adults were more abundant from July through December 2015 (19.9 ± 19.70) but their number was small from January through June 2016 (7.5 ± 9.43) (Table 3).

The relationship in seasonal abundance between *D. citri* pests and its predator *C. arcuatus* was analyzed from July 2015 to June 2016 (Figure 1). The number of *D. citri* adults and *C. arcuatus* caught on yellow sticky traps were the largest from July through December 2015 then gradually decreased from January through June 2016 (Figure 1). A moderate positive correlation (*r*= -0.42) in ODF grove was noted and a very strong positive correlation (*r*= -0.91) in the BOK grove between mean abundance of the *D .citri* and its predator *C. arcuatus* adults caught on sticky traps.

Table 2: Dates of first emergence of *D. citri* adults during the sampling period (July 2015-June 2016)

Generation	Citrus grove	Approximate emergence period of earliest adults		Citrus growth flush	T Max (°C)	T Min (°C)	H (%)	P (mm)
1st	Oued fodda	2015	28 August	Autumn growth flush	37	24	63	0
	Boukadir		1 September					
2nd	Oued fodda	2016	20 April	Spring growth flush	26	13	65	0
	Boukadir		23 April					

T Max (°C): mean maximum daily temperature during emergence period of *D. citri*
T Min (° C): mean minimum daily temperature during emergence period of *D. citri*
H (%): mean daily humidity during emergence period of *D. citri*
P (mm): daily precipitation amounts during emergence period of *D. citri*

Table 3: Composition and mean abundance of the *D. citri* and their predators *C. arcuatus, C. carnea* and *S. aleyrodiformis* caught on sticky traps in citrus orchards (July 2015-June 2016)

				Citrus groves	
Orders	Family	Species	Sampling period	ODF	BOK
Hemiptera	Aleyrodidae	*Dialeurodes citri* (Ashmead, 1885)	July-Dec 2015	598.4 ± 527.95	1135.3 ± 1652.13
			Jan-June 2016	309.6 ± 421.75	421.4 ± 612.01
Coleoptera	Coccinellidae	*Clitostethus arcuatus* (Rossi,1794)	July-Dec 2015	19.4 ± 13.19	6.2 ± 7.05
			Jan-June 2016	16.5 ± 16.81	1.7 ± 2.14
Neuroptera	Chrysopidae	*Chrysoperla carnea* (Stephens, 1836)	July-Dec 2015	1.9 ± 4.20	0.9 ± 0.81
			Jan-June 2016	0.3 ± 0.39	0.4 ± 0.66
	Coniopterygidae	*Semidalis aleyrodiformis* (Stephens, 1836)	July-Dec 2015	19.9 ± 19.70	17.42 ± 17.40
			Jan-June 2016	7.5 ± 9.43	1.06 ± 2.42

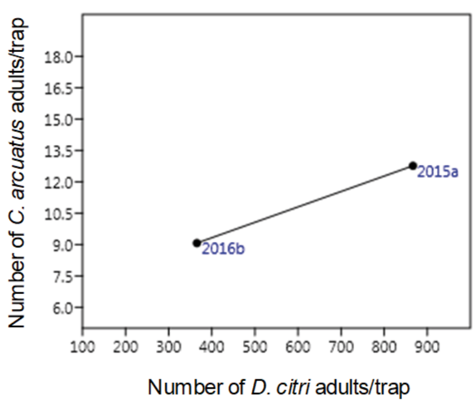

Figure 1: Relationship between the *D. citri* pests and its predator *C. arcuatus* caught on yellow sticky traps in the semi-arid citrus region of Chlef from July 2015 to June 2016. a, Sampling conducted from July through December; b, Sampling conducted from January through June

3.3 RELATIONSHIP BETWEEN TEMPERATURE, HUMIDITY, PRECIPITATION AND NUMBER OF *D. citri* ADULTS

In the two citrus groves, the mean number of *D. citri* adults caught on sticky traps was the highest during September and October (2015) and during April and May (2016), when monthly average temperatures varied from 18 to 30 °C, monthly average humidity varied from 46 to 69 % and with a low precipitation (0 to 27 mm). No *D. citri* adults were trapped in ODF and BOK citrus grove from December 2015 to March 2016 when monthly average temperatures did not exceed 13 °C and with a high precipitation (59 to 130 mm), and a high relative humidity (72 to 77 %)(Figure 2).

In ODF and BOK orchards, the number of adults of *D. citri* was correlated positively with monthly average

Table 4: Correlation between mean adult abundance and climatic parameters

	ODF citrus grove		BOK citrus grove	
	Mean adult abundance			
Climatic parameters	*r*-Value	*p*-Value	*r*-Value	*p*-Value
Monthly average temperature (°C)	0.5219	0.0208*	0.4397	0.0116*
Monthly average humidity (%)	-0.4491	0.0936	-0.4549	0.0463*
Monthly precipitation (mm)	-0.5659	0.0903	-0.4378	0.0366*

$p < 0.05$ (significant) *

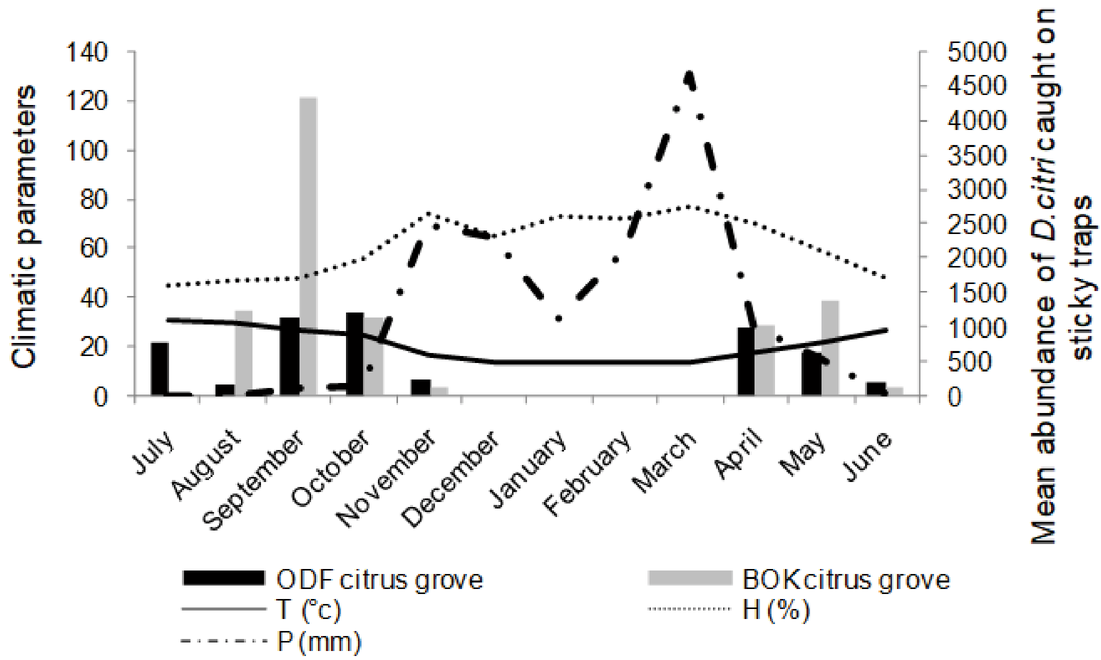

Figure 2: Evolution of *D. citri* captured in relation to monthly average temperatures (T °C), relative humidity (H %) and rainfall (P mm), during the one-year period of study

temperatures ($r_{(ODF)} = 0.52$, $p_{(ODF)} = 0.021$, $r = 0.43$) (Table 4). Whereas the correlation was moderate and negative between monthly data of average humidity, rainfall and mean number of *D. citri* adults ($r_{(BOK)} = -0.45$ & $r_{(BOK)} = -0.43$) in BOK citrus grove.

3.4 RELATIONSHIP BETWEEN TEMPERATURE, HUMIDITY, PRECIPITATION, AND *D. citri* NYMPHAL INFESTATION RATES

The population dynamics of *D. citri* nymphs from July 2015 to June 2016 in the ODF citrus grove showed three infestation peaks appearing respectively in September 2015 (37 %), January 2016 (49 %) and April 2016 (48 %) corresponding to periods of high activity of nymphal populations(Figure 3). Indeed, in the BOK citrus grove, three infestation peaks were recorded in August 2015 (29 %), January (25 %) and April 2016 (26 %) (Figure 3). Additionally, during peaks of the nymphal infestation, the monthly average temperatures varied between 13 and 27 °C, where average monthly humidity data varied between 47 % and 73 % and average monthly rainfall ranged from 3 to 30 mm.

In ODF orchards a significant strong negative correlation (r= -0,72) was noted between monthly average temperatures and nymphal infestation rates of *D. citri*. However, a significant moderate positive correlation(r= 0,62)was recorded between nymphal infestation rate and monthly average humidity. Results were not significant for nymphal infestation rate in BOK orchard, probably due to a low nymphal infestation rate (Table 5). The relationship between climatic parameters and nymphal infestation rates in the semi-arid citrus region of Chlef from July 2015 to June 2016 are shown in Figure3.

Table 5: Correlation between nymphal infestation rate and climatic parameters

	ODF citrus grove		BOK citrus grove	
	Nymphal infestation rate			
Climatic parameters	*r*-Value	*p*-Value	*r*-Value	*p*-Value
Monthly average temperature (°c)	-0.7221	0.0080*	-0.2682	0.3992
Monthly average humidity (%)	0.6209	0.0312*	0.2485	0.4362
Monthly precipitation (mm)	0.4444	0.1477	0.1810	0.5734

$p < 0.05$ (significant) *

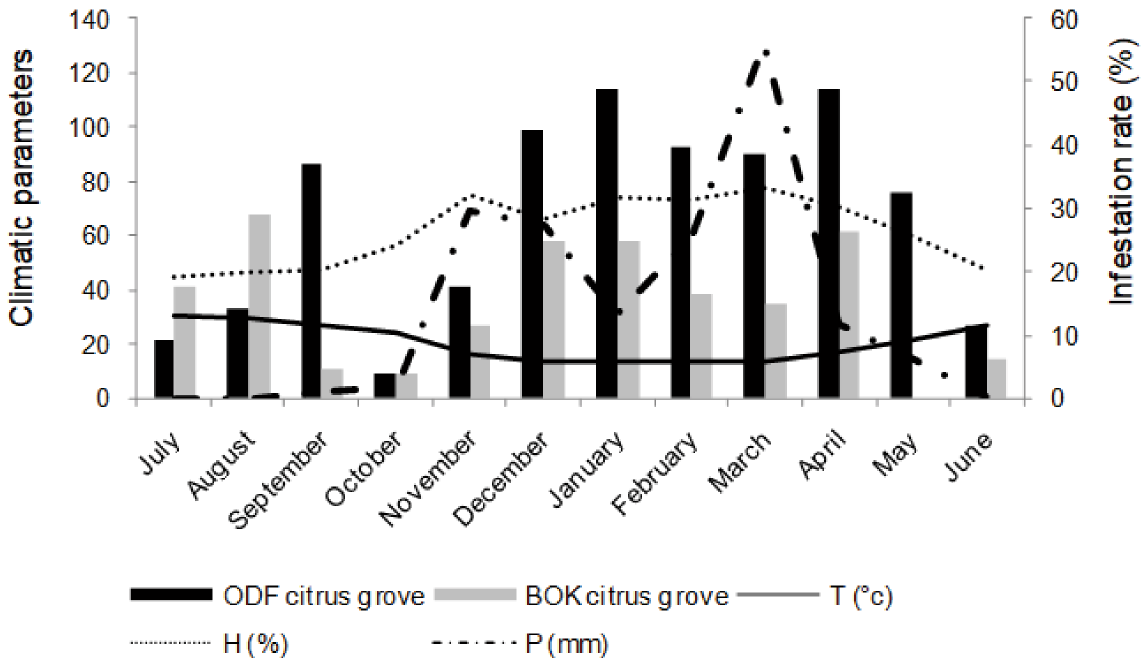

Figure 3: Relationships between climatic parameters and nymphal infestation rate in the semi-arid citrus region of Chlef from July 2015 to June 2016. The principal x-axis illustrates the values of climatic parameters (T (°C), H (%) and P (mm))

3.5 ANALYSIS OF OCCURRENCE OF POLYVOLTINISM & DIAPAUSE FOR *D. citri* PESTS IN CASE STUDY ORCHARDS

The current findings showed two egg laying periods for *D. citri* leading to the appearance of two homogeneous peaks of population clearly separated from each other by a diapause period of 5 to 6 months from November to March. This period of suspended or arrested development, diapause, is usually caused by environmental changes such fluctuations in daylight, temperature, or humidity. These were observed with the current investigation and were like those reported by Saini et al. (2016) in India. Benmessaoud-Boukhalfa & Chebrou (2014) described that the dynamics of whitefly populations is complex because of their polyvoltinism (*i.e.,* having several broods per year), and their interactions with climatic factors such as temperature, humidity, and precipitation, as well as their host plant. Similarly, polyvoltinism was seen by Onillon (1976); Lloréns (1994); Bellows & Meisenbacher (2007),who counted two annual generations of *D.citri* in France, in California and in Spain while three generations per year were found in Japan (Kaneko, 2017). However, unlike in Japan in the sub-humid citrus region in Mitidja plain (north of Algeria), Benmessaoud-Boukhalfa (1987) estimated that the *D. citri's* diapause period has a duration of 4 to 5 months which would make it unlikely for the pest to have more than two broods a year.

It has been found from the current investigation of the orchards in the Chlef region, that the daily average temperature of 19.5 °C and 30.5 °C in the spring and autumn period, respectively, with a daily average humidity of 65 % with no rainfall, were favorable for the early emergence of the *D. citri* adults. Boukhalfa & Bonafonte (1979) also noted that the beginning of emergence started from mid-April in the sub-humid citrus region of Mitidja. However, in France and Spain, the earliest adults were observed in May (Onillon, 1975, Soto 1999), while Ohgushi & Ohkubo (2005) reported that the first adult emergence appeared in early May to mid-June in Japan. The earliest adults of *D. citri* were found during citrus flushing cycles over April-August and September (2015-2016). It was observed, that the females of *D. citri* lays eggs on young shoots of citrus flush growth. Singh et al. (2021) reported that exclusively during the flush period, *D. citri* population was very active. All of this suggests that colder temperatures increase the diapause phase by suppressing emergence.

3.6 EFFECTIVENESS OF PREDATORS *Chrysoperla carnea*, *Clitostethus arcuatus* AND *Semidalis aleyrodiformis* IN REDUCING POPULATIONS OF CITRUS WHITEFLY PESTS

The authors reported three species of predators:

Chrysoperla carnea, Clitostethus arcuatus and *Semidalis aleyrodiformis*. Khan et al. (2020) reported that several insect species belonging to Neuroptera order are a natural enemy of great numbers of pests. *C. carnea* adults however were rarely observed, and their activity was limited as light reduction in the number of adults and eggs of *D. citri*. It can be argued that the effect of the predator *C. carnea* is not significant in decreasing the populations of the citrus whitefly pest. This conclusion is supported by the results of the current study as shown in Table 3. In contrast, large captures of *C. arcuatus* and *S. aleyrodiformis* adults were observed in the citrus orchards in the present investigation (Table 3). Benmessaoud-Boukhalfa (1987) reported that *C. arcuatus* is the most efficient predator of *D. citri* in Algeria. The ladybird beetle *Clitostethus arcuatus* is one of the most effective predators of the whitefly. This ladybird feeds on all development stages of Aleyrodidae (Yazdani and Samih, 2012). A single beetle consumes more than 50 *D. citri* eggs per day (Onillon, 1975).

In Egypt, *C. arcuatus* is considered as the most important predator of *D. citri* with more of 41.9 % of the total number of predators (El-Husseini et al., 2018).

A positive correlation was found between the *D. citri* count and the predator *C. arcuatus* adults, which means that this predatory coccinellids feeds on whitefly but without significantly reducing their abundance. Thus, it can be reasoned that *C. arcuatus* can be efficient against *D. citri* but only when this whitefly lays few numbers of eggs (EPPO, 2004). In addition, the influence of predators on *D. citri* populations decreased with increasing temperature (Bale et al., 2002, Thomson et al., 2010). Increased precipitation also greatly reduces their ability to move and search for prey (Thomson et al., 2010). However, Thöming & Knudsen (2021) reported that the diversity of beneficial insects was dependent on vegetation diversity.

3.7 DYNAMICS OF EFFECT OF TEMPERATURE, PRECIPITATION & HUMIDITY ON INSECT INFESTATION RATES

Dreistadt (2012) reported that the honeydew secreted by *D. citri* nymphs attracts ants, which can disrupt the activity of whitefly predators. According to Byrne (1991), whitefly population dynamics depends on different climatic factors and natural enemies. Climatic parameters such as temperature, rainfall and relative humidity are known to impact the population dynamic of *D. citri* (Kumar, 2001; Rashid et al., 2003; Saini et al., 2016). The present study revealed that temperature may influence the dynamics of *D. citri* nymphal population because a strong negative correlation was found between nymphal infestation rate and monthly average temperatures. This correlation revealed that the *D. citri* nymphal infestation peaks occurred in winter and autumn which were cold to mild periods with monthly average temperatures varying between 13 and 27 °C. The same results were obtained by Saini et al. (2016) who reported a negative correlation between nymphal population of *D. citri* and the mean minimum and maximum temperature.

From the correlation results, a decrease in monthly average temperature resulted in an increase in the *D. citri* nymphal infestation rate (Figure 3). Similarly, Bernardo et al. (2006) also signaled the strong effect of temperature on the development rate of insects. However, a moderate positive correlation was found between *D. citri* nymphal infestation rate and monthly average humidity. Unlike the temperature, increased monthly average humidity resulted in an increase of *D. citri* nymphal infestation rate with values varying between 47 % and 73 % during the *D. citri* nymphal infestation peaks (Figure 3). These results were consistent with a study by Kumar (2001) that demonstrated that humidity varying between 60-75 % was favorable for development of *D. citri*. Likewise, several investigations have also focused on the impact of relative humidity on populations of *D. citri* (Mansour et al., 2021; Zeb et al., 2011; Saini et al., 2016).

The correlation analysis between weather parameters and mean adult abundance of *D. citri* revealed that the monthly average temperatures exhibited a significant moderate positive influence. Two peaks of abundance were recorded in spring and autumn period, seasons which are characterized by higher monthly average temperatures (18 to 30 °C) with less rainfall. In particular, an increase in monthly average temperature resulted in an increase of *D. citri* adult abundance. While monthly average humidity and monthly precipitation had a significant moderate negative correlation with the number of *D. citri* adults caught on sticky traps, this indicated that a decreased monthly average humidity and monthly precipitation resulted in an increase of *D. citri* adult abundance. In addition, the absence of *D. citri* adults on the sticky traps was observed in the winter period when monthly precipitation exceeds 130 mm and monthly average humidity varies between 72 to 77 %.

In closing, with the present study a decrease in *D. citri* adult count was noted during rainy periods and an increase was seen during dry periods. This was like that cited by Rashid et al.(2003) who reported that precipitation and humidity affect the abundance of whitefly populations. Insects can be affected in their development by environmental factors (Bale et al., 2002). Indeed, global warming causes critical changes in population abundance of insects (Sharma, 2014). Finally, there is a need to set up an integrated control system against insect pests (Saini et al., 2016). This must be coordinated with studies of their population dynamics.

4 CONCLUSIONS

This article may facilitate the implementation of an integrated pest management program in Chlef citrus region by understanding the tri-trophic interaction between host plants, *D. citri* populations and theirs predators under the impact of the particular climatic conditions of the region.

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4.2 LIST OF ABBREVIATIONS

BOK: Boukadir; ODF: Oued fodda; FAO: Food and Agriculture Organisation; *D. citri: Dialeurodes citri; C. arcuatus: Clitostethus arcuatus; S. aleyrodiformis: Semidalis aleyrodiformis; C. carnea: Chrysoperla carnea;* T Max: maximal temperature; T Min: minimal temperature; H (%): humidity; P (mm): precipitation. EPPO: European and Mediterranean Plant Protection Organization.

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The history and current state of flax (*Linum usitatissimum* L.) cultivation and use in Japan

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The history and current state of flax (*Linum usitatissimum* L.) cultivation and use in Japan

Abstract: Flax (*Linum usitatissimum* L.) is an ancient crop that has been cultivated for thousands of years for its fiber as well as for its oil rich seeds. The crop was introduced into Japan in the end of the 17th century, but its popularity did not begin to increase until the 1890s. The production of domestic fiber flax exceeded 45,000 metric tons in the late 1910s, and Japan had a well-developed linen industry for flax processing. In the 1960s, the mass production of synthetic fibers led to the collapse of Japanese linen industry, and consequently fiber flax culture in the country disappeared. In Japan, flaxseed oil with rapid drying property was once exclusively used for manufacturing a variety of industrial products such as paints, varnishes, linoleum, and printer's ink. Recently, this vegetable oil has gradually attracted the attention of Japanese consumers because of its health benefits mainly attributed to its high content of alpha-linolenic acid (omega-3 fatty acid). Over the past decade, the consumption of edible flaxseed oil has been increasing rapidly, but domestic oilseed flax production remains small. In this review, we describe the history of fiber flax cultivation in Japan. The current state, problems, and perspectives of oilseed flax culture and use in the country are also presented.

Key words: alpha-linolenic acid, cultivar development, cultivation history, cultural practices, fiber flax, germplasm, oilseed flax, omega-3 fatty acid

Zgodovina in trenutno stanje gojenja in uporabe navadnega lanu (*Linum usitatissimum* L.) na Japonskem

Izvleček: Navadni lan (*Linum usitatissimum* L.) je starodavna poljščina, ki se goji že tisočletja zaradi vlaken kot tudi zaradi na olju bogatih semen. Poljščina je bila uvedena na Japonsko proti koncu 17. stoletja, toda njena priljubljenost se je začela povečevati še le po 1890. Domača pridelava lanenih vlaken je v letu 1910 presegla 45.000 metričnih ton in Japonska je imela v tem času dobro razvito industrijo za predelavo lanu. V obdobju šestdesetih let (1960) je masovna proizvodna plastičnih vlaken povzročila propad industrije lanu na Japonskem, posledično se je izgubila tudi kultura gojenja lanu za vlakna v državi. Potem se je laneno olje na Japonskem , ki se hitro suši, skoraj izključno uporabljalo za izdelavo različnih industrijskih izdelkov kot so barve, laki, linolej in tiskarsko čenilo. V zadnjem času postaja to rastlinsko olje na Japonskem vse bolj priljubljeno pri potrošnikih zaradi zdravilnih lastnosti, ki jih v glavnem pripisujejo veliki vsebnosti alfa-linolenske kisline (omega-3 maščobne kisline). V zadnjih desetletjih se potrošnja jedilnega lanenega olja hitro povečuje, a pridelava ostaja majhna. V tem pregledu je opisana zgodovina pridelave lanu za vlakna na Japonskem. Prikazano je trenutno stanje, problemi in perspektive gojenja lanu za olje v deželi.

Ključne besede: alfa linolenska kislina, razvoj sort, zgodovina gojenja, načini gojenja, lan za vlakna, genetske osnove, lan za olje, omega-3 maščobne kisline

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1 INTRODUCTION

Flax (*Linum usitatissimum* L.) belongs to the Linaceae family. The crop may have originated in the Near-Middle East from where it spread to Europe, the Nile Valley, and over the rest of the world (Allaby et al., 2005; You et al., 2017). Flax is among the oldest crop plants: this crop has been cultivated for thousands of years for its fiber as well as for its oil rich seeds, and the ancient Egyptians already produced fabrics from flax fiber (Ehrensing, 2008; Jhala and Hall, 2010). In the Bible, flax or linen is mentioned many times, e.g., Exodus 9:31, the Book of Proverbs 31:13, and Jeremiah 13:1. For a long time, flax has been selected for production of either fiber or flaxseed (or often called linseed) oil. Consequently, the crop has diversified into two main types, namely, fiber flax and oilseed flax which are the same species but differ considerably in morphology, growth habits, and agronomic characteristics (Soto-Cerda et al., 2013). Fiber flax usually grows up to ca.120 cm tall and is scarcely branched. Oilseed flax is generally shorter, multiple branched, and produces more seeds. Its seed is larger, and higher in oil content than that of fiber flax (Fu et al., 2002).

Currently, oilseed flax is more economically important than fiber flax. Oilseed flax is grown mainly for seed oil, flaxseed meal, and flax straw (Ehrensing, 2008). According to the FAO statistics (FAO, 2023), global flaxseed production reached 3.34 million metric tons harvested from 4.14 million ha in 2021. Russia is the largest producer of flaxseed with 1.3 million tons, followed by Kazakhstan, Canada, China, India, Ethiopia, and France. What makes flaxseed so exceptional is the high alpha-linolenic acid (omega-3 fatty acid) content. Flax is well-known to be the richest botanical source of alpha-linolenic acid along with oilseed perilla [*Perilla frutescens* (L.) Britton var. *frutescens*] (Nakui and Mikami, 2023). Flaxseed oil with high amount of alpha-linolenic acid is highly susceptible to oxidation and polymerization. These properties make it suitable for various purposes in industry, including paint and flooring (linoleum) industries (Jhala and Hall, 2010; Hall et al., 2016). In addition to industrial applications, this omega-3-enriched vegetable oil has been used for human consumption (Čeh et al., 2020). Alpha-linolenic acid is believed to provide health benefits, because it is a potent inhibitor of pro-inflammatory mediators (Oomah, 2001; Jhala and Hall, 2010), and its adequate intake has been increasingly recommended. The positive human health impact is also attributed to high content of lignans, high-quality proteins, and dietary fiber; lignans are phenolic compounds that act as both antioxidants and phytoestrogens (Jhala and Hall, 2010; Bekhit et al., 2018).

Flaxseed oil has gradually attracted the attention of Japanese consumers because of its healthful properties (Nakano et al., 2009; Enokido and Ohashi, 2016). It is also worth mentioning that fiber flax was produced commercially in Japan until the mid-1960s. As far as we know, however, there is no scientific review regarding flax culture in the country. In this paper, we aim to summarize the history, current status, and perspectives of flax cultivation and use in Japan.

2 FIBER FLAX

2.1 CULTIVATION HISTORY

It is said that flax was introduced into Japan from China in the end of the 17th century, and grown for medicine (Hara, 1980). However, its cultivation did not spread until the latter half of the 19th century. In the 1870s, flax seeds were imported into Japan from the United States, Russia, and the United Kingdom, to grow flax plants on a trial basis in the northernmost island, Hokkaido (Hara, 1980). The results showed that the crop was well adapted to Hokkaido where the climate generally corresponds to Central European weather conditions. Around that time, a method used to separate fiber from flax stem was brought to Japan, and commercial flax production commenced in Hokkaido (Hara, 1980).

In 1900, a total of 11 domestic flax factories were in operation, and flax fibers obtained were used in manufacturing high-value linen products such as fine clothing, sheets, fire hoses, fishing nets, and canvas (Hara, 1980). Linen products were also requisite for military equipments including uniforms, tents, rope, and parachute harnesses. With the start of the Sino-Japanese War in 1894, domestic flax production actually grew rapidly; the

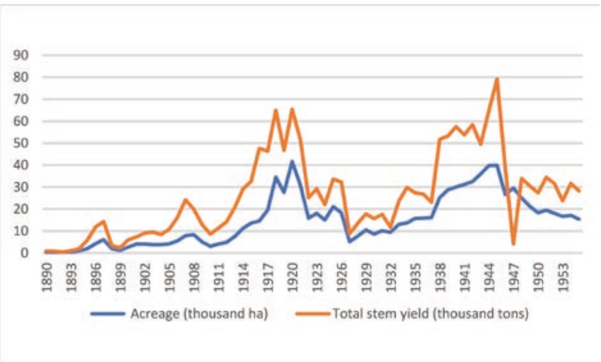

Figure 1: Production (total stem yield) and cultivation area of fiber flax from 1890 to 1955 in Japan. Source: The Commemorative Committee for the 70th Anniversary of Flax Business Development in Hokkaido (1957)

acreage under flax cultivation increased from 239 ha in 1891 to 4,275 ha in 1896 (Figure 1). The Russo-Japanese War broke out in 1904, which triggered a further rise in flax cultivation. In 1907, flax production amounted to 24,245 tons from an area of 7,918 ha (Figure 1).

During World War I, flax became a vital material in the production of a new weapon of warfare, viz., the airplane in Europe (Stamper, 2018). Linen was extensively utilized to cover wings and aircraft frames. A great amount of flax was inevitably needed to meet the demands of the linen and aviation industries. Japan exported flax fibers and linen products to Europe (Hara, 1980), and the total flax acreage in Japan increased by 4.4 times between 1907 and 1918 (Figure 1). Flax production reached 64,977 tons harvested from 34,530 ha in 1918. Following the war, the flax acreage dropped to 15,857 ha in 1922, chiefly due to the loss of military demand, and this decreasing tendency continued until 1937. Flax production peaked again during World War II (Figure 1).

Japan was defeated in the war in 1945. One third of the nation's wealth was destroyed, and food became extremely scarce. For a while after the war, the Japanese government gave priority to increasing food production (Moen, 1999), and consequently, flax growers reduced flax planting (Figure 1) and diverted more land under food crops such as rice, potato, soybean, and wheat. In the 1960s, the mass production of synthetic fibers led to a decline in the linen industry; flax culture in the country disappeared completely in 1967 (Hara, 1980).

2.2 CULTIVAR DEVELOPMENT

The establishment of the Japanese linen industry in the 1890s was solely dependent on imported flax cultivars or landraces, notably from France or Russia. The cultivation of these introductions increased year by year; however, within-cultivar variation in growth characteristics was viewed as problematic (Hara, 1980). In the late 1910s, the Hokkaido National Agricultural Experiment Station became significantly involved in flax breeding. The first domestic cultivar 'Pernau 1', a selection from a Russian landrace, was developed in 1923 (The Commemorative Committee for the 70th Anniversary of Flax Business Development in Hokkaido, 1957). 'Pernau 1' demonstrated high fiber quality and uniformity in growth characteristics though it was susceptible to flax rust and *Fusarium* wilt. This cultivar dominated domestic flax production for two decades from 1932 (Hara, 1980).

The next cultivars developed by the Hokkaido National Agricultural Experiment Station included 'Saginaw 1' (released in 1935) and 'Saginaw 2' (released in 1937), which were selected from the US cultivars 'Saginaw' and 'Washington 14', respectively (Hara, 1980). The release of these two cultivars provided the industry with high-yielding and disease-resistant flax, and both cultivars were widely grown for a decade from 1945 (Hara, 1980).

Attempts were also made to create high-yielding cultivars with better disease resistance and uniformity in growth, through controlled crossing. As a result, several prospective cultivars such as 'Unryu' (released in 1949) and 'Aoyagi' (released in 1955) were bred, but their fiber quality was not satisfactory (Hara, 1980). After 1957, the most popular cultivar was 'Wiera', which was introduced from the Netherlands and characterized by resistance to lodging and diseases as well as high fiber quality (Hara, 1980).

3 OILSEED FLAX FOR INDUSTRIAL USE

The seed oil of flax readily polymerizes upon exposure to air. As a result, it forms a soft and durable film (Jhala and Hall, 2010). This property is known as drying quality of flaxseed oil, and is responsible for extensive use in manufacturing various industrial products including paints, resins, varnishes, oilcloth, soap, linoleum, and printing inks.

At one time in Japan, flaxseed oil was exclusively utilized for industrial purpose. According to Kimoto (1963), 45,900 tons of flaxseed oil were used in 1962 to manufacture industrial products. About 30,000 tons of flaxseed oil were consumed for industrial applications annually in the 1990s (Saiwai Shobo, 2011); all the flaxseeds for oil extraction were imported from overseas. In the 2000s, however, the use of other vegetable oils (e.g., soybean oil) and petroleum products in place of flaxseed oil led to the considerable decline in flaxseed oil consumption for industrial purpose. The consumption reduced to less than 10,000 tons at the end of the 2000s, despite a general awareness of high quality of paints and coatings (varnishes) containing flaxseed oil (Saiwai Shobo, 2011).

4 FLAX FOR EDIBLE OIL

4.1 CONSUMPTION AND CULTIVATION

Both flaxseed and flaxseed oil had long been thought to be poisonous and were not used as a food source in Japan (Ono, 2009). Flaxseeds indeed contain anti-nutrients, particularly cyanogenic glycosides, which can be hydrolyzed to produce toxic hydrogen cyanide upon ingestion (Bekhit et al., 2018; Dzuvor, et al., 2018). Cyanogenic glycosides occur in more than 2,500 plant species such as almond, wheat, barley, cassava, apples, and stone fruits (Cho et al., 2013), and the levels of cyanogenic glycosides in flaxseed (300-500 mg per 100 g of seeds: Singh et al., 2011) are considered too low to adversely affect the health of humans. Parikh et al. (2019) described that humans would need to consume the unrealistic amount of 1 kg of flaxseed daily for cyanide toxicity to ever manifests itself. We would also like to add that no adverse effect including food poisoning due to flaxseed consumption has been reported in the literature (Bekhit et al., 2018).

In the early 1990s, a small amount of flaxseed oil was first imported to Japan from North America with the aim of utilizing as a nutritional supplement (Ono, 2009). Recently, flaxseed oil has gained an established reputation as a high-value food ingredient thanks to healthful properties of this vegetable oil with high content of alpha-linolenic acid. Alpha-linolenic acid comprises ca. 63 % of the total fatty acids in the flaxseed oil available in Japan, whereas linoleic acid (omega-6 fatty acid) comprises ca. 16 % (Enokido and Ohashi, 2016). During the past decades, Japanese people have increased the Western diets which are characterized by a higher omega-6 and a lower omega-3 fatty acid intake (Tanaka et al., 2010; Simopoulos, 2010). A balanced ratio of omega-6/omega-3 fatty acids is thought to be important for human health (Simopoulos, 2010), and the Ministry of Health, Labour and Welfare, Japan recommends a ratio of 4 : 1 to 5 : 1 (MHLW, 2020). Consuming flaxseed oil is thus expected to improve omega-6/omega-3 ratio.

The domestic market of flaxseed oil for home use grew from US$ 34 million in 2016 to US$ 70 million in 2020; the market size of the total vegetable oils for home use in 2020 was estimated to be US$ 1,150 million (Nisshin Oillio, 2021). In the early 2000s, oilseed flax cultivation started in Hokkaido. On November 13, 2009, the Ministry of Health, Labour and Welfare, Japan announced the detection of transgene from an unapproved genetically-modified (GM) flax cultivar in a shipment of Canadian flaxseed exported to Japan (MHLW, 2009). The GM flax cultivar in question, FP967 (CDC Triffid), was not authorized for food or feed in Japan; it had tolerance to soil residues of sulfonylurea-based herbicides (Ludvíková and Griga, 2015). This incident led to rigorous and extensive testing guaranteeing the absence of GM seeds in imported flaxseed. In this context, it was hoped that oilseed flax plantings would increase in Japan (Kimura, 2017). The oilseed flax acreage, however, has remained below 50 ha for the last few years (JSAPA, 2021). There are several reasons why the plantings fail to increase, of which the most important one must be that domestic flaxseed is more than twice as expensive as imported flaxseed, owing to relatively low productivity of Japanese oilseed flax (see below).

4.2 CULTIVAR SELECTION

Nowadays, Japan's oilseed flax production consists mostly of the following three cultivars (JSAPA, 2021). Their agronomic characteristics are described below.

4.2.1 'Batsman'

It is an early maturing, mid tall, and high-yielding cultivar with average oil content. This brown-seeded cultivar was developed by Van de Bilt zaden en vlas, the Netherlands.

4.2.2 'Brighton'

A brown-seeded and mid tall cultivar bred by Van de Bilt zaden en vlas, the Netherlands. The cultivar is also characterized by average oil content and medium maturity.

4.2.3 'York'

A brown-seeded and medium /late maturing cultivar with good oil yield and oil quality. It has resistance to flax rust and *Fusarium* wilt. 'York' was developed at the North Dakota Agricultural Experiment Station in the United States.

4.3 CULTURAL PRACTICES

Oilseed flax requires moderate to cool temperatures during its growing season (You et al., 2017). It is also known that cool temperatures after flowering tend to increase oil (particularly linolenic acid) content (Čeh et al., 2020). Japanese oilseed flax is now cultivated exclusively in Hokkaido; temperatures in the main flax-producing areas range from 6 to 28°C during growing season (Japan Meteorological Agency, 2023).

Oilseed flax can be raised in almost all types of soils provided plenty of moisture is available, but it prefers well-drained silty loam, clay loam, and silty clays (Hall et al., 2016). In Japan, the crop is typically sown from the end of April through early June. The seed sowing depth should be around 2 cm for best germination. Seeding rate of 400-450 g per 100 m^2 is common in the country. Flax competes poorly with weeds, so weed control is important during its early development. Weeds are controlled more easily by herbicides in the seedling stage and early treatment usually minimizes yield decreases. Oilseed-flax flowers generally bloom from mid-June to late July, each developing into a round seed capsule or boll. Ripening of the boll begins 20 to 25 days after flowering, and the boll contains eight or less small, smooth, and shiny seeds. The seeds should be harvested when a majority of the bolls turn brown. Edible oil is commonly extracted from flaxseed by mechanical cold pressing. Cold pressing has some advantages such as its capability of producing high-quality oil, low equipment cost and energy requirement, and avoidance of usage of chemicals, though the main drawback of this method is low oil yield when compared with solvent extraction method (Mikołajczak et al., 2023).

In Japan, it is recommended that oilseed flax be rotated with other crops (e.g., common buckwheat, wheat, and potato) to reduce disease potential and improve yields. Flax should not be grown in the same field more than once every three or four years. Few insect or disease pests affect oilseed flax in the country. Flax rust [caused by *Melampsora lini* (Ehrenb.) Desmaz.], *Fusarium* wilt [caused by *Fusarium oxysporum* f. sp. *lini* (Bolley) Snyder et Hansen], and anthracnose (caused by *Colletotrichum destructivum* O'Gara) can occur (NARO Genebank, 2023a). Insect pests such as army worm (*Spodoptera frugiperda* (Smith, 1797)) occasionally attack this crop, but seldom do significant damage.

5 CONCLUDING REMARKS

In Japan, several sources of vegetable oils are widely used for human food, but the market of vegetable oils for home use is practically dominated by canola, olive, and sesame oils (Nisshin Oillio, 2021). Over the past decade, the demand for flaxseed oil has been increasing as a result of its health benefits. Despite having much value, oilseed flax cultivation in the country has not received good research attention. It should also be pointed out that the average yield (455 kg ha^{-1} in 2019) of Japanese oilseed flax is surpassed by that in major oilseed-flax growing countries, viz., France (2,083 kg ha^{-1} in the same year), Canada (1,433 kg ha^{-1}), China (1,308 kg ha^{-1}), and the United States (1,243 kg ha^{-1}) (JSAPA, 2021; FAOSTAT, 2023). To make oilseed flax an attractive farm crop, there is a need to put more research and funds in the cultivar development and crop management practices for improving yield and on the value-added potential of this crop.

Germplasm is the basis of plant breeding programs, and enrichment of plant genetic resources is necessary to broaden the genetic base and invigorate breeding stocks (Diederichsen and Raney, 2006; You et al., 2017). Kiryluk and Kostecka (2020) mentioned that in Poland, the flax grain yield increased from 500 kg ha^{-1} in 1993 to as much as 1,500 kg ha^{-1} in 2016, due to the introduction of new cultivars and modern cultivation technologies. Unfortunately, the flax germplasm collections in Japan are currently limited (NARO Genebank, 2023b). Much effort is required to introduce new germplasm accessions and to evaluate the accessions for yield-relating characteristics such as seed mass and oil concentration in the seeds, as well as other agronomic traits. Additionally, cultural manipulations need to be improved in order to enhance the productivity of Japanese flax crop.

Historically, the straw residue from oilseed flax was considered a waste product, and burned or left in the field (Ehrensing, 2008). In recent years, attempts have been made to develop high-value products from oilseed-flax stems with applications in the pulp and paper, erosion control mats, reinforced plastic materials, and biofuel industries (Soto-Cerda et al., 2013). New industrial uses of oilseed-flax fiber will increase the demand for this multi-use oilseed crop.

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