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VSEBINA / CONTENTS

Izvirni znanstveni članki / Original research articles

Pregledni znanstveni članki / Review articles

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Insure them and improve their welfare: effect of Hygeia Community Health Insurance on households' welfare in Kwara State, Nigeria

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ABSTRACT

The Hygeia Community Health Plan was designed such that agriculture-based households can have access to affordable healthcare services. It is also aimed at providing financial risk protection against catastrophic healthcare costs which if persistent, could possibly drive them into poverty. This paper used a well-structured questionnaire to solicit responses on the effect of the Hygeia Community Health Plan on the welfare of farming households in Kwara State, Nigeria. A two-stage sampling technique was used to sample 175 farming households comprising of 115 beneficiaries and 60 non-beneficiaries from Shonga, Bacita and Lafiagi districts of Edu local government area of Kwara State, Nigeria. The ordinary least square and logit model were used in the analysis of the data for this study. The results of the analysis showed that the Hygeia community health plan was positively and statistically significant in influencing the per capita income, per capita calorie intake and the food security status of farming households in the area. Therefore, it was recommended that the government should create an enabling environment or partner with private insurance organizations. This will help them work out a plan to help rural households in other parts of the country access affordable healthcare services easily. This will help in the attainment of the universal access to health services in Kwara State and country Nigeria at large.

Key words: Hygeia; health plan; community; welfare and farming; Kwara state; Nigeria

IZVLEČEK

ZAVARUJ JIH IN IZBOLJŠAJ NJIHOVO ZDRAVSTVENO VARSTVO: UČINEK ZDRAVSTVENEGA ZAVAROVANJA HYGEIA NA DOBROBIT GOSPODINJSTEV V DRŽAVI KWARA, NIGERIA

Zdravstveni plan skupnosti Hygeia je bil zasnovan tako, da imajo pretežno kmetijska gospodinjstva dostop do ugodnih zdravstvenih storitev. Njegov namen je bil tudi zaščita pred finančnimi tveganji, ki nastajajo ob naraščajočih stroških zdravstvenega zavarovanja, ki bi zavarovance lahko pahnili v revščino. Prispevek je nastal na osnovi dobro zasnovanega vprašalnika za preučitev odgovorov, ki so jih dali izprašanci na učinke zdravstvenega plana skupnosti Hygeia na dobrobit kmečkih gospodinstev v državi Kwara, Nigeria. Uporabljena je bila dvostopenjska vzorčevalna tehnika, v kateri je sodelovalo 175 kmečkih gospodinjstev, 115 upravičencev in 60 neupravičencev zdravstvenega sklada iz okrožij Shonga, Bacita in Lafiagi, Edu lokalne vladne enote v državi Kwara, Nigeria. Za obdelavo podatkov sta bila v tej raziskavi uporabljena linearni regresijski model (OLS-ordinary least squaeres) in logistični model. Rezultati so pokazali, da je Hygeia komunalni zdrastveni plan statistično značilno pozitivno vplival na prihodek na prebivalca in vnos kalorij, kar je izboljšalo prehransko varnost kmečkih gospodinjstev na območju. Zaradi tega priporočamo vladi, da oblikuje ustrezno vspodbudno okolje ali se poveže z zasebnimi zavarovalnicami. To bi pomagalo izdelati plan dostopnega zdravstvenega servisa za kmečka gospodinjstva na podeželju tudi v drugih predelih dežele. Pripomoglo bi tudi doseči univerzalen dostop do zdravstvenih storitev v državi Kwara in širše v Nigeriji.

Ključne besede: Hygeia; zdravstveni plan; skupnost; dobrobit in kmetovanje; država Kwara, Nigerija

1 INTRODUCTION

Farming households in Nigeria constitute over 70 per cent of the country's rural population, most of which are deprived of access to quality health facilities that are essential for good living (Ajilowo, 2007). Some of the

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major consequences of this have been the migration to urban areas for medical treatment, the loss of about 25 per cent of their annual income to the treatment of various grades of sicknesses, increase risk of mortality of both children and adult, impaired productivity of able men and women among others. The World Health Assembly in 1988 mandated provision of sound health for all people by 2000 as the main target for all governments (WHO, 1997). This is because sound health is a fundamental requirement for leading a socially and economically productive life. However, many low-income countries have not been able to meet the basic healthcare needs of their people, especially those in the rural areas. In Nigeria, persistently low quality and inadequacy of health services provided in public facilities are some of the problems facing the health sector. Similarly, the state of the Nigerian health system can be said to be dysfunctional and grossly under-funded with a per capita expenditure of US$ 9.44 (World Bank, 2005). As a result, Nigeria still has one of the worst health indices in the world and sadly accounts for 10 per cent of the world's maternal deaths in childbirth. The National health management information system is still weak, without an integrated system for disease surveillance, prevention and management (UNICEF, 2008).

Poor access to healthcare by farming households is not only due to inadequate or absence of health facilities. It can also be attributed to low purchasing power evidenced by their earnings and expenditure patterns. This is because they predominantly finance healthcare services out -of -pocket (Ogbimi, 2004 and Ataguba et al., 2006). Out-of-pocket costs are those health-care expenses paid that are not reimbursed by any health insurance company. Examples of common out-of-pocket costs include deductible, co-pay, and co-insurance. A health plan therefore is expected to "cap" out-of-pocket expenses. This means that once the maximum out-of-pocket cost for plan is reached, health plan takes over and provides coverage.

The Hygeia Community Health Plan is a form of health insurance plan designed to reduce economic difficulties following illness or injury. It evolved out of the aspirations of Hygeia Nigeria Limited and PharmAccess Foundation (A Dutch Non-Governmental Organization) to scale up HIV and AIDS care in Africa. Both parties decided that HIV and AIDS care should be provided within an integrated healthcare delivery framework as opposed to the more prevalent vertical disease models. It was also conceptualized that this integrated healthcare delivery framework would best be sustained and maintained within the context of a health insurance scheme. The scheme subsidizes premiums which were intended to facilitate the entry of individuals who were usually poor or had been impoverished by the disease. This subsequently gave rise to Hygeia Community Health Plan (HCHP); a demand-drive, donor subsidized community health insurance scheme for low to medium income populations of Nigeria. The scheme was launched in January 2007 and it commenced operations in February 2007. The HCHP is currently the local implementation partner of the Dutch Health Insurance Fund in Nigeria. The Fund has pledged funding for the co-premiums of 115,000 low income individuals over a period of 5 years. The HCHP worked with the Fund, the World Bank and some state governments such as Kwara state on extending coverage to the low income people which comprises of farming households.

The success of any micro-insurance program such as the Hygeia Community Health Plan depends on its ability to improve economic outcomes among others while maintaining financial sustainability. It also assures donors that their money is being spent in the most efficient way possible. The Hygeia Community Health Plan focuses specifically on those rural households that are engaged in agricultural and non-agricultural production as a form of livelihood. At the household level, farming household are expected to have at least a source of capital which may be natural, physical, social, human, or financial (cash, credit/debit, savings) capital. Together these assets constitute a stock of resources used to generate well-being (Rakodi, 1999). Thus, this is expected to have significant impacts on their welfare and other resultant outcomes (Jansen et al., 2005). For example, some of these households may combine their assets with the benefits from Hygeia Community Health Plan to ensure an improvement in productivity and income that will result in improvement in their welfare.

Improved welfare which refers to a state of being happy, healthy and successful cannot be accomplished when households still have to pay exorbitant prices for healthcare. Agricultural production results in various degrees of hurt and illnesses which usually increase medical expenses and reduce income (Gertler et al., 2003). Thus resulting in a situation where households forgo qualitative care, yet they still pay substantial sums for low quality care (Das et al, 2008). High health care expenditures mean short-term health shock and can lead to debt, asset sales thus further plunging them in poverty (Annear, 2006). Furthermore, higher incidences of poverty in most rural areas in Nigeria have been traced to lack of appropriate insurance against income shocks. This is even worse because some farmers dispose their productive assets to meet immediate health consumption needs (Alayande and Alayande, 2004). This article therefore examines the extent to which enrolment in Hygeia Community Health Plan affects the welfare of farming households in Kwara State, Nigeria. This will add to the growing body of evidence on the effect of health insurance on households' welfare in Nigeria.

Some of the available studies on the impact of coping strategies (Insurance inclusive) on household livelihoods outcomes have generally focused on food security as the livelihood indicator. Households were found to respond to food insecurity caused by shocks and stresses through reduction in quantity, composition and quality of foods consumed and the collection of wild foods. Others are the reduction in daily meal frequency, borrowing from relatives, and inter-household food transfer to name a few (Mishra, 2007; Smucker & Wisner, 2008). Against these background, the research question addressed in this paper are; Does enrollment health insurance plan (such as Hygeia Community Health plan) have any effect on the per capita income, per capita calorie intake and food security status of households? If yes, to what extent?

2 MATERIALS AND METHODS

2.1 Study Area

The study was conducted in Kwara State whose capital is Ilorin which has total land area of about 32,500 km^2, and an estimated population of about 2.37 million people (NPC, 2008) out of which farmers account for about 70 per cent. The average population density of the state in 2006 was about 73 people per square kilometer. The farming system in the state is characterized by low quality but surplus land, low population density and cereal based cropping pattern. Agricultural production is largely peasant and small scale relying heavily on the use of manual labour equipped with crude implements. Landholding in the state is very small and most of the households have less than two hectares of land for farming. The output from this land is low and most households have to buy food when their own production is insufficient. Some of the rural households also participate in credit programs to supplement their household's income (KWSG, 2006).

2.2 Data and Sample Size

Study used primary data collected in 2014 through a proportionate sampling of 175 farming households from Shonga, Bacita and Lafiagi districts of Edu local government of the state. This comprises of 115 beneficiaries and 60 non-beneficiaries. Edu local government area of Kwara state was selected because it is one of the areas currently benefitting from the Hygeia Community Health Plan in the State. Data were collected on a wide range of variables using well-structured questionnaire and personal interview method where appropriate.

2.3 Analytical tools

Descriptive statistics: The descriptive statistics used include measures of mean and frequency distribution. The mean is a measure of central tendency.

Ordinary least square (OLS) regression method: This was used to analyze the effect of the Hygeia health plan on two welfare indicators. The indicators considered are the Per Capita Income (PCI) and Per Capita Calorie Intake (PCCI). The econometric model that was employed is implicitly stated as follow:

$$Y = f(X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, \ldots\ldots U)$$

Where,
Y = Per Capita Income/ Per Capita Calorie Intake
Per capita calorie intake is expressed in (Kcal/AE/Day)
Per capita income is expressed in Naira
X_1 = Gender of Household Head ($F = 0, M = 1$)
X_2 = Educational status of Household Head (Years of schooling)
X_3 = Age of Household Head (Years)
X_4 =Farm Size (Hectares)
X_5 =Farming experience (Years)
X_6 = Household size (Adult Equivalent)
X_7 = Total monthly per capita expenditure of household (Naira)
X_8 = Access to credit (yes = 1, 0 otherwise)
X_9 = Hygeia insurance scheme (Beneficiary = 1, 0 otherwise)
U = Random error term

Logit model: This was used to analyze the effect of the Hygeia health plan on the food security status of the farming households. To determine the food security status of households, a daily recommended per capita calorie intake of 2500 kcal /AE /day was adopted by the study as the food security line (FAO, 2005). In line with this, households that consumes less than the recommended calorie intake were classified as being food insecure while, households that consumes at least the recommended value were classified as food secure. The food security indicator (FSI) was measured in such a way that a food secure household takes the value of 1 while food insecure household takes 0.

$$Y = f(X_1, X_2, X_3, X_4, X_5, X_6, \ldots\ldots U)$$

Where,
Y = Food Security Status (Food secure = 1, 0 otherwise)
X_1 = Age of Household Head (years)
X_2 = Years of schooling of household head
X_3 = Household size (Adult Equivalent)

X_4 = Total monthly per capita expenditure (Naira)
X_5 = Farm size (hectares)
X_6 = Hygeia health plan (Yes = 1, No = 0).
U = Error term

3 RESULTS AND DISCUSSION

3.1 Socio-economic Characteristics

Table 1: Socio-economic Characteristics

Variables	Frequency	Percentage
Age (years)		
≤ 30	43	24.6
31 – 45	70	40.0
46 – 60	51	29.1
> 60	11	6.3
Gender		
Female	4	2.3
Male	171	97.7
Educational level		
No formal Education	43	24.6
Primary Education	65	37.1
Junior Secondary	7	4.0
Senior Secondary	35	20.0
Tertiary	25	14.3
Marital Status		
Single	10	5.7
Married	160	91.4
Widowed/Separated	5	2.9
Household size (Adult Equivalent)		
< 3	33	18.9
3 – 6	106	60.6
> 6	36	20.5
Farm size (hectares)		
< 3	102	58.3
3 – 6	70	40.0
> 6	3	1.7
Farm Experience (years)		
≤ 10	35	20.0
11 – 20	66	37.7
21 – 30	48	27.4
> 30	26	14.9
Membership of Cooperative Societies		
Non-member	109	62.3
Member	66	37.7
Monthly Per Capita Income (₦'000)		
< 5	130	74.5
5 – 10	37	21.1
> 10	8	4.6
Monthly Per capita Health Expenditure (₦)		
< 500	72	41.2
500 – 1000	77	44.0
1001- 1500	17	9.7
> 1500	9	5.1

Source: Field Survey, 2014: Observation N = 175

Table 1 shows that 97.7 per cent of the respondents are male-headed households within the ages of 30 to 60 years. Only a little above 10 per cent of them have post-secondary education with majority representing 37 per cent who have just primary education. 91.4 per cent of the respondents are married with a household size (adult equivalent) of between 3 and 6 persons. About 58 per cent have a farm size of less than 3 hectares which implies that most of them are subsistence farmers with an average farming experience of 22 years. Also, majority representing 62.3 per cent are not members of any form of cooperative society. The mean per capita income and monthly health expenditure of these households are N4452.55 and N676 respectively. This result is consistent with those of Babatunde et al (2011) for North-central Nigeria, Oyekale &Eruwa (2009) for rural households in Osun State and Oriakhi &Onemolease (2010) for Edo state.

3.2 Hygeia Community Health Plan and Per Capita Income

Table 2: Hygeia Community Health Plan and Per Capita Income

Variables	Coefficient	Standard Error	t-value
Age (years)	-26.012	44.253	-0.588
Years of Schooling	3.860	54.595	0.071
Household size (AE)	-1780.249***	179.616	-6.003
Farm size (hectares)	966.484***	186.945	5.170
Hygeia Health Plan (yes = 1)	**909.695***	**522.734**	**1.740**
Household Asset (₦'000)	0.003***	0.001	2.895
Farm Experience (years)	31.739	45.530	0.697
Credit Access (yes = 1)	1733.432**	705.258	2.458
Constant	5354.719**	2467.382	2.170

Source: Field Survey, 2014; *Significant at $p > 0.10$, ** Significant at $p > 0.05$ *** Significant at $p > 0.01$, AE = Adult Equivalent.

The Hygeia Health Plan, farm size, household asset and access to credit facilities were found to be positively significant at 10 per cent, 1 per cent and 5 per cent respectively. This implies that a beneficiary of the Hygeia health plan will have a higher per capita income of about 910 units compared to the non-beneficiaries. This is likely because a beneficiary of the health plan will be able to save more money thereby reducing out-of-pocket expenses and increasing their per capita income. The farm size that was significant at 1 per cent implies that households with large farm size will be able to produce large output and as such realize more farm income. This will in-turn increase the per capita income compared to households with smaller farm sizes. This result is in consonance with that of Ibekwe (2010) for Imo state.

Access to credit and the household assets were also positively significant at 5 per cent and 1 per cent respectively. This implies that access to credit facilities and possession of more household asset will increase the potential to expand production activities thereby increasing their per capita income. The household size was found to be negatively significant at 1 per cent, which implies that large households will spend more thereby reducing the per capita income that will be available to them compared to smaller households. All this agrees with a priori expectations.

3.3 Hygeia Community Health Plan and Per Capita Calorie Intake

Table 3: Hygeia Community Health Plan and Per Capita Calorie Intake

Variables	Coefficient	Standard Error	t-value
Age (years)	24.608**	11.6204	2.12
Gender (male = 1)	570.732	467.622	1.22
Years of Schooling	-14.311	14.372	1.00
Household size (AE)	-41.782	48.973	-0.85
Farm size (hectares)	101.452**	48.972	2.09
Hygeia Health Plan (Yes = 1)	**1083.471***	**140.705**	**7.70**
Farm Experience (years)	20.687*	11.896	1.74
Credit Access (yes = 1)	396.254*	201.598	1.97
Per Capita Expense	0.053**	0.026	2.05
Total Asset	0.001***	0.0003	2.09
Constant	828.3459	696.321	1.19

Source: Field Survey, 2014; *Significant at $p > 0.10$, ** Significant at $p > 0.05$ *** Significant at $p > 0.01$

As shown in Table 3, the factors that were found to significantly influence the per capita calorie intake by farming households in the area are the age of household head, farm size, the Hygeia community health plan, years of farming experience, access to credit facilities, per capita expenditure and the total assets of the household. The age of the household head, farm size and per capita expenditure were positively significant at 5 per cent. This implies that older household head and those with larger farm size and higher per capita expenditure will in-turn consume more calories than the younger ones with smaller farm sizes. This may be because households with large farm sizes will be able to produce more thereby increasing their income and are in-turn able to spend more especially on food to stay healthy. This result is consistent with the findings of Orewa & Iyanbe (2010).

Also, Hygeia community health plan and total household asset were positively significant at 1 per cent. This implies that all things being equal, a beneficiary of the Hygeia community health plan will increase its calorie consumption by 1083 units compared to a non-beneficiary. This can be attributed to the fact that a beneficiary of the health plan spends less on healthcare services thereby able to save more. These savings can therefore be used in ensuring higher calorie intake. A non-beneficiary on the other hand is burdened with health expenditures which are paid mainly out-of-pocket with only little left for consumption purposes.

The years of farming experience and access to credit facilities were also positively significant at 10 per cent. This implies that, the more experienced the household head, the more he is able to ensure that farming activities are done efficiently and as such increasing output and calorie intake of his household. Also, increased access to credit facilities will also increase production and the financial capacity needed for the household to afford the required calorie intake. This is also consistent with the findings of Orewa & Iyanbe (2010) for urban households in Nigeria.

3.4 Variables' Ranking by Welfare indices

Table 4: Variables' ranking by welfare indices

Variables	Low	Medium	High
Per capita calorie (Kcal/AE/day)	2318.90	3019.90	4129.90
Monthly per capita income	3595.00	4529.70	5247.50
Total Household Asset (₦'000)	336	393	3333
Farm Size (hectares)	2.57	2.89	2.77
Monthly Health expense	2591.53	3110.34	2.860.69
Years of Schooling	7.34	7.14	7.43

Source: Field Survey, 2014; N = Naira, Kcal/AE/day = Kilocalorie/Adult Equivalent/day

Table 4 shows that households with low per capita calorie intake are characterized by low monthly per capita income, lower asset base and a relatively small (2.57 hectares) farm size. On the other hand, households with large asset base (N3, 333, 000.00) were found to have a higher per calorie intake and a higher per capita income but they spend relatively small amount on health expenditure than those in the medium class. This might be because most of the households in the higher class are able to benefit from the health plan as oppose to the others. Therefore they have more to save since they spend less on healthcare.

3.5 Hygeia community health plan and food security status

Table 5: Hygeia community health plan and food security status

Variables	Coefficient	Standard Error	t-value
Age (years)	-0.0178	0.0251	-0.71
Per Capita Expense	0.0001*	0.0000	1.83
Years of Schooling	0.0064	0.5003	0.13
Household size (AE)	-0.0635	0.2001	-0.32
Farm size (hectares)	101.4516	0.1654	0.73
Hygeia Health Plan (Yes = 1)	3.4944***	0.5780	6.38
Constant	-1.2617	1.2733	0.99

Source: Field Survey, 2014; *Significant at $p > 0.10$, ** Significant at $p > 0.05$ *** Significant at $p > 0.01$

Table 5 shows that the Hygeia health plan was found to be positively significant, influencing the food security status of the households at 1 percent. This implies that, a beneficiary of the Hygeia health plan is more likely to be food secured than a non-beneficiary. This may be attributed to the fact that a beneficiary of the health plan would have been able to reduce out-of-pocket expenses. This would enable the household to spend more on food to complement own production so as to ensure food security.

The household's monthly per capita expenditure was also positively significant at 10 per cent. This implies that the more a particular household spends, the more it is likely to spend on food related items and hence become food secured. That is all things being equal, the more a household spends, the better are its chances of attaining food security. This result is similar to those of Bashir et al (2012) for Pakistan and Mitiku et al (2012) for Southern Ethiopia.

4 CONCLUSIONS

This study examined the effects of the Hygeia community health plan on farming households' welfare using the ordinary least square and the logit regression models. The major findings showed that the households that benefitted from the Hygeia community health plan had higher and significant per capita income, per capita calorie intake and were more food secured than those who did not. Other factors that were found to significantly influence the welfare of the farming households' are the age of the household head, household size, farm size, years of farming experience, total household asset, access to credit facilities and the per capita expenditure of the households. All these would result in a healthier workforce thereby increasing the production capacities of the farming households. This would lead to increase in household income and by extension generate improvement in households' welfare. Therefore, it is recommended that the design and implementation of community-based health plan should be encouraged for the rural farmers. Also, Nutrition-oriented programs can be organized in an attempt to improve the food and dietary diversity of these rural people and the nation at large.

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Some important aspects in *Moringa oleifera* Lam. micropropagation

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ABSTRACT

Type and source of explant as well as the type of cytokinin were important factors for successful moringa micropropagation. Explants obtained from *in vitro* grown plant materials were better than others obtained from soil growing seedlings. In addition, nodal segments were better than shoot cuttings in terms of number of shoots/ explants, frequency of shoot formation and number of nodes/shoot. While callus formation on the base of nodal segment on BAP containing media were higher than those of KIN, especially under the influence of high concentration as an aspect of vitrification, BAP was better than KIN in moringa multiplication. Low nutrient medium (half strength MS) supplemented with 0.5 mg l^{-1} IAA was essential for successful root formation. The suitable conditions for moringa micropropagation on full strength MS or SH may exert low stress and low need to raise the expression of SOD and POX. On the other side, stress due to over increase of chemical components of double MS medium or low nutrient content of half strength MS, B5 or WPM expressed the highest number and staining intensity of SOD and POX bands, vice versa was detected in case of CAT.

Key words: medium type; shoot multiplication; cytokinins; gene expression; isoenzymes; micropropagation; antioxidant enzymes; type of explant

Abbreviations: benzyl amino purine (BAP), kinetin (KIN), esterases (ESTs), glutamate oxaloacetate transaminases (GOTs), superoxide dismutases (SODs), peroxidases (POXs)

IZVLEČEK

NEKATERI POMEMBNI VIDIKI V MIKROPROPAGACIJI MORINGE (*Moringa oleifera* Lam.)

Vir in vrsta izsečkov kot tudi vrsta citokininov sta pomembna dejavnika za uspešno mikropropagacijo moringe. Izsečki, pridobljeni iz *in vitro* vzgojenih rastlin so bili boljši kot tisti pridobljeni iz sejank vzgojenih v tleh. Dodatno so bili izsečki nodijev boljši kot iz ostalih delov poganjka glede na število nastalih poganjkov na izseček, pogostost tvorbe poganjkov in število nodijev na poganjek. Tvorba kalusa na bazi nodialnega segmenta je bila boljša v gojišču z BAP kot v gojišču s KIN, še posebej zaradi vpliva večjih koncentracij je bil pri mikropropagaciji moringe z vidika vitrifikacije BAP boljši kot KIN. Gojišče z majhno vsebnostjo hranil (polovični MS) z dodatkom 0,5 mg l^{-1} IAA je bilo odločilno za tvorbo korenin. Primerne razmere za mikropropagacijo moringe na polnomočnih gojiščih MS ali SH so manj stresne in ne vzpodbudijo tvorbo SOD in POX. Po drugi strani je stres zaradi povečanja spojin v dvojnem gojišču MS ali v polovičnih gojiščih MS, B5 ali WPM z majhno količino hranil vzpodbudil največjo ekspresijo SOD in POX , kar se je pokazalo z največjim številom in močnejšo obarvanostjo eletroforeznih trakov, obratno je bilo ugotovljeno v primeru CAT.

Ključne besede: tip gojišča; razmnoževanje poganjkov; citokinini; izražanje genov; izoencimi; mikropropagacija; antioksidacijski encimi; vrsta izsečkov

Okrajšave: benzil amino purin (BAP), kinetin (KIN), esteraze (ESTs), glutamat oksaloacetat transaminaze (GOTs); superoksid dizmutaze (SODs); peroksidaze (POXs)

1 INTRODUCTION

Moringa oleifera Lam., commonly known as moringa, is a distinguished member of the monogeneric family Moringaceae. Moringa is a perennial soft wood tree, native to the western and sub-Himalayan tracts including India, Pakistan, Asia Minor, Africa and Arabia. Moringa is now being cultivated in many other

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places like the Caribbean Islands, Central America, North and South America, Cambodia, the Philippines and Egypt (Morton, 1991; Fahey, 2005). Moringa is considered the most nutrient-dense plant yet discovered where fruits, leaves, flowers and immature capsules of this tree are highly nutritious and used in many countries all over the world (Anwar and Bhanger, 2003). Various types of antioxidants such as ascorbic acid, flavonoids, phenolics and carotenoids present in moringa leaves (Dillard et al., 2000; Siddhuraju et al., 2003). Besides its food uses, moringa is used as animal feed (Sanchez et al., 2006). Seeds powder can be used for water purification due to its antimicrobial and coagulant properties (Ndabigengesere and Narasiah, 1998; Suarez et al., 2003; Bhatia et al., 2007). Moringa is also reported to possess anti-inflammatory, antimicrobial, antioxidant, anticancer, cardiovascular, hepatoprotective, anti-ulcer, diuretic and anticholestrolmic properties. (Cáceres et al., 1992; Murakami et al., 1998; Guevara et al., 1999; Siddhuraju et al., 2003; Stephenson and Fahy, 2004).

A large scale production of moringa is needed because of its nutritional and medicinal importance. Germination of moringa seeds decreased with the increase in time between harvesting and sowing where it reaches 7.5 % in three months (Sharma et al., 1982). This hinders the use of seeds for moringa cultivation. Also, trees propagated from seeds showed genotypic and phenotypic variations that resulted in variation in production and nutritional values (Riyathong et al., 2010; Salem 2016). Propagation through stem cuttings results in trees with inferior fruits and shallow root system making them more drought-susceptible (Church World Service, 2000). This method of propagation reduces the yield and life of mother plant (Islam et al., 2005). Consequently, micropropagation was essential prerequisite of propagation of elite tree. Moringa micropropagation was accomplished using tissues obtained from seedling or mature trees (Islam et al., 2005; Riyathong et al., 2010; Förster et al., 2013; Salem, 2016; Zhang et al., 2017). Vitrification and the associated somaclonal variation are two aspects retarding the application of *in vitro* techniques as effective vegetative micropropagation tools in moringa (Hassanein et al., 2008; Mirzai et al., 2015; Salem, 2016; Hassanein et al., 2018).

In vitro culture conditions exert stress which need (Chen and Ziv, 2001; Rojas-Martínez et al., 2010) induction of some antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) to help the cultured tissues to avoid the exerted stress (Pawar and Panneerselvam, 2012; Devi et al., 2013). Under stress conditions, SOD plays its role by catalyzing the dismutation of excess O_2^- into O_2 and H_2O_2. Toxic H_2O_2 is further catalyzed by CAT, POX and other enzymes to form H_2O and O_2 (Ueda et al., 2013; Rout et al., 2013; Zhang et al., 2017).

The response of different types of explant to the same culture condition is varied due to the difference in endogenous plant hormones they have (Kumar and Reddy, 2011). Explants derived from *in vitro* grown plant materials are recommended for micropropagation, where they have better potential to organogenesis as compared to explants obtained from *in vivo* grown plants (Kumar and Reddy, 2011). While different types of plant media such as B5 (Gamborg et al., 1968), SH (Schenk and Hildebrandt, 1972), WPM (Lloyd and McCown, 1980) and MS (Murashige and Skoog, 1962) have been used for regeneration of different plant species. MS was used to induce regeneration on different types of explants belongs to different plant species (Hassanein et al., 2008; 2015; Kumar and Reddy, 2011). While some plant species response similarly to several media, generally plant species express preference response for a specific medium when certain explant is used (McCown and Sellmer, 1987). For example, weaker salt medium such as WPM promoted the formation of axillary bud development in forest plant species (McCown and Sellmer, 1987) and it was probably due to lower concentration of salts and sucrose than those of B5 and MS (Bhatt and Dhar, 2004). In the other side, application of double strength MS medium resulted in increase the number of formed shoots compared to full strength MS (Gnamien et al., 2013).

The best concentration and type of growth regulators which are necessary to stimulate organogenesis on *in vitro* cultured explant should be determined for establishment of tissue culture protocol (Parzymies and Dąbsk, 2012). In addition, determination of optimal media type, and type and source of explants are important factors for successful micropropagation. The best condition for moringa micropropagation is not correctly determined and still needs several studies. Consequently, the aim of the present work was to determine the best type of media, cytokinin and explant to establish successful in vitro propagation protocol in moringa. In addition, the effect of nutrient strength of the cultured media on isoenzyme patterns of the cultured explant during shoot multiplication was the second aim of this work.

2 MATERIALS AND METHODS

2.1 Shoot multiplication using soil-obtained explants under the influence of different concentrations of BAP or KIN

To establish shoot culture of moringa plant, seeds were germinated in plastic pots filled with clay soil in Central Laboratory of Genetic Engineering, Sohag University for six days. Nodal segments and shoot cuttings (upper part of shoots including shoot tip) obtained from these soil growing seedlings were subjected for sterilization using 70 % ethyl alcohol for three min and 0.1 % $HgCl_2$ for further three min with continuous shaking. Plant materials were rinsed 3 times in sterilized deionized water, three minutes each. After sterilization, the ends of each explant were discarded. Segments in length of 1.0- 1.5 cm long were transferred to MS medium (Murashige and Skoog, 1962) supplemented with different concentrations of BAP or KIN (0.28, 0.56, 1.12 and 2.24 mg l^{-1}).

2.2 Shoot multiplication using *in vitro* obtained nodal segments under the influence of different concentrations of BAP or KIN

Sterilized moringa seeds were aseptically placed in 250 ml glass jars contained sterilized cotton pieces wetted with deionized water. Cultures were incubated for 4 days at 28 ± 2 ºC under dark conditions, then they were transferred to 16-h photoperiod at the same temperature for further 2 days. After six days, seedlings were used to establish shoot culture.

Nodal segments obtained from *in vitro* grown seedlings were cultured on MS medium supplemented with 0.56 mg l^{-1} BAP for 2 weeks then nodal segments were used as plant materials. They were subcultured on MS medium supplemented with different concentration of BAP or KIN (0.28, 0.56, 1.12 and 2.24 mg l^{-1}). MS basal medium was used as a control.

2.3 Effect of media type and strength on shoot multiplication

Nodes of about 1.0-1.5 cm were excised from aseptically grown seedlings and cultured on half, double, full MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972) and B5 (Gamborg et al., 1968) media and Woody Plant Medium (Lloyd and McCown, 1980). Each medium was supplemented with 0.56 mg/ l BAP.

2.4 Incubation conditions

All cultures were incubated at 28 ± 2°C under 16-h daily light at 100 µmol m-2 s-1 for 3 weeks. Number of shoots per explants, length of shoots (cm) and number of nodes per shoots were determined.

2.5 Protein extraction and isoenzyme

Shoots grown for 21 days under the influence of the different media were collected and subjected for gene expression analysis. One gram of fresh plant materials was ground in 1 ml extraction buffer in a mortar at 4 ºC. Extraction buffer consisted of 0.1 µM Tris-HCl, pH 7.0 and 0.002 M cysteine. The homogenate was centrifuged at 13500 rpm at 4 ^{o}C for 15 min. Supernatants were collected for immediate electrophoresis in 7.5 % polyacrylamide slab gels. Gels were run at 24 mA for 6-h at 10 ^{o}C in 0.025 M Tris + 0.129 M glycine buffer pH 8.9. Peroxidase (POX) was stained according to the method of Siegel and Galston, 1967, superoxide dismutase (SOD) according to Beauchamp and Fridovich, 1971, glutamate oxaloacetic transaminase (GOT) and esterase (EST) according to the method of Brewer, 1970.

2.6 Statistical analysis

Three replicates with thirty explants for each treatment were used in all experiments. Data were statistically analyzed by ANOVA and compared using the least significant difference (LSD) test at 5 % (*) and 1 % (**) levels as described by Snedecor and Cochran, (1980).

3 RESULTS

Two types of explants obtained from soil growing seedling were used to establish protocol for moringa micropropagation. When shoot cuttings were used as an explant and cultured on MS medium supplemented with different concentrations of BAP (Table 1) for three weeks, 0.56 mg l^{-1} was the best, where the highest regeneration frequency, number of shoots/explant and growth parameters were obtained. The lowest response was detected when shoot segments were cultured on growth regulators free medium but they showed the highest number of nodes/shoot and the formation of adventitious roots (Fig. 1). Increasing BAP concentration more than 0.5 resulted in decrease in number of shoots/explant especially when 2.24 mg l^{-1}

BAP was used). Fresh mass/cluster increased significantly in shoot cuttings cultured on MS medium supplemented with different concentrations of BAP up to 1.12 mg l^{-1}. Shoot cuttings cultured on MS medium supplemented with different concentrations of BAP showed significant increase in shoot length, fresh mass/shoot and number of nodes/explant compared to control.

Table 1: Shoot segments from six-day old soil growing seedling cultured for three weeks on MS medium supplemented with different concentrations of BAP or Kin.

(mg l^{-1})	PGR	Freq. of shoot formation %	Number of shoots/ explants	Fresh mass/ Cluster (g)	Length of shoot (cm.)	Fresh mass/ Shoot (g)	No nodes/shoot
0	control	40.0	1.67±0.57	0.27±0.06	2.77±0.71	0.02±0.00	2.33±0.57
0.28	BAP	70.0	1.67±0.58 [a]	0.57±0.06 [b]	2.73±0.25 [a]	0.09±0.01 [c]	1.33±0.58 [c]
	Kin	63.3	1.33± 0.57 [a]	0.09±0.01 [b]	2.80±0.68 [a]	0.03±0.01 [a]	1.80±0.25 [a]
0.56	BAP	86.7	2.00±0.00 [a]	0.57±0.09 [b]	2.87±0.23 [a]	0.05±0.01 [c]	1.66±0.58 [c]
	Kin	83.3	2.67± 0.57 [c]	0.52±0.08 [b]	4.80±0.75 [c]	0.07±0.02 [c]	4.30±0.86 [c]
1.12	BAP	76.7	1.67±0.56 [a]	0.26±0.06 [a]	2.83±0.47 [a]	0.05±0.01 [c]	1.00±0.00 [c]
	Kin	70.0	2.67±0.57 [c]	0.83±0.10 [c]	5.30±0.72 [c]	0.08±0.03 [c]	2.67±0.67 [a]
2.24	BAP	63.3	1.00±0.00 [c]	0.21±0.05 [a]	2.40±0.44 [a]	0.10±0.01 [c]	1.33±0.58 [c]
	Kin	76.7	3.33± 0.57 [c]	0.36±0.08 [a]	2.93±0.20 [a]	0.05±0.01 [c]	4.67±0.57 [c]
	LSD at 5 %		0.40	0.17	3.33	0.03	0.54
	LSD at 1 %		0.61	0.49	4.21	0.04	0.65

Values are means of three replicates ± standard deviation (SD). Different letters indicate statistically significant differences between groups (mean ± SD, a not significant, b significant at $p < 0.05$, c highly significant at $p < 0.01$).

Figure 1: Photograph shows *in vitro* shoot regeneration on shoot segments under the influence MS free of hormones

When nodal segments were used (Table 2) as explants, the frequency of shoot formation, number of shoots/explant, fresh mass/cluster and number of nodes/explant were higher than those of shoot cuttings (Table 1). The increase of fresh mass/cluster was associated with decrease in shoot length and appearance of callus on the base of nodal segment where its size increased with increase of BAP (Fig. 2).

Table 2: Nodal segments excised from six-day old soil growing seedling cultured for three weeks on MS medium supplemented with different concentrations of BAP or Kin.

(mg l^{-1})	PGR	Freq. of shoot formation %	Number of shoots/ explants	Fresh mass/ Cluster (g)	Length of shoot (cm.)	Fresh mass/ Shoot (g)	No nodes/shoot
0	control	53.3	2.00±0.00	0.51±0.03	2.83±0.57	0.06±0.01	2.33±0.57
0.28	BAP	76.7	2.33±0.58 [a]	0.45±0.09 [a]	3.00±0.20 [a]	0.05±0.01 [a]	2.00±0.00 [a]
	Kin	76.7	1.67± 0.57 [a]	0.16±0.05 [c]	2.50±0.54 [a]	0.02±0.00 [c]	1.30±0.30 [c]
0.56	BAP	100.0	5.75±0.50 [c]	0.88±0.14 [c]	2.73±0.25 [a]	0.06±0.01 [a]	2.33±0.58 [a]
	Kin	100.0	3.75± 0.50 [c]	0.66±0.07 [c]	6.53±0.45 [c]	0.09±0.04 [c]	4.67±0.57 [c]
1.12	BAP	86.7	4.00±1.00 [c]	0.84±0.12 [c]	1.77±0.21 [c]	0.05±0.02 [a]	1.66±0.58 [c]
	Kin	86.7	2.00±0.00 [a]	0.23±0.06 [c]	2.50±0.52 [a]	0.03±0.01 [c]	2.33±0.57 [a]
2.24	BAP	90.0	2.67±0.58 [a]	0.99±0.24 [c]	1.23±0.25 [c]	0.04±0.01 [b]	1.66±0.58 [c]
	Kin	93.3	3.67± 0.57 [c]	0.56±0.05 [a]	3.67±0.58 [c]	0.06±0.01 [a]	3.33±0.57 [c]
	LSD at 5 %		0.80	0.11	0.40	0.01	0.50
	LSD at 1 %		1.56	0.26	1.33	0.02	0.98

Values are means of three replicates ± standard deviation (SD). Different letters indicate statistically significant differences between groups (mean ± SD, a not significant, b significant at $p < 0.05$, c highly significant at $p < 0.01$).

Figure 2: Photographs show in vitro shoot regeneration from nodal cuttings under the influence of different concentrations of BAP for three weeks; 0.28 (A), 0.56 (B), 1.12 (C) and 2.24 mg. l^{-1} (D)

When shoot segments of soil grown seedlings were used as explants and cultured on MS medium containing different concentration of KIN, they expressed higher values of the all measured parameters (Table 1), especially under relatively high concentrations (1.12 and 2.24 mg l^{-1}), than those of BAP. Nodal segments expressed higher values of shoot number/explant than shoot cutting irrespective the concentration of KIN (Table 2). Irrespective the type of explant, shoot length and number of nodes/shoot on MS medium supplemented with KIN was higher than those of BAP. On the other side, when the best concentration of both cytokinins (0.56 mg l^{-1}) was used, the number of shoots on MS with BAP was higher that of KIN (Table 2). Callus at the base of nodal segments on KIN containing medium (Fig. 3) was lower than that of BAP, and it was not strongly influenced by the concentration of KIN.

Figure 3: Photograph shows in vitro shoot regeneration under the influence of different concentrations of Kin: (A) nodal segment were cultured for three weeks on 0.56 (A) or 2.24 mg. l^{-1}Kin (B)

For moringa multiplication, the best source of explants were *in vitro* grown shoots where plant materials were established as shoot culture on MS medium supplemented with different concentrations of BAP or KIN (Tables 3 and 4). Number of shoots obtained from *in vitro* grown plant materials (Tables 3 and 4) was higher than that obtained from soil grown plant materials (Tables 1 and 2). The best cytokinin concentration was 0.56 mg l^{-1} of BAP or KIN, where it expressed the highest number of shoots/explant. Shoot multiplication using 0.56 mg l^{-1} BAP was better than that of KIN. On the other side, explants subcultured on media containing the highest concentration of BAP or KIN (2.24 mg l^{-1}) expressed high values of fresh mass/cluster but low values of shoot length as an aspect of vitrification.

Table 3: Nodal segments explants obtained from in vitro grown shoots were cultured for three weeks on MS medium supplemented with different concentrations of BAP

(mg l^{-1})	PGR	Number of shoots/ explants	Fresh mass/ Cluster (g)	Length of shoot (cm.)	Fresh mass/ Shoot (g)	Number of nodes/ shoot
0	control	2.33±1.52	0.22±0.07	4.60±0.79	0.09±0.02	5.00±1.00
0.28	BAP	4.33±0.57 [a]	0.28±0.08 [a]	2.10±0.26 [c]	0.07±0.02 [a]	4.30±1.52 [a]
	Kin	2.33±0.57 [a]	0.74±0.16 [c]	2.77±0.25 [c]	0.07±0.02 [a]	3.33±0.57 [b]
0.56	BAP	9.67±2.08 [c]	0.76±0.18 [c]	2.87±0.83 [c]	0.06±0.04 [a]	3.33±0.57 [b]
	Kin	4.33±0.57 [a]	0.84±0.05 [c]	3.10±0.36 [c]	0.06±0.01 [a]	3.00±1.00 [c]
1.12	BAP	6.00±1.00 [b]	0.65±0.08 [c]	3.37±0.55 [b]	0.08±0.01 [a]	3.33±0.57 [b]
	Kin	2.67±0.57 [a]	0.68±0.03 [c]	3.10±0.36 [c]	0.05±0.00 [a]	3.33±0.57 [b]
2.24	BAP	5.33±1.52 [b]	0.70±0.11 [c]	0.97±0.20 [c]	0.05±0.01 [a]	2.33±0.57 [c]
	Kin	4.00±1.00 [a]	0.85±0.18 [c]	1.83±0.15 [c]	0.04±0.00 [a]	3.33±0.57 [b]
LSD at 5 %		4.67	0.18	0.98	0.06	0.92
LSD at 1 %		6.80	0.39	2.30	0.11	1.94

Values are means of three replicates ± standard deviation (SD). Different letters indicate statistically significant differences between groups (mean ± SD, a not significant, b significant at $p < 0.05$, c highly significant at $p < 0.01$).

To study the effect of medium strength and type on shoot multiplication, *in vitro*-obtained nodal segments were cultured for three weeks on different strengths of MS medium [0.5 (half strength), 1.0 (full strength), 2.0 (double strength)] as well as full strengths of SH, WPM and B5, each of them was supplemented with 0.56 mg l^{-1} BAP. The highest shoot number (8.33) and the best growth were obtained when explants were multiplied on full strength MS medium. The data indicated that the number of formed shoots was decreased when the concentrations of MS components were more or less than MS full strength. WPM or B5 stimulated poor shoot multiplication and growth. On the other side, explants cultured on SH medium showed better shoot regeneration more than WPM or B5 but still lower than that of full strength MS. The formation of callus at the base of explant was essential prerequisite for the formation of valuable number of regenerated shoots. In

this concern, MS in full strength and SH media showed the same effect. The mass of callus on SH medium was larger than that of MS and it associated with shorten the length of formed shoots.

Expression of SOD was influenced by chemical components of the cultured media supplemented with 0.56 mg l^{-1} of BAP (Fig. 4, Table 5). Two medium (double MS medium and B5) expressed SOD-5 but it was not detected under the other conditions. In addition, SOD-3 was detected only when WPM medium was used (lane 5). The appearance of these extra bands (SOD-3 or SOD-5) was associated with the increase of staining intensity of all the detected isoforms. Generally, the staining intensity of SOD bands decreased when the culture conditions was suitable for moringa multiplication, on full strength MS and SH media.

Table 4: Effect of medium type and MS strengths on shoot multiplication and growth of in vitro grown shoots for three weeks, each type of medium containing 0.56 mg.L^{-1}BAP

Medium Type	Number of shoots/ explants	Fresh mass/ Cluster (g)	Length of shoot (cm.)	Fresh mass/ Shoot (g)	Number of nodes/ shoot
MS (control)	8.33±1.52	1.29±0.40	2.70±0.30	0.05±0.01	5.00±1.00
Half MS	5.00±1.73 ^{b}	0.66±0.30 ^{b}	1.13±0.15 ^{c}	0.03±0.01 ^{a}	4.00±1.00 ^{a}
Double full MS	3.00±1.00 ^{c}	0.56±0.06 ^{b}	2.13±0.25 ^{a}	0.05±0.01 ^{a}	3.33±0.57 ^{b}
SH	6.67±2.08 ^{a}	1.09±0.51 ^{a}	2.07±0.36 ^{b}	0.06±0.01 ^{a}	4.33±0.57 ^{a}
WPM	1.33±0.57 ^{c}	0.22±0.05 ^{c}	1.47±0.25 ^{b}	0.03±0.01 ^{a}	2.33±0.57 ^{c}
B5	2.33±0.57 ^{c}	0.27±0.22 ^{c}	1.20±0.36 ^{c}	0.06±0.02 ^{a}	3.00±1.00 ^{b}
LSD at 5 %	1.80	0.32	0.59	0.03	1.40
LSD at 1 %	4.33	0.74	1.33	0.06	2.20

Values are means of three replicates ± standard deviation (SD). Different letters indicate statistically significant differences between groups (mean ± SD, a not significant, b significant at $p < 0.05$, c highly significant at $p < 0.01$).

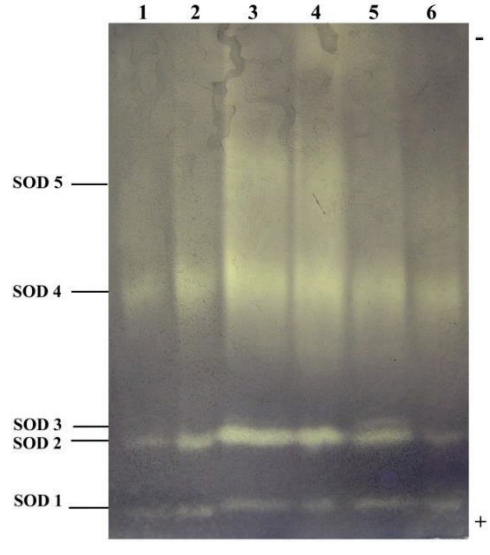

Figure 4: Native gel electrophoresis of SOD isoenzyme pattern of moringa shoots subcultured on full (lane 1), half (lane 2) and double MS medium (lane 3) as well as B5 (lane 4), WPM (lane 5) and SH media (lane 6) for three week, each of them was supplemented with 0.56 mg. l^{-1} BAP.

Table 5: SOD isozyme electrophoretic patterns of moringa shoots subcultured on full (lane 1), half (lane 2) and double MS medium (lane 3) as well as B5 (lane 4), WPM (lane 5) and SH media (lane 6) for three week, each of them was supplemented with 0.56 mg. l^{-1} BAP

	Full MS	Half MS	double MS	B5	WPM	SH
SOD 5			++	++		
SOD 4	+	+	++	++	+	+
SOD 3					+	
SOD 2	+	++	+++	+++	+++	+
SOD 1	+	+	+	+	+	+

+ = low intensity ++ = intermediate +++ = high intensity

Eleven different peroxidase isoenzyme forms were detected (Fig. 5, Table 6). Ten of eleven isoenzyme forms were detected when shoots were subcultured on different media. Two isoenzyme forms (POX-2 and POX-4) disappeared when shoots were cultured on WPM. Shoots subcultured on full strength MS (lane 1) showed the lowest staining intensity of most isoenzyme forms. It's worthy to mention that staining intensity increased when the concentrations of MS components were more than or less than full strength MS.

Catalase pattern (data not shown) showed that all of the subcultured shoots on different media expressed the same isoenzyme forms but two of them disappeared in shoots subcultured on double MS. High staining intensity was detected when shoots were cultured on full strength MS or SH.

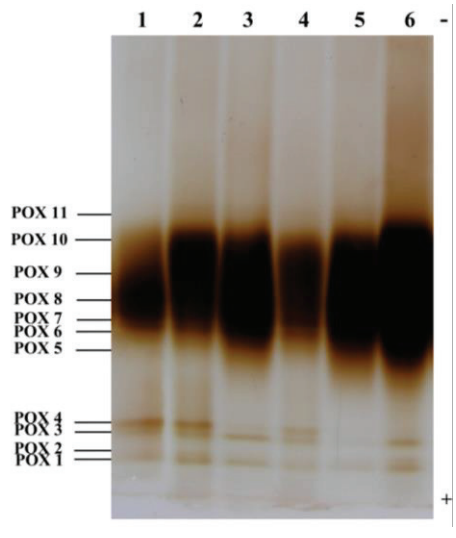

Figure 5: Native gel electrophoresis of POX isoenzyme pattern of moringa shoots subcultured on full (lane 1), half (lane 2) and double MS medium (lane 3) as well as B5 (lane 4), WPM (lane 5) and SH media (lane 6) for three week, each of them was supplemented with 0.56 mg. l^{-1} BAP

Table 6: POX isozyme electrophoretic patterns of moringa shoots subcultured on full (lane 1), half (lane 2) and double MS medium (lane 3) as well as B5 (lane 4), WPM (lane 5) and SH media (lane 6) for three week, each of them was supplemented with 0.56 mg. l^{-1} BAP

	Full MS	Half MS	double MS	B5	WPM	SH
POX 11		+	+		+	+++
POX 10	+	+++	+++	+++	+++	+++
POX 9	+++	+++	+++	+++	+++	+++
POX 8	+++	+++	+++	+++	+++	+++
POX 7	+++	+++	+++	+++	+++	+++
POX 6	++	+++	+++	+++	+++	+++
POX 5			+		+	+
POX 4	+	+	+	+		+
POX 3	+	+	+	+	+	+
POX 2	+	+	+	+		+
POX 1	+	+	+	+	+	+

+ = low intensity ++ = intermediate +++ = high intensity

Staining for GOT isoenzyme (Fig. 6, Table 7) indicated that isoenzyme form GOT-3 was expressed when shoots were cultured on full MS (lane 1), WPM (lane 5) and SH media (lane 6). Isoenzyme form GOT-6 was only detected in shoots cultured on double MS (lane 3). Generally, strong reduction of nutrient in the medium (half strength MS) resulted in disappearance of two bands (GOT-2 and GOT-3) and the lowest staining intensity.

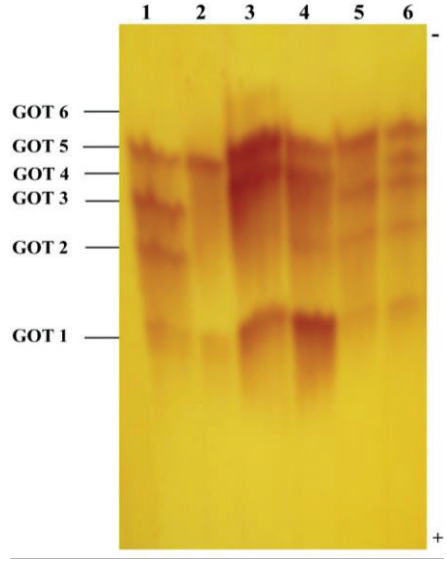

Figure 6: Native gel electrophoresis of GOT isoenzyme pattern of moringa shoots subcultured on full (lane 1), half (lane 2) and double MS medium (lane 3) as well as B5 (lane 4), WPM (lane 5) and SH media (lane 6) for three week, each of them was supplemented with 0.56 mg. l^{-1} BAP

Table 7: GOT isozyme electrophoretic patterns of moringa shoots subcultured on full (lane 1), half (lane 2) and double MS medium (lane 3) as well as B5 (lane 4), WPM (lane 5) and SH media (lane 6) for three week, each of them was supplemented with 0.56 mg. l^{-1} BAP

	Full MS	Half MS	double MS	B5	WPM	SH
GOT 6			+			
GOT 5			++	+		
GOT 4	+	+	++	+	+	+
GOT 3	+				+	+
GOT 2	+		+	+	+	+
GOT 1	+	+	++	++	+	+

+ = low intensity ++ = intermediate +++ = high intensity

EST expression under the influence of different media was visualized in (Fig. 7, Table 8). A total of 18 different EST isoenzyme forms were detected especially when nodal segments were cultured on double strength MS medium. The lowest number of bands and staining intensity were detected when nodal segments were cultured on full strength MS or SH medium. Low number of EST bands with low staining intensity was detected in shoots subjected for low nutrients where they were cultured on half strength MS medium.

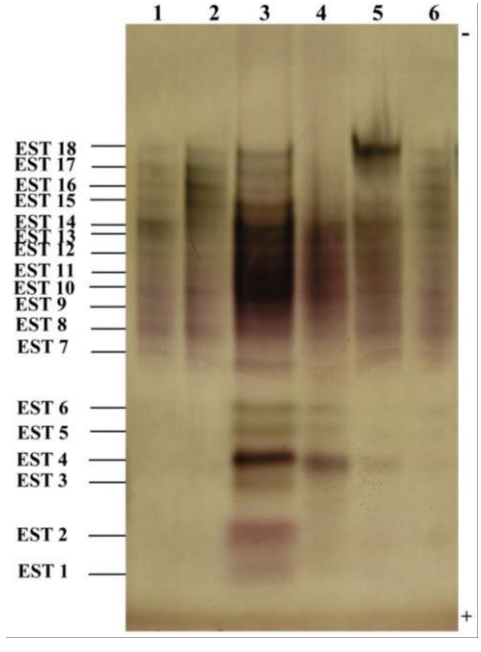

Figure 7: Native gel electrophoresis of EST isoenzyme pattern of moringa shoots subcultured on full (lane 1), half (lane 2) and double MS medium (lane 3) as well as B5 (lane 4), WPM (lane 5) and SH media (lane 6) for three week, each of them was supplemented with 0.56 mg. l^{-1} BAP

Table 8: EST isozyme electrophoretic patterns of moringa shoots subcultured on full (lane 1), half (lane 2) and double MS medium (lane 3) as well as B5 (lane 4), WPM (lane 5) and SH media (lane 6) for three week, each of them was supplemented with 0.56 mg. l^{-1} BAP

	Full MS	Half MS	double MS	B5	WPM	SH
EST 18	+	+	+		+++	+
EST 17	+	+	+		+	+
EST 16	+	+	+		+	+
EST 15	+	+	+++	+	+	+
EST 14	+	+	+++	+	+	+
EST 13	+	+	+++	++	++	+
EST 12	+	+	+++	++	++	+
EST 11	+	+	+++	++	++	+
EST 10	+	+	+++	++	++	+
EST 9	+	+	+++	++	++	+
EST 8	+	+	+++	+	+	+
EST 7	+	+	+	+	+	+
EST 6			+	+		
EST 5			+	+		
EST 4			+++	+++	+	+
EST 3			+	+		
EST 2			+++	+		
EST 1			+	+		

+ = low intensity ++ =intermediate +++ = high intensity

4 DISCUSSION

Two types of explants obtained from soil growing seedlings, shoot and nodal cuttings, were used to establish shoot culture of moringa on MS medium supplemented 0.65 mg l^{-1} BAP. Nodal segments were better than shoot cuttings where they expressed higher values of frequency of shoot formation, number of shoots/explant, fresh mass/cluster and number of nodes/explant than those of shoot cuttings. Explants cultured on growth regulators free medium showed the formation of roots as was previously reported (Förster et al., 2013). Supplementing the medium with different concentrations of BAP resulted in callus formation and it increased with the increase of BAP concentrations. The variable response of different types of explant to the same culture conditions may be due to the difference in endogenous plant hormones they have (Kumar and Reddy, 2011).

On KIN containing medium, shoot formation and growth on nodal segments were better that those on shoot segments. Plants cultured on KIN medium were taller with higher number of nodes/shoot compared to BAP containing medium. Medium supplemented with BAP resulted in greater ability to form adventitious shoots. There was opposite relationship between shoot length and formation of callus at the base of nodal segments. In moringa, callus formation increased with the increase of BAP concentration (Förster et al., 2013). It was clear that both of the applied cytokinins were able to induce morphogenesis and growth in moringa. Cytokinins stimulate cell division and morphogenesis (Taiz and Zeiger, 1991), enhance the lateral bud growth due to break the apical dominance (George, 1993), and induction of adventitious bud formation. In moringa and other plant species, two of them have been using widely, they are KIN and BA (Pierik, 1997; Förster et al., 2013; Salem, 2016).

Source of explant was also important factor during moringa shoot multiplication may be due to the different values of endogenous phytohormones they contain (Reddy et al., 2008; Förster et al., 2013). Moringa

explants obtained from established shoot culture were better than others obtained from soil growing seedling as they have better potential for organogenesis (Reddy et al., 2008). The results showed that BAP was superior in multiplication compared with KIN. In moringa as well as other plant species, specific BAP concentration led to multiplication and vegetative growth but other applied concentrations was strongly resulted in induction of callus formation (Ibrahim et al., 2013). Callus formation on the base of nodal segment on BAP containing media were higher than those of KIN, especially under the influence of high concentration as an aspect of vitrification. Vice versa, the superiority of kinetin over other BAP was sometimes proven (Parzymies and Dąbsk, 2012).

Moringa shoot multiplication was strongly influenced by the concentration of nutrients in culture media, where it expressed different values on different media or different strengths of MS medium. Between the different types of the used media, full strength MS medium was best one because it contains all the elements which inducted the best cells dedifferentiation leading to the highest adventitious shoot formation (Thorpe, 1978). In comparison to other media, MS is referred to as a high salt medium where it has high contents of nitrogen and potassium (Cohen, 1995). Reducing the salt concentration of MS medium due to application of its half strength resulted in reduction of shoot multiplication and shoot growth as was reported by Bhatt and Dhar (2004). This poor performance with regard to the determined parameters was also detected when WPM was used where it characterized by low mineral content. Mineral content of SH was lower than MS but higher than B5 and WPM, consequently, the efficiency of moringa micropropagation was in parallel to mineral content of the culture media.

Under the influence of *in vitro* culture conditions, moringa explants as well as other plant species were subjected to extreme conditions such as wounding, medium components and others (Kumar and Reddy, 2011; Wojtania and Skrzypek, 2014; Salem, 2016). These conditions resulted in ROS accumulation leading to different physiological disorders including vitrification (Rojas-Martínez et al., 2010). Also these conditions, induce acclimation responses which allows the plants to survive under unfavorable conditions (Kevers et al., 2004). The suitable medium for micropropagation of certain plant, such as full strength MS in case of moringa, exerted low stress and low need to rise the activities of antioxidant enzymes such as SOD, CAT and POX. Increase in the activity of an enzyme is expressed as increase in the number of bands or the staining intensity of all or some isoenzyme forms. Generally, the staining intensity of SOD bands decreased when the culture conditions was suitable for moringa multiplication, on full strength MS and SH media. On the other side, over increase of chemical components of the cultured medium (as in case of double MS medium) or on medium with low mineral component (as in case of B5 or WPM) moringa shoots expressed the highest number and staining intensity of SOD bands. Both POX and CAT create cooperation with SOD to overcome the toxic effect of H_2O_2. In this work, the lowest staining intensity of most isoenzyme forms of POX was detected under favorite condition of moringa *in vitro* culture. On the other side, high staining intensity of CAT was detected when shoots were cultured on full strength MS or SH. These enzymes established efficient system to regulate the elevated oxidative stress due to *in vitro* culture condition which stimulate *in vitro* shoot multiplication.

Since the high salt content of the medium may inhibit root formation and growth irrespective the type and concentration of auxin, half strength MS medium containing 0.5 mg l^{-1} IAA was usually used to induce root formation in moringa as was reported by (Sauer et al., 1985). The plants were successfully transferred to open condition after three weeks acclimatization.

5 CONCLUSION

In vitro obtained nodal segments cultured on full strength MS medium containing 0.56 mg l^{-1} of BAP is recommended for efficient moringa micropropagation without severe verification or retardation of root formation and successful acclimatization.

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Growth and antioxidant system responses of maize (*Zea mays* L.) seedling to different concentration of pyrene in a controlled environment

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic pollutants effecting different aspects of plants physiology. To assess the physiological responses of plants to PAHs, maize (*Zea mays*) was treated with 25, 50, 75, and 100 ppm of pyrene and after 21 days, the activity of some antioxidant enzymes, malondialdehyde (MDA), total flavonoid, total anthocyanin, and soluble sugar contents were measured in shoots and roots of plants. Pyrene led to increase MDA content as well as CAT, POD, and SOD activities. Increase in pyrene concentration reduced all studied growth variables and significantly increased photosynthetic pigments contents of plants. Soluble sugar content was significantly higher in the shoot, while that was reduced in the roots through increasing of pyrene concentration ($p < 0.05$). Also, the increase of pyrene concentration decreased total flavonoid content compared to anthocyanin content. In conclusion, these findings supported the hypothesis that pyrene toxicity induces oxidative stress in the maize plant and it also increases the antioxidant systems in order to moderating stress condition. However, the antioxidant system of maize was not strong enough to eliminate all produced ROS at high concentrations, thus this caused oxidative damage to the plant and decreased its growth variables.

Key words: PAHs; physiological responses; pollution; toxicity

IZVLEČEK

RASTNI IN ANTIOKSIDACIJSKI ODZIV SEJANK KORUZE (*Zea mays* L.) NA RAZLIČNE KONCENTRACIJE PIRENA V NADZOROVANIH RAZMERAH

Policiklični aromatski ogljikovodiki (PAHs) so organska onesnažila, ki vplivajo na različne fiziološke procese v rastlinah. Za ovrednotenje fiziološkega odziva koruze na PAH-e so bile njene sejanke tretirane s 25, 50, 75, in 100 ppm pirena, po 21 dneh so bile izmerjene v koreninah in poganjkih aktivnost nekaterih antioksidacijskih encimov, vsebnost malondialdehida (MDA), celokupnih flavonoidov, antocianinov in topnih sladkorjev. Piren je povečal vsebnost MDA kot tudi aktivnosti CAT, POD in SOD. Povečanje koncentracije pirena je zmanjšalo vse merjene rastne parametre in povečalo vsebnost fotosinteznih barvil v rastlinah. Vsebnost topnih sladkorjev je bila s povečanjem koncentracije pirena značilno večja v poganjkih in manjša v koreninah ($p < 0.05$). Povečanje koncentracije pirena je zmanjšalo vsebnost celokupnih flavonoidov v primerjavi z antocianini. Zaključimo lahko, da toksičnost pirena inducira v rastlinah koruze oksidacijski stres in poveča odziv antioksidacijskega sistema na stresne razmere. Kljub temu odziv antioksidacijskega sistema koruze ni zadoščal za preprečitev tvorbe reaktivnih zvrsti kisika v večjih koncentracijah, kar je povzročilo oksidativne poškodbe v rastlinah in zmanšalo njihovo rast.

Ključne besede: PAH-i; fiziološki odziv; onesnaženje; toksičnost

1 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are made up of only carbon and hydrogen (Gong et al., 2007) and comprised of two or more fused benzene cycles (Watts et al., 2006; Li et al., 2014). PAHs include a large and heterogeneous group of organic contaminants that are mainly formed and emitted because of the incomplete combustion of organic materials (Lundstedt, 2003). PAHs are divided into two groups including low molecular mass (LMM) compounds containing 2-3 rings and high molecular mass (HMM) compounds

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containing 4-7 rings (Wilson & Jobes, 1993). Pyrene is one of the high molecular mass PAH which is made of four benzene rings and thus it is determined as one of the important pollutants listed in the Environmental Protection Agency (Khan et al., 2008). Some PAHs are toxic to living organisms and their mutagenic and carcinogenic effects are well known. Hence, their fate and transport in the environment is of worldwide attention (Fuxing et al., 2010).

Plants can uptake PAHs through roots and leaves (Gao & Zhu, 2004; Collins et al., 2006), and subsequently can transfer them into food chains (Hung & Mackay, 1997). A number of factors such as concentration and physicochemical properties of the compound, soil type, temperature, plant species and stage of ontogenesis, and lipid content of plants can influence the rate of PAHs uptake by plants (Binet et al., 2000). Indeed, all stages of plant growth can be affected by PAHs from germination to production (Tomar & Jajoo, 2014). PAHs also have harmful effect on plants in terms of decrease in photosynthesis and respiration, changes in enzyme activities, photosynthetic pigments content (Alkio et al., 2005), and injury to membranes by lipid oxidation (Branquinho et al., 1997; Chiang et al., 1996). Previously, some researchers investigated the effects of PAHs on maize plants (Kummerova et al., 2013; Dupuy et al., 2015; Liao et al., 2015) and this plant was introduced as a good choice for remediation of soil contaminated with PAHs (Liao et al., 2015; Kosnar et al., 2018). However, while the negative effects of PAHs on the plants growth and development is well known, but the all aspects of those effects on plants, and the precise mechanisms of plants response to PAHs toxicity is not completely clear and still remaining ambiguous. Accordingly, in this study, the effects of the different concentrations of pyrene as one of the abundant PAHs in the environment (Wilcke, 2000; Xu et al., 2007) on the growth of maize (*Zea mays* L.) were studied. Moreover, the evaluation of the biochemical and physiological responses of plants to pyrene toxicity was another aim of this study.

2 MATERIALS AND METHODS

2.1 The treatment

In order to prepare different concentrations of pyrene (25, 50, 75 and, 100 ppm), the appropriate amount of pyrene for each treatments were dissolved in ethanol. Then, the solutions were sprayed on sterile perlite in pots. Treated perlite was used for plant cultivation after evaporation of ethanol for 72 h.

2.2 Experimental design

Experiments were conducted as pot culture of plants under controlled conditions using a completely randomized design (CRD) with three replications for each treatment.

2.3 Plant culture

The seeds of maize (*Zea mays* L. var. single crosses 704) were obtained of the East Azerbaijan Research and Education Centre for Agriculture and Natural Resources (Tabriz, Iran) and stored at 4 °C until cultivation. Appropriate numbers of seeds were selected based on their vigor and uniformity, disinfected using 1 % (v/v) sodium-hypochlorite solution for 5 minutes, and sufficiently washed using sterile distilled water. Then, the sterilized seeds were planted in uncontaminated (control) and pyrene-contained perlite. After 3 days, all germinated seeds were transferred to growth chambers with controlled conditions (25-30 °C, 16/8 h light/dark photoperiod, light intensity of 75 µmol $m^{-2}s^{-1}$ provided by common day light fluorescent lamps, and relative humidity of 60 %) for 3 weeks. The water content of the pots was adjusted to 100 % field capacity every two days using sterile distilled water. After 4 and 10 days, the water of pots was replaced with 50 % and 100 % Hoagland solution, respectively.

2.4 Harvesting of plants and assays

The cultivation period of plants was 21 days when the PAHs toxicity symptoms such as chlorosis, necrosis, and reduced leaf size were observed in plants. Biochemical and physiological assays were performed using fresh samples before the harvesting of plants. After the estimation of shoot height and root length, the harvested plants were divided into the roots and shoots. The samples were sufficiently washed with water, immediately dried on the towel paper, and transferred to 70 °C after determining of the fresh mass. The dry mass of samples was measured after 72 h.

2.5 Measurement of photosynthetic pigments content

Photosynthetic pigments content (chlorophyll a, b, total chlorophyll, and total carotenoids) was measured according to the method of Hartmut (1987). Briefly, a quantity of 0.1g of fresh leaf samples was homogenized with 5 ml of acetone using a mortar and pestle on ice bath. Homogenates were filtered using a number 42 Whatman filter paper and the absorbance of extracts was recorded at 645, 663, and 470 nm by Spectrophotometer (Analytic Jena, Specol 1500, Germany).

2.6 Measurement of total protein content and antioxidant enzyme assays

An amount of 0.1 g of samples was homogenized in ice-cold phosphate- buffered solution (PBS, 50 mM, pH = 7) using mortar and pestle. Homogenates were centrifuged at 10000 g for 10 min at 4 °C. The supernatants were used immediately for determination of the total soluble protein content (Bradford, 1976) as well as the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT).

SOD activity was evaluated by determination of nitro-blue-tetrazolium (NBT) photoreduction inhibition by extracts (Winterbourn et al., 1976). The reaction mixture pH (3 ml) contained 2.7 ml sodium phosphate solution (1 M, pH = 7.8), 100 µl NBT (1.5 mM), NaCN (0.3 mM) EDTA (1 M), 50 µl of riboflavin (0.12 mM) and 50 µl of enzyme extract. The mixtures were illuminated at light intensity of 75 µmol $m^{-2}s^{-1}$ for 12 minutes and the absorbance of the solutions was recorded at 560 nm. The amount of the enzyme causing 50 % protection of NBT photoreduction was considered as one unit and SOD activity expressed as U mg^{-1} protein.

The activity of POD was determined by recording the increase in absorbance at 470 nm during polymerization of guaiacol to tetraguaiacol for 3 minutes (Obinger et al., 1997). The reaction mixture (1 ml) encompassed 300 µl of guaiacol (4 mM), 350 µl of phosphate buffer (10 mM, pH = 7), 300 µl of H_2O_2 (50 mM) and 50 µl of enzyme extract. The reaction was initiated by adding H_2O_2 to reaction mixture and POD specific activity was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1} for guaiacol. One unit of POD activity was considered as the enzyme amount capable of oxidizing 1 µM guaiacol to tetraguaiacol per minute and POD activity expressed as U mg^{-1} protein.

CAT activity was assayed according to the methods of Chance and Maehly (1955). The activity of CAT was measured at 240 nm by following the decomposition of H_2O_2 for 3 min. The reaction mixture contained 2.5 ml potassium phosphate buffer (50 mM, pH = 7), 1 ml H_2O_2 (10 mM) and 500 µl of enzyme extract. CAT specific activity (expressing as U mg^{-1} protein) was calculated using the extinction coefficient of 27 M^{-1} cm^{-1} for H_2O_2 and one unit of enzyme activity was considered as the amount of enzyme necessary for the reduction of 1 µM H_2O_2 per minute.

2.7 Measurement of malondialdehyde content (MDA)

Malondialdehyde (MDA) content measured by a method described by Boominathan and Doran (2002). Approximately, 0.1 g of samples were homogenized with 0.1 % (W/V) trichloroacetic acid (TCA, Merck, Germany) and centrifuged for 5 minutes at 10000 g. Then, 0.5 ml of supernatants was mixed with 2 ml of 20 % TCA containing 0.5 % of 2-thiobarbituric acid (Merck, Germany) and heated in hot water at 95 °C for 30 minutes. Mixtures were immediately transferred to ice bath and then centrifuged at 10000 g for 15 min. Finally, the absorbance of supernatants was recorded at 532 nm and MDA concentration were calculated according to a standard curve prepared using 3,1,1,3-tetraethoxy propane (0-100 nM) and expressed as µmol g^{-1} FM.

2.8 Measurement of total flavonoid and anthocyanin contents

For measurement of total flavonoid, 0.1 g of samples was homogenized in methanol 80 % using mortar and pestle. Homogenates were centrifuged at 10000 g for 5 min and then the 500 µl of supernatants, 1.5 ml of 80 % methanol, 100 µl of 10 % aluminum chloride solution, 100 µl of 1 M potassium acetate, and 2.8 ml of distilled water were added to 500 µl of each extract. After 40 minutes, absorbance of the mixture was measured at 415 nm compared to the control. Quercetin was used for the preparation of calibration curve (20-200 mg l^{-1}). The total flavonoid content of the extract was reported as milligram quercetin equivalents (QE) g^{-1} FM (Chang et al., 2002).

To measure the total anthocyanin content, 0.02 g of dried plant sample was pulverized with 4 ml of hydrochloric acid containing 1 % methanol in a porcelain mortar. The solution was kept in the refrigerator for 24 hours and then, centrifuged for 10 minutes at 13000 g. The supernatant was removed and absorbance of the extract was measured at 530 and 657 nm against the control (hydrochloric acid containing 1 % methanol). The anthocyanin content of each extract was calculated using the following equation (Mita et al., 1997).

$A = A_{530} - (0.25 \times A_{657})$

Where, A is absorbance of the solution (subscripts indicate the wavelength at which the absorbance is measured).

2.9 Measurement of soluble sugar contents

The soluble sugar content was determined by the phenol-sulfuric acid method (Kochert, 1978). A quantity of 5 ml of ethanol (70 %) was added to 50 mg of dry sample and incubated in refrigerator for one week. The samples were centrifuged at 10000 g for 15 minutes at room temperature. Then, 0.5 ml of the plant extract was made to 2 ml with distilled water and then 1ml of 5 % phenol and 5 ml of concentrated sulfuric acid were added. The mixture was vortexed and incubated for 30 minutes at room temperature. The absorption of solution

was recorded at 485 nm, and glucose was used to preparation of standard curve. The data were expressed as milligram per gram of plant dry mass and showed as mg g^{-1} DM in the text.

2.10 Statistical analysis

All measurements were conducted with three replications and data were reported as mean ± standard deviation (SD). Data normality was assessed using the Kolmogorov-Smirnov test. The data were analyzed using GLM procedure by SPSS software (Ver.16) and Tukey's multiple range tests was used for mean comparisons at 1 % probability level. SPSS software was used to calculate the correlation coefficient (Pearson) between characteristics. Microsoft excel 2013 software was used for the preparation of figures.

3 RESULTS

3.1 Growth variables

The results showed that the increase in pyrene concentration significantly reduced all studied growth parameters in comparison to the control ($p < 0.05$) (Table 1). Treatment of plants with 100 ppm of pyrene led to 73.66 and 74 % reduction in the shoot and root length, respectively. In addition, fresh mass of root and shoot decreased to 81 and 77 %. Similarly, treatment with 100 ppm of pyrene led to 62 and 61.29 % decrease in shoot and root dry mass in comparison to the control. Although the highest decrease in all growth parameters was shown in plants treated with 100 ppm of pyrene, no significant difference was seen among plants treated with 50, 75, and 100 ppm of pyrene.

Table 1: The effect of concentrations of pyrene on the growth variables of maize

Concentration of pyrene (ppm)	Shoot Length (cm)	Root Length (cm)	Shoot FM)mg(	Root FM)mg(	Shoot DM)mg(	Root DM)mg(
0	0.25^{a}±39.1	1.59^{a}±28	64.75^{a}±995	35.45^{a}±888	16.99^{a}±197	24.03^{a}±155
25	1.03^{b}±26.3	0.03^{b}±12.6	37.45^{b}±435	33.4^{b}±559	3.57^{b}±93	28.32^{b}±131
50	0.06^{c}±15.2	0.51^{bc}±9.66	34.16^{c}±425	37.45^{c}±239	1.59^{c}±87.1	3.23^{c}±94.6
75	0.21^{d}±12.6	0.51^{bc}±9.66	37.26^{d}±243	17.02^{c}±222	0.93^{c}± 75	1.06^{c}±63.2
100	0.26^{e}±10.3	0.26^{c}±7.27	25.01^{d}±188	9.86^{c}±196	1.25^{c}±74.3	1.58^{c}±60

The data represent the mean of three replications ± SD and similar upper case letters indicates no significant difference at $p < 0.05$. DM: Dry Mass, FM: Fresh Mass.

3.2 Photosynthetic pigments content

Chlorophyll a content was significantly high in plants treated by 50, 75, and 100 ppm of pyrene in comparison with the control plants and the highest values of chlorophyll a (73.87 and 81.55 %) were observed in plants treated by 75 and 100 ppm, respectively ($p < 0.05$). In contrast, all applied levels of pyrene significantly decreased chlorophyll b content. Such content in the plants treating with 25, 50, 75, and 100 ppm of pyrene were decreased to 57.70, 74.27, 70.63, and 73.74 %, respectively. Moreover, the highest value of carotenoids (93.93 %) was observed at 100 ppm of pyrene (Fig. 1).

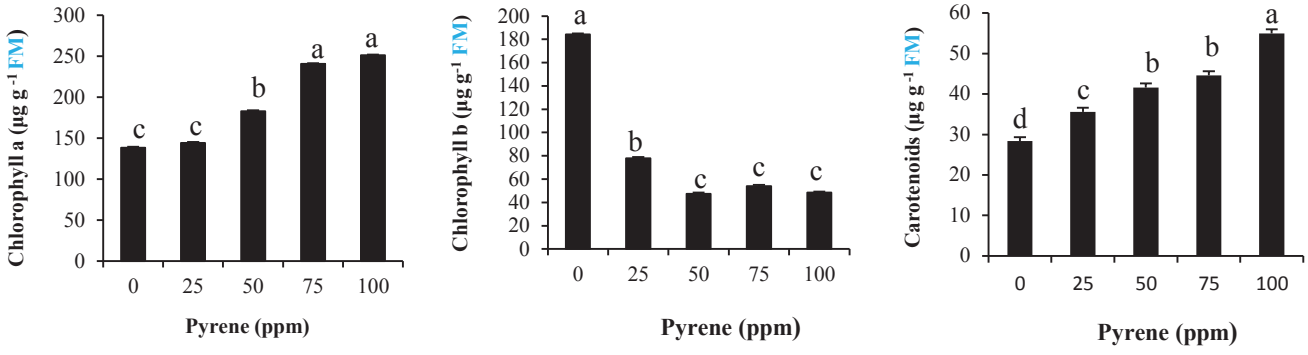

Figure 1: The effect of concentrations of pyrene on photosynthetic pigments contents of maize plants. The data represent the mean of three replications and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

3.3 MDA content

Pyrene had a significant effect on the malondialdehyde content. The MDA content increased by the increasing of pyrene concentrations ($p < 0.05$). The highest amount was measured in plants treated with 100 ppm of pyrene in which MDA contents of shoot and root were 3.6 and 2.33 times higher than its contents in control, respectively (Fig. 2).

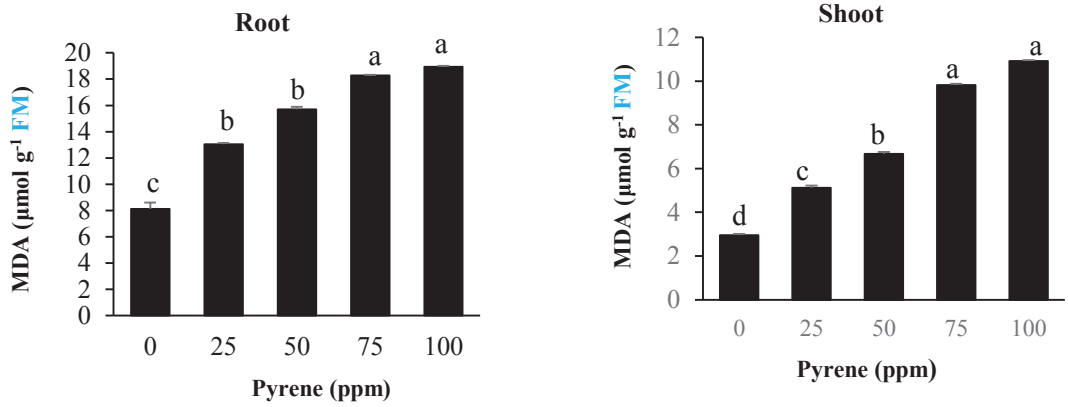

Figure 2: The effect of concentrations of pyrene on malondialdehyde (MDA) content of maize plants. The data represent the mean of three replications and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

3.4 Soluble sugars content

The results showed that pyrene had significant effect on the soluble sugar content in the plant treated ($p < 0.05$). Soluble sugar contents of shoots significantly increased in plants treated with 25 and 50 ppm of pyrene compared with the control ($p < 0.05$). There was no significant difference between controls and plants treated by 75 ppm of pyrene. 100 ppm of pyrene led to significant reduction in shoot soluble sugar content ($p < 0.05$). Regarding root soluble sugar content it was reduced by increasing of pyrene concentration and significantly lower content was observed in plants treated by 50, 75 and 100 ppm of pyrene in comparison with the control ($p < 0.05$) (Fig 3).

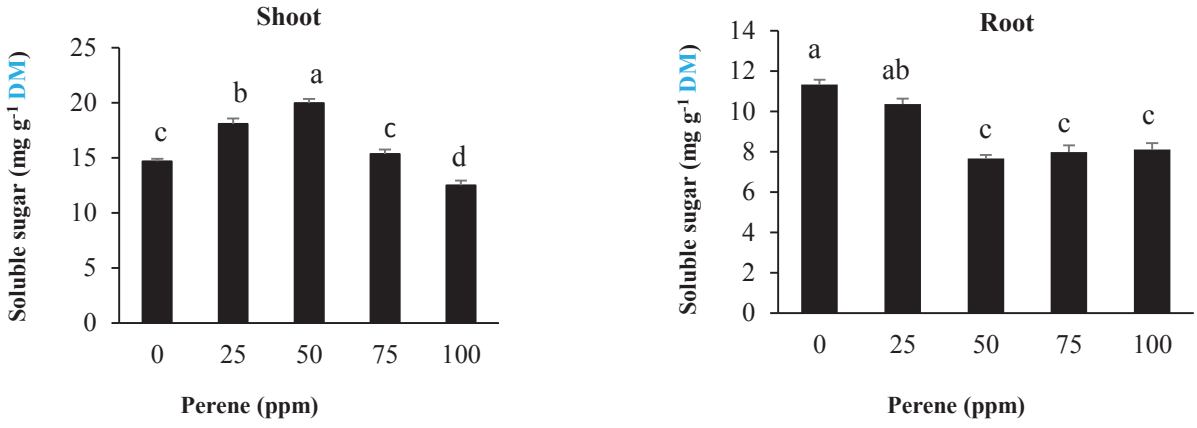

Figure 3: The effect of concentrations of pyrene on soluble sugar contents of maize plants. The data represent the mean of three replications and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

3.5 Activity of antioxidant enzymes

CAT activity significantly decreased in shoots of plants treated by 25 and 50 ppm of pyrene in comparison with the control, but there were no significant difference between plant treated with 75 and 100 ppm of pyrene and control ($p < 0.05$). In the roots, CAT activity in the plants treated by 25, 50, and, 75 ppm of pyrene was increased in comparison with the control (Table 2).

POD activity in shoots of plants treated by different concentrations of pyrene was significantly higher compared to the control and the highest activity (5.75 times) was observed in shoots of plants treated by 75 ppm of pyrene ($p < 0.05$). Moreover, POD activity in root (especially in plants treated by 25 and 50 ppm of pyrene) was higher than that in control plants, but there was no significant difference in plant treated with 75 and 100 ppm of pyrene and control ($p < 0.05$) (Table 2).

SOD activity in shoot was significantly higher in plants treated with different concentrations of pyrene in comparison to control ($p < 0.05$). Plants treated by 75 ppm of pyrene showed the highest SOD activity in the shoots (2.45 times). Such increase in root was observed in concentration levels of 25 and 50 ppm, but a decrease was observed in concentration of 100 ppm in comparison to the control plant.

Table 2: The effect of the concentrations of pyrene on antioxidant enzymes activity (U mg $^{-1}$ protein) in the shoot and root of maize plant

pyrene	Root			Shoot		
(ppm)	CAT	POD	SOD	CAT	POD	SOD
0	0.03^{c}±0.45	7.69^{c} ± 80.52	0.6^{b}± 47.81	0.002^{a}±0.183	0.05^{d}±2.5	$0.\ 1^{c}$± 15.9
25	0.03^{a}±0.94	5.98^{ab}±117.5	0.5^{a}±102.5	0.01^{b}±0.133	0.03^{c}±8.5	0.4^{b}± 22.8
50	0.01^{b}±0.75	14.65^{a}±126.7	0.9^{a}± 97.12	0.001^{b}± 0.130	0.02^{b}±11.7	0.3^{a}± 36.9
75	0.002^{bc}±0.66	10.26^{c}±72.71	1.2^{b}± 51.61	0.023^{a}±0.173	0.04^{a}±14.6	0.9^{a}± 38.9
100	0.04^{c}± 0.40	9.23^{c}± 73.92	0.8^{c}±25.92	0.004^{a}±0.193	0.02^{c}±8.1	0.1^{b}± 24.3

The data represent the mean of three replications ±SD and similar upper case letters indicates no significant difference at $p < 0.05$. CAT: catalase, POD: peroxidase and SOD: superoxide dismutase.

3.6 Total flavonoid and anthocyanin contents

With increasing pyrene concentration total flavonoids content in shoot and root was significantly reduced compared to control plants ($p < 0.05$). However, there was no significant difference among plants treated with different concentration of pyrene in roots. Treatment of plants with pyrene led to an accumulation of anthocyanins in roots and shoots. The highest anthocyanins content in roots and shoots were observed in plants treated with 75 and 25 ppm of pyrene, respectively (Table 3).

Table 3: The effect of the concentrations of pyrene on total flavonoid and anthocyanins content (mg EQ g^{-1} FM) in the shoot and root of maize plant

pyrene	Root		Shoot	
(ppm)	Total Flavonoid	Total Anthocyanin	Total Flavonoid	Total Anthocyanin
0	0.001^{a}±0.270	0.001^{ab}±1.01	0.002^{a}± 0.292	0.002^{c}±0.61
25	0.003^{b}±0.180	0.003^{ab}±0.99	0.009^{b}±0.179	0.001^{a}±0.80
50	0.005^{b}±0.178	0.005^{a}±1.16	0.004^{b}± 0.169	0.001^{b}±0.68
75	0.002^{b}±0.186	0.001^{a}± 1.32	0.003^{c}±0.126	0.003^{b}±0.76
100	0.004^{b}± 0.153	0.002^{c}± 0.72	0.005^{c}±0.103	0.002^{b}± 0.73

The data represent the mean of three replications ± SD and similar upper case letters indicates no significant difference at $p < 0.05$.

3.7 Correlation analysis

The analysis of correlation between MDA content and POD and SOD activity in shoot and root (at 1 and 5 % levels) showed negative correlation coefficient between enzymes activities and MDA content. These findings indicate that POD and SOD involved in plants resistance to oxidative stress are induced by pyrene toxicity. Moreover, no correlation was seen between CAT activity and MDA content of the shoot and root of maize (Table 4).

Table 4: Statistical analysis for correlation between the activity of antioxidant enzyme and MDA content in the shoot and root of maize plant.

	SOD Root	SOD Shoot	POD Root	POD Shoot	CAT Root	CAT Shoot	MDA Root	MDA Shoot
MDA Shoot	0.685** -	0.356^{ns}	0.287^{ns}-	0.574*-	0.259^{ns}	0.047^{ns}	0.885**	1
MDA Root	0.579*-	0.352^{ns}	0.422^{ns}-	0.573*-	0.266^{ns}-	0.143^{ns}	1	
CAT Shoot	0.299^{ns}	0.04^{ns} -	- 0.138^{ns}	0.052^{ns}-	0.106^{ns} -	1		
CAT Root	0.002^{ns}	0.118^{ns}	0.722**	0.235^{ns}-	1			
POD Shoot	0.372^{ns}	0.834**	0.021^{ns}	1				
POD Root	0.065^{ns}-	0.092^{ns}	1					
SOD Shoot	0.031^{ns}-	1						
SOD Root	1							

Notes: **Correlation is significant at 0.01 levels, *Correlation is significant at 0.05 levels, ^{ns} correlation is not significant.

4 DISCUSSION

The results of this study showed that pyrene had a negative effect on the growth variables of maize plants. The results indicated that the growth variables were smaller by increasing of the concentrations of pyrene compared to the control plant. The reduction of the growth variables in the presence of PAHs had been reported previously in plants such as wheat (Tomar & Jajoo, 2014; Salehi & Deljoo, 2015), maize, pea seedlings (Kummerova et al., 2012), and *Arabidopsis thaliana* (L.) Heynh (Liu et al., 2009). Pyrene and possibly other compounds of this group by disrupting the development and function of the roots in the early stages of plant growth are playing an important role in mineral nutrition, will disrupt the growth and also decrease it. (Dupuy et al., 2016). The main mechanism could have been that there was increased sensitivity of maize to pyrene as indicated by high concentration of MDA with increasing pyrene concentration. This could have been caused by disruption in electron transportation and elicitation of ROS. Therefore, MDA accumulation resulting from oxidative stress and ROS accumulation was a reliable marker for determining of the negative effect of pyrene on the growth of maize plants.

For detoxification a plant would need a protective system equipped with enzymatic and non-enzymatic mechanisms for scavenging of reactive oxygen species (ROS) accumulated under oxidative stress (Alscher et al., 1997). Based on the results of the present study, CAT, POD, SOD activity, and anthocyanins content were increased by the different levels of pyrene leading to ROS detoxification and oxidative stress reduction. Therefore, these enzymes are important tools involved in the ROS detoxification and plant resistance to oxidative stress. Also, the analysis of correlation (Table 4) showed that there are a negative correlation between MDA content and POD and SOD activity in the shoot (r^2 = -0.574 & r^2 = -0.685, respectively) and the root (r^2 = -0.573 & r^2 = -0.579, respectively) indicating the role of these enzymes in ROS detoxification and plant resistance to oxidative stress. Moreover, no correlation was found between CAT activity and MDA content of the shoot and the root of maize (Table 4). Similar results were reported for sunflower, alfalfa and wheat plants (Salehi-Lisar & Deljoo, 2015).

Photosynthetic pigments content changed in a different way by pyrene concentrations. Accordingly, chlorophyll a and carotenoids contents were increased and chlorophyll b content decreased. High ROS levels can decline chlorophyll levels leading to photosynthesis decline. Generally, this is result from light harvesting complex protein in photosystem II drasticaldamage under stress conditions. The chlorophyll b is a part of this protein complex,embedded in the chloroplast membrane and by the increasing of ROS levels within chloroplast its content is reduced by the oxidative stress. The destruction rate of chloroplast membrane is also increased. Thus, the destruction of protein complex will occur under stress and chlorophyll b level will be also decreased (Liu et al., 2009; Alberet & Thornber, 1977).

Pyrene had significant influence on soluble sugar content ($p < 0.05$). 25 and 50 ppm of pyrene effectively increased the soluble sugar content in the shoots, but the increase of pyrene in the roots decreased its content. The increased concentration in carbohydrate in shoots could be the result of higher concentration of anthocyanins and better photosynthesis. The higher concentration of anthocyanins in shoots of plants treated by pyrene may acts as a protective pigments for photosynthesis apparatus, finally leading to increase in the soluble sugar content. Reduced level of soluble sugars in roots may be due to the low requirement for photosynthetic materials due to the reduced growth of maize roots (Table 1). In addition, lower carbohydrate content in roots can be due to higher consumption of energy for resistance to pyrene toxicity. According to our results, higher accumulation and degradation rate of pyrene was occurring in maize roots (Houshani et al., 2019). Carbohydrates in plants, in addition to energy production, lead to the regulation of various gene expressions (Rolland et al., 2006) and may have antioxidant activity (Lang-Mladek et al., 2010).

5 CONCLUSION

According to the obtained results, similar to other PAHs, pyrene especially at higher concentrations had a negative effect on growth and chlorophyll content of *Zea mays*. Pyrene induced oxidative stress in maize as shown by MDA accumulation in the plant. POD and SOD as well as anthocyanins could be an important antioxidant system involved in detoxification of ROS and plant resistance to pyrene toxicity. Therefore, these findings supported this fact that pyrene toxicity induces oxidative stress in the maize plant and it also increases the antioxidant systems activity in order to moderating stress condition. However, the antioxidant system of maize was not strong enough to eliminate all produced ROS at high concentrations, thus this caused oxidative damage to the plant and decreased its growth variables.

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The effect of weed frequency in the overall alfalfa (*Medicago sativa* L.) productivity, case study from Kosovo

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ABSTRACT

Alfalfa (*Medicago sativa* L.) represents an important leguminous forage crop in farming as well as in systems of animal husbandry. It is known for its wide usage for grazing, hay, silage as well as in the form of green manure and cover crop. Our study aims to assess the influence of weeds on quality and quantity of three different aged alfalfa plots. A list of registered weed plant species divided by harvesting periods on each of the surveyed plots is offered. The biomass productivity and its correlation to the effects of artificial fertilizers, alfalfa age and the frequency of weeds are provided. We concluded that using mineral fertilizers in a controlled manner will help to reduce considerably the amount of weeds and also that alfalfa crops will be best to be replaced after four years as its productivity will start afterwards to drop sharply, whereas the number and frequency of weeds will increase conversely.

Key words: alfalfa; weed management; forage crops

IZVLEČEK

UČINEK PLEVELOV NA PRIDELEK LUCERNE (*Medicago sativa* L.), VZORČNA ŠTUDIJA S KOSOVA

Lucerna (*Medicago sativa* L.) predstavlja v kmetijstvu pomembno krmno metuljnico kot integralni del živinorejskih sistemov. Njena uporabnost je zelo široka, od paše, priprave sušene krme, silaže, kot zeleno gnojilo in pokrovna rastlina. Namen te raziskave je oceniti vpliv plevelov na količino in kakovost pridelka lucerne na treh različno starih posevkih. Vključena je lista plevelov, ki so se pojavljali na teh ploskvah v odvisnosti od časa košnje. Produkcija biomase je prikazana v odvisnosti od učinkov gnojenja z mineralimi gnojili, starosti posevka in pogostosti plevelov. Na osnovi rezultatov lahko zaključimo, da lahko uporaba mineralnih gnojil na primeren način znatno zmanjša količino plevelov in, da je potrebno posevek lucerne zamenjati po štirih letih, ker njegova donosnost potem obdobju znatno upade, poveča pa se zapleveljenost.

Ključne besede: lucerna; upravljanje plevelov; krmne rastline

1 INTRODUCTION

Among all of the forage crops, alfalfa is considered to be one of the most important one due to being source of proteins, minerals, particularly vitamin A (Raoofi et al., 2014; Karimi, 2007) and also due to its verified role in improving soil structure as well as multiplier of other ecological functions and its unique ability to grow in semiarid areas (Gu et al., 2018; Zhao et al., 2004; Jefferson & Cutforth, 1997). Alfalfa's forage yield is entirely dependent on a variety of factors, like soil conditions, rainfall availability and soil moisture (Fan et al., 2016). This indicates its high water demand and also reflects its biological ability of nitrogen fixation (Shabani et al., 2017; Wang et al., 2018). Anyhow, one of the biggest challenges in alfalfa's yield productivity still remains the presence of weeds, which harshly compete with the main plant for sources of light, moisture and nutrients (Wilson, 1997). Besides of suppressing the overall alfalfa yield, weeds can also impact the densities of alfalfa stands (Becker et al., 1998). In many studies it has been proven that weed interference with alfalfa also causes reduction in quality as well as yield quantity, decreasing its trade price for about 46 % (Boschetti et al., 1998; Wilson & Burgener, 2009; Riley & Bradley, 2014) and its overall density by 20-30 % (Temme et al., 1979), all this being followed by drastic reduction of alfalfa nutritional values (Doll,

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1986). Weeds also do interfere with alfalfa in other aspects, like reducing alfalfas' edibility (Marten et al., 1987), seedling efficiency (Fischer et al., 1988) and also altering the forage normal composition, increasing as a result the drying time (Doll, 1986).

It has been noted that in particular, alfalfa seedlings are vulnerable to weed competition, as they are not impetuous enough to compete with weeds, and this, as with many other crops, results in reduced yields (Zimdahl, 2004; Wilson, 1981).

Knowing all this, regrettably, weeds in forage crops have not had sufficient attention and this will surely lead to serious quality and quantity damages of alfalfa crop. Additionally, crop nutritional values for livestock feeding will also decrease, an issue that particularly in Kosovo has never been a subject of attention.

This study brings an insight of the weed effect and interference with alfalfa, identification of weed species and their distributional frequency before first, second and third harvest accordingly. Weeds direct affect alfalfa productivity, biomass levels and correlation of these values with alfalfa age, soil and ecological conditions of the given studied site.

2 MATERIAL AND METHODS

Weed survey on alfalfa land parcels was conducted during 2016, starting from April until the end of September. All of the recorded plant species (Annex 1) were identified based on Flora Europaea (Tutin et al., 1964 - 1980) and species naming has followed the Euro Med Checklist (Euro+Med, 2006) nomenclature. We have choosen three alfalfa plots (*Medicago sativa* L. em Vass. – K-22 'Kruševaćka' commercial cultivar) in the village of Zotaj (42º27.051 N, 021º06.854 E) – 20 km south from capital Prishtina. The site is characterized with continental climate, with a mean annual temperature of 10.5 ºC and mean monthly temperatures of 20.6 ºC (July) and –1.4 ºC (January). The mean annual precipitation from 1999 to 2016 was 590 mm, of which months with the highest amount of rainfall are May, October and November. We have analyzed in particular: weed species present, biomass at $1m^2$, alfalfa mass at $1m^2$, weed mass at $1m^2$, overall yield per land parcel as well as weed species composition in floristic terms. Plant material has been surveyd and collected in three different time perioids (first – April/May; second – June/July; third – August/September) – always just before the harvest.

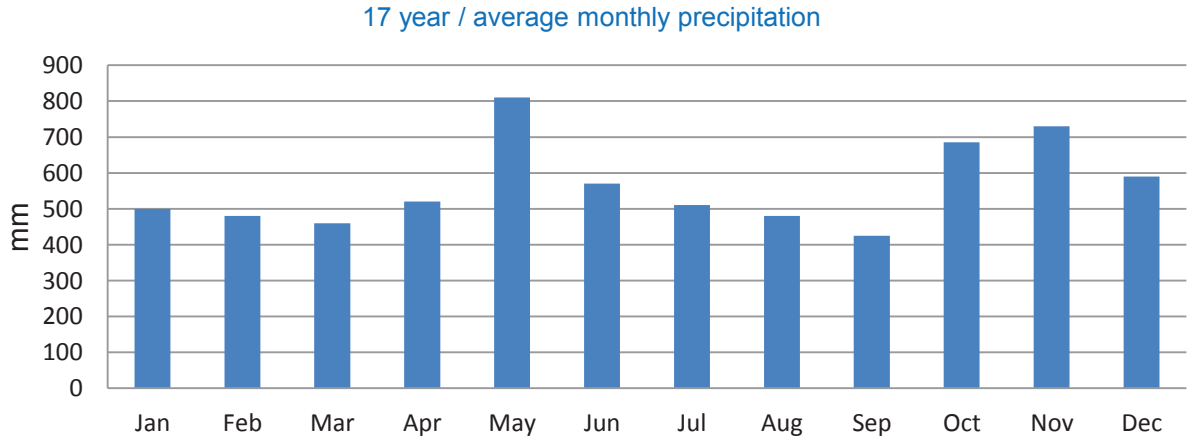

Figure 1: Rainfall data overview for a period of 17 years in Zotaj.

We have selected three alfalfa land parcels and on each of them we have made a total of ten measurements at $1m^2$ – repeatedly before first, second and third harvest. Parcel details are as following: Parcel 1 – sown with alfalfa 6 years ago, has not been treated with mineral fertilizers, previous agricultural crop was wheat, 240 m above sea level (a.s.l). Parcel 2 – sown with alfalfa 4 years ago, treated with mineral fertilizers, previously was ploughed land, 220 m a.s.l. Parcel 3 – sown with alfalfa 2 years ago, treated with mineral fertilizers, previously was ploughed land, 222 m a.s.l. All three parcels were really flat, so we could not indicate their exposition.

3 RESULTS AND DISCUSSION

Knowledge on abundance and distribution of weed species within a given landscape of an agroecosystem is a valuable goal for weed science. Abundance and distribution as measures of the number of individuals in an area and a measure of the geographical range of a weed species accordingly are essential units in this context. The study of weed population's abundance and distribution is helpful in determining how a population changes over time in response to discerning pressures applied by agronomic practices.

During our survey we continuously measured biomass level, alfalfa mass and weed mass at three selected plots of alfalfa and the obtained results are presented in three parts, according to the harvest. We have continually made ten relèves of $1m^2$ on each of the three parcels, at three different pre-harvest periods.

3.1 First preharvest

During the first preharvest period, we made our survey from 27th until the 29th of May 2016. Parcel one (alfalfa 6 years old), after ten relèves had on average 22.5 % weeds and 77.5 % alfalfa. Parcel two (alfalfa 4 years old), after ten relèves had on average 13.8 % weeds and 86.2 % alfalfa. Parcel three (alfalfa 2 years old), after ten relèves had on average 8.2 % weeds and 91.8 % alfalfa. Biomass production at the pre-first harvest period was as following: 1st parcel: 8.2 kg m^{-2}, 2nd parcel: 9.1 kg m^{-2}, 3rd parcel: 11.07 kg m^{-2} (Figure 2).

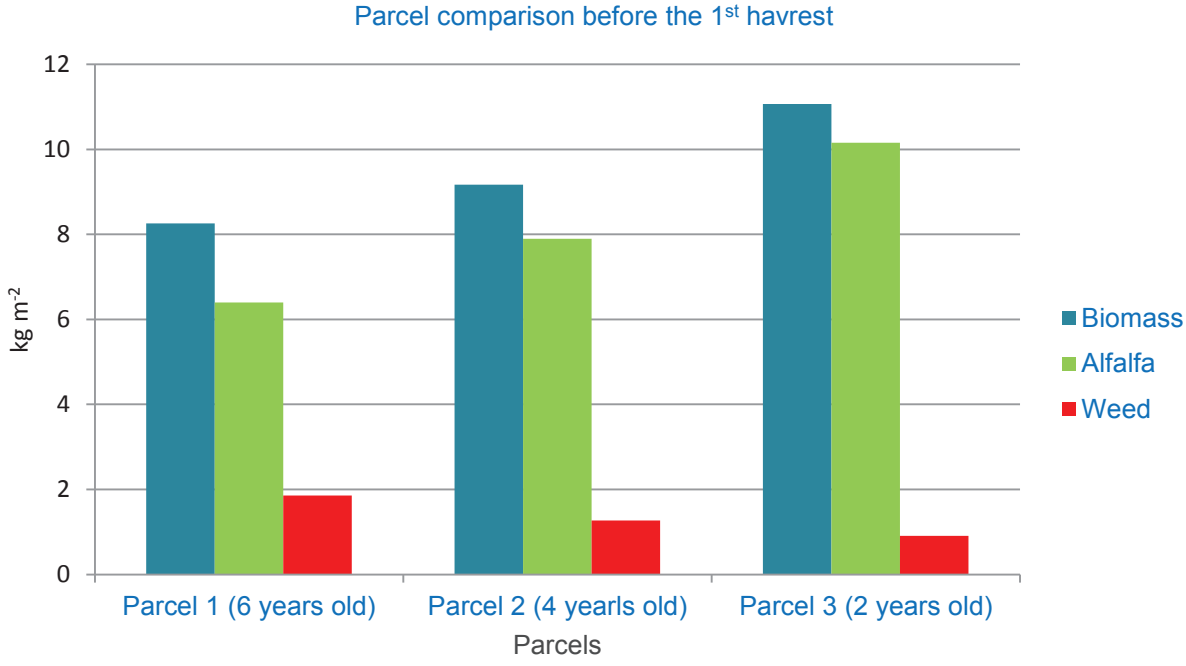

Figure 2: Parcel productivity comparison before the first harvest (kg m^{-2}).

3.2 Second preharvest

During the second preharvest period, we made our survey from 10th until the 12th of July 2016. Parcel one (alfalfa 6 years old) after ten relèves had on average 21.5 % weeds and 78.5 % alfalfa. Parcel two (alfalfa 4 years old), after ten relèves had on average 12.1 % weeds and 87.9 % alfalfa. Parcel three (alfalfa 2 years old), after ten relèves had on average 7.5 % weeds and 92.5 % alfalfa. Biomass production at the second preharvest period was as following: 1st parcel: 7.6 kg m^{-2}, 2nd parcel: 8.2 kg m^{-2}, 3rd parcel: 10.2 kg m^{-2} (Figure 3).

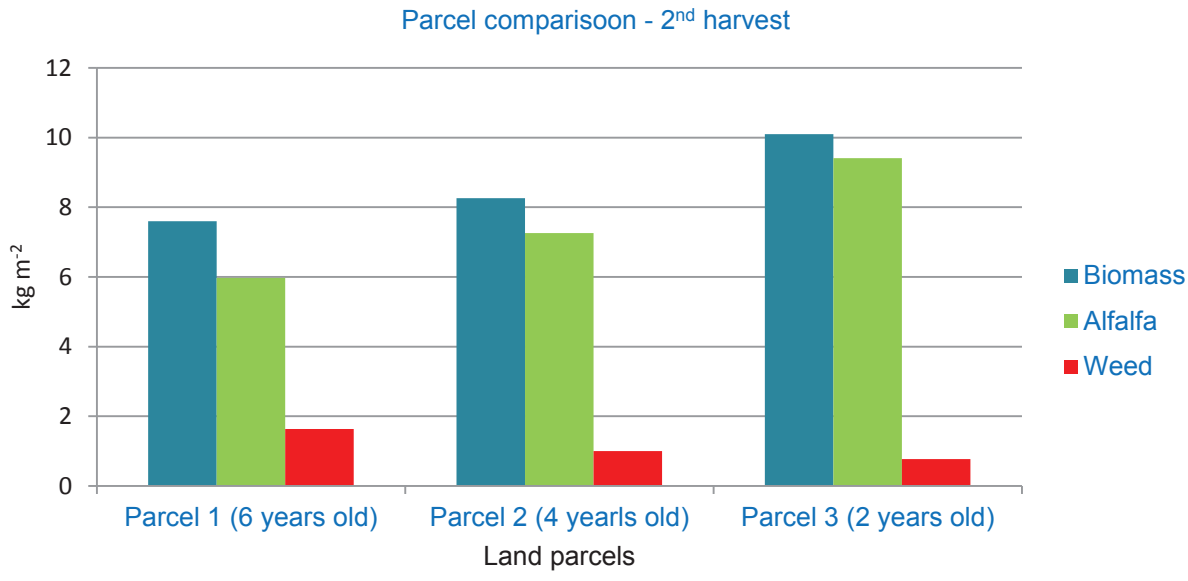

Figure 3: Parcel productivity comparison before the second harvest (kg m^{-2}).

3.3 Third preharvest

During the third preharvest period, we made our survey from 13th until the 15th of September 2016. Parcel one (alfalfa 6 years old), after ten relèves had on average 17 % weeds and 83 % alfalfa. Parcel two (alfalfa 4 years old), after ten relèves had on average 9.8 % weeds and 90.2 % alfalfa. Parcel three (alfalfa 2 years old), after ten relèves had on average 6.05 % weeds and 93.9 % alfalfa. Biomass production at the prethird harvest period was: 1st parcel: 5.8 kg m^{-2}, 2nd parcel: 6.4 kg m^{-2}, 3rd parcel: 8.7 kg m^{-2} (Figure 4).

Significant differences between parcels and preharvest times have been observed (Table 1) regarding the weed species composition, their distribution frequency and the distribution patterns as well as overall yield of alfalfa. Regarding the weed species present, significant differences have been observed between parcel one and parcel three, the same applies to their distribution frequency – and their frequency and presence was higher in the first preharvest time, compared to the third preharvest time..

Table 1: ANOVA statistical table

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	12.6	2	6.3	0.50	0.03	2.2
Within Groups	261.6	21	12.4			
Total	274.3	23				

Differences were also observed in the following aspects: there was much higher productivity of alfalfa in the first preharvest and a drastic reduction in the third preharvest. We have also noticed that the amount and frequency of weeds directly affects the reduction of alfalfa yield.

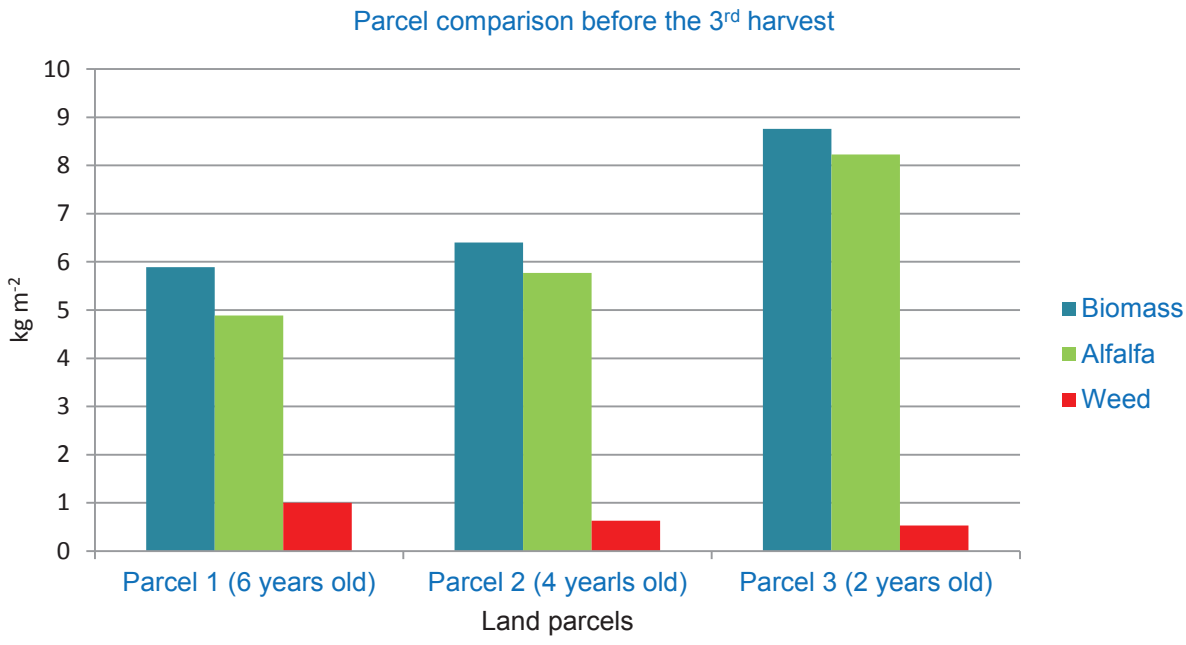

Figure 4: Parcel productivity comparison before the third harvest (kg m^{-2})

It was observed that the number and frequency of weed distribution is directly related to the age of alfalfa stands. As older the alfalfa stand is, the greater will be the number of weeds on that given parcel. The role of mineral fertilizers is also important to be noted here, as it has been observed that alfalfa parcels that were not treated with fertilizers, the frequency of weeds vas much higher.

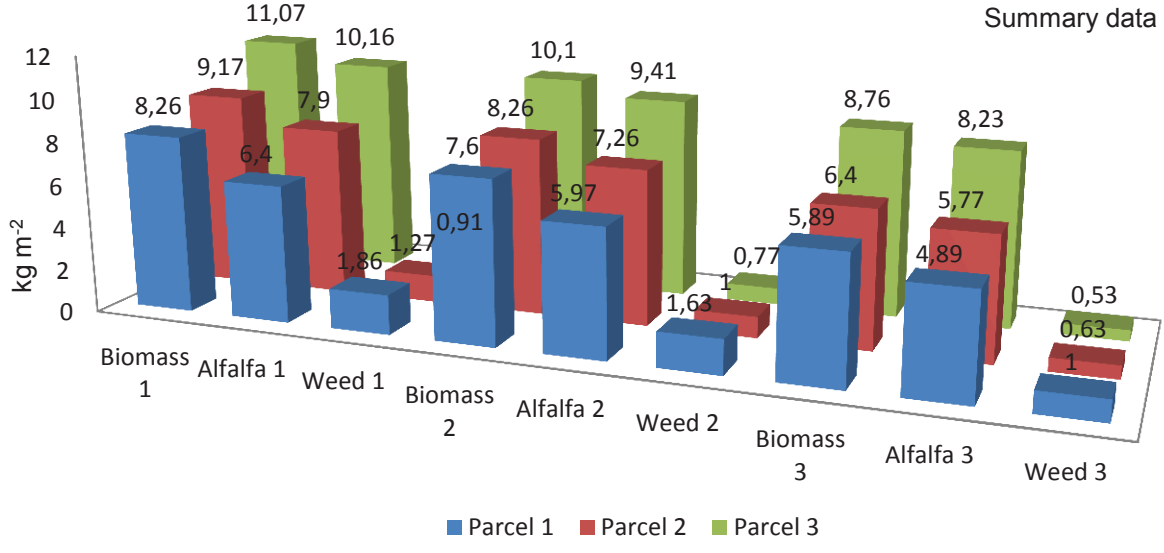

Figure 5: Summary chart of biomass, alfalfa and weed data for all three parcels during three (1, 2, 3) preharvesting periods

4 CONCLUSIONS

Weed frequency is higher during the spring months due to the favorable ecological conditions such as sufficient humidity, solar radiation and biology of weed species. Higher alfalfa yield is expected during the four years from sowingand afterwards a yearly based decrease on yield will be noticed. During the all three pre-harvest periods, the number and frequency of weeds was always higher at the parcel one (alfalfa 6 years old), compared to parcels two and three. Alfalfa productivity was higher at parcel three (alfalfa 2 years old) compared to parcels two and especially parcel one. Predominant weed plant families were: Poaceae (1st pre-harvest), Fabaceae (2nd pre-harvest) and Asteraceae (3rd pre-harvest) while less represented families were Caryophyllaceae (2 %) and Violaceae (1 %) – Figure 5. In total 71 plant species of weeds were recorded (Annex 1) in the surveyed plots. A correlation (Figure 5 & Figure 6) between the increased presence of weeds and the lack of mineral fertilizers has been noted. Biomass productivity was for 29.9 % higher at parcel three, compared to parcel one – which indicated that younger alfalfa is far more productive. As a general conclusion of this work we can state that based on the obtained results in our selected parcels, alfalfa is recommended to be cultivated for up to four years but no longer due to the optimal productivity rates.

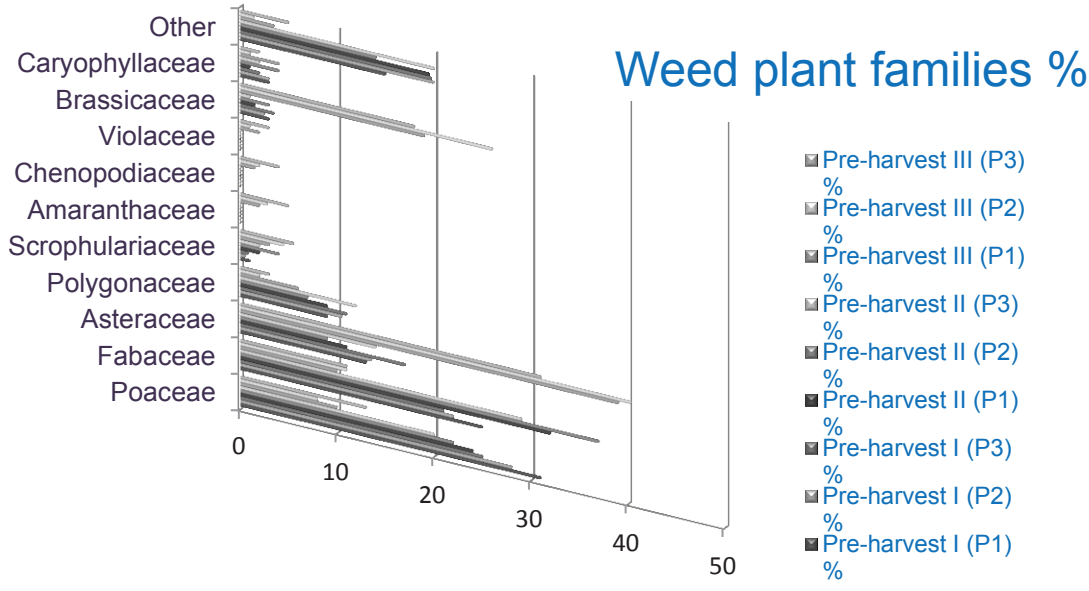

Figure 6: Plant families of weed species according to their pre-harvest time

Annex 1. List of registered weed plant species in three studied alfalfa parcels

Family	Species	Parcel 1			Parcel 2			Parcel 3		
Asteraceae	*Taraxacum officinale* Web.	+			+			+		
	Artemisia vulgaris L.	+		+	+			+		
	Anthemis arvensis L.	+					+			
	Erigeron canadensis (L.) Cronquist	+			+			+		
	Crepis capillaris (L.) Wallr.	+		+		+				
	Matricaria chamomilla L.	+			+			+		+
	Sonchus oleraceus L.	+			+			+		+
	Ambrosia artemisiifolia L.		+					+		
	Cirsium arvense (L.) Scop.	+			+					
	Achillea millefolium L.	+		+				+		
	Cichorium intybus L.		+							
	Galinsoga parviflora Cav.	+			+			+		
Poaceae	*Agropyrum repens* Beauv.				+					
	Bromus inermis Leyss.	+				+				
	Bromus sterilis L.	+			+					
	Cynodon dactylon Pers.							+		
	Digitaria sanguinalis Scop.	+			+					
	Dactylis glomerata L.	+			+					
	Hordeum murinum L.	+					+			
	Lolium perenne L.		+		+					
	Poa annua L.	+			+					
	Poa trivialis L.	+			+			+		
	Poa pratensis L.	+								
	Triticum aestivum ssp. *aestivum* L.		+		+				+	
	Avena sativa L.				+					
	Bromus hordeaceus L.	+			+					
	Setaria viridis (L.) P. Beauv.	+		+	+					
	Festuca pratensis Huds.	+								
	Sorghum halepense (L.) Pers.	+				+				
Polygonaceae	*Persicaria lapathifolia* (L.) Del.		+							
	Rumex crispus L.	+			+					
	Fallopia convolvulus (L.) Á. Lö.	+		+			+			
	Polygonum aviculare L.				+					
Amaranthaceae	*Amaranthus retroflexus* L.		+		+			+		
	Beta vulgaris L.	+			+			+		
Convolvulaceae	*Convolvulus arvensis* L.	+		+		+		+		
Chenopodiaceae	*Chenopodium album* L.	+					+		+	
	Chenopodium hybridum L.	+			+					
Brassicaceae	*Capsella bursa-pastoris* (L.) Medik.	+		+	+	+			+	
	Myagrum perfoliatum L.	+			+					
	Barbarea vulgaris W.T. Aiton		+			+				+
Fabaceae	*Trifolium repens* L.	+			+			+		
	Trifolium pratense L.	+			+			+		
	Onobrychis arenaria (Kit.) DC.	+					+		+	
	Melilotus officinalis (L.) Pallas	+		+	+			+		
	Vicia lutea L.	+				+				
Caryophyllaceae	*Silene vulgaris* (Moench.) Garcke	+	+	+	+		+	+		
	Stellaria media (L.) Vill.	+		+	+			+		
	Silene alba Mill.	+		+	+				+	
Asparagaceae	*Hosta plantaginea* (Lam.) Asch.	+			+					
Plantaginaceae	*Plantago major* L.	+		+	+	+		+		
	Plantago lanceolata L.		+			+				+
	Veronica persica Poirr.	+			+			+		
	Veronica agrestis L.	+		+	+			+		
Geraniaceae	*Geranium sanguineum* L.		+			+				+
	Erodium cicutarium L.		+			+			+	
	Geranium dissectum L.	+	+		+		+	+		
Lamiaceae	*Stachys palustris* L.	+			+			+		
	Salvia nemorosa L.	+			+		+		+	
	Glechoma hederacea L.		+			+		+		
	Stachys annua L.	+			+			+		
Solanaceae	*Solanum nigrum* L.	+			+			+		
Malvaceae	*Malva sylvestris* L.	+			+		+	+		
	Hibiscus trionum L.		+		+					
Apiaceae	*Orlaya grandiflora* (L.) Hoffm.	+			+			+		
	Conium maculatum L.		+			+		+		
Verbenaceae	*Verbena officinalis* L.	+	+		+			+		
Papaveraceae	*Papaver rhoeas* L.	+			+			+		
Violaceae	*Viola sororia* Willd.		+			+				
Euphorbiaceae	*Euphorbia salicifolia* Host.			+					+	
	Euphorbia esula L.		+			+			+	
Total no. of species	**71**									

* Each column within a parcel indicates the pre-harvest period.

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Seed yield of two new quinoa (*Chenopodium quinoa* Willd.) breeding lines as affected by sowing date in Central Italy

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ABSTRACT

Research on the introduction of quinoa in Italy is currently lacking. The present research was aimed at identifying the correct sowing period. Field experiment was consucted in Cesa, Tuscany, in 2017. Two new breeding lines coded as DISPAA-Q42 and DISPAA-Q47-CB were utilized. Three sowing dates (SD) were implemented: February 23; March 17 and April 27. Results showed that the most successful SD was February 23. A significant decrease in both seed yield and a delay in phenological phases, relating to plant maturation and flowering was associated with the sequential delay in SD in both lines. Results also showed a significant effect of lines on yield, true-leaf stage development, flower development and maturity. Only DISPAA-Q42 was considered suitable for cultivation in the Tuscan environment. DISPAA-Q47-CB was the more susceptible line, due to the sequential delay in SD and delayed plant maturation. No effect between lines was evident for protein and saponin content. The present study clearly shows the potential for the successful cultivation of quinoa in Central Italy, and highlights the necessity of taking into consideration both breeding lines and SD in order to accomplish this goal.

Key words: Central Italy; *Chenopodium quinoa;* new breeding lines; quinoa; sowing date; Tuscany

IZVLEČEK

VPLIV DATUMA SETVE NA PRIDELEK SEMENA DVEH NOVIH LINIJ KINOJE (*Chenopodium quinoa* Willd.) V OSREDNJI ITALIJI

Raziskav o uvajanju kinoje v Italiji trenutno ni. Namen te raziskave je bil ugotoviti primeren čas setve. V ta namen je bil leta 2017 izveden poljski poskus v Cesi, Toskana. Uporabljeni sta bili dve novi žlahtniteljski liniji kinoje, 'DISPAA-Q42' in 'DISPAA-Q47-CB'. Setev (SD) je bila opravljena v treh terminih: 23 februarja; 17 marca in 27 aprila. Rezultati so pokazali, da je bila najuspešnejša setev 23 februarja. Pri obeh linijah je bil pri kasnejših terminih setve opazen značilen upad pridelka in zastoj v fenoloških fazah kot sta cvetenje in zorjenje rastlin. Rezultati so pokazal značilni učinek linije na pridelek, razvoj pravih zelenih listov, cvetenje in zrelost. Samo linija DISPAA-Q42 se je izkazala primerna za gojenje v okoljskih razmerah Toskane. Linija DISPAA-Q47-CB je bila bolj občutljiva na kasnejšo setev zaradi zakasnelega zorjenja rastlin. Med obema linijama ni bilo nobenih razlik v vsebnosti beljakovin in saponinov. Raziskava jasno nakazuje potencial uspešnega gojenja kinoje v osrednji Italiji in poudarja potrebo po upoštevanju tako žlahtniteljskih linij kinoje kot časa setve za doseganje zastavljenih ciljev.

Ključne besede: osrednja Italija; *Chenopodium quinoa;* nove žlahtniteljske linije; kinoja; datum setve; Toskana

1 INTRODUCTION

The nutritional qualities of quinoa (*Chenopodium quinoa* Willd.), rich in both proteins and essential amino acids, together with its suitability for use by people with celiac disease, has resulted in an increased worldwide demand for food products. Among the world markets, the European market has registered the greatest increase. The Italian market for gluten-free products currently ranks second in the world, with a shares of 13 % corresponding to an annual turnover of approximately 145 million Euros (Euromonitor International, 2015). Although there are no official data, it was estimated, in 2015, that Italy imported approximately 2.5 % of the world production in quinoa, an equivalent of 2500 t. In addition to the alimentary

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benefits (De Feo et al., 1997; Repo-Cardoso et al., 2003), the potential introduction of quinoa as an alternative crop has attracted the attention of farmers internationally, even within areas outside the geographical origin of this species. This is especially evident for temperate environments.

The concept of introducing quinoa in Italy originated in the early twentieth century, in view of the excellent nutritional properties already recognized (Racah, 1917; Anonymous, 1936; Maugini, 1936; Massa, 1936). However, the actual introduction of quinoa in Italy (approximately 500 ha) occurred more recently. Noteworthy, this introduction was performed in a disorganized manner, in that the preliminary phase of experimentation, necessary to identify both suitable agronomic varieties and cultivation techniques, was lacking. Initially, it was naively thought that it was merely sufficient to introduce the varieties in Italy. However, as could have been predicted scientifically (Christiansen et al., 2010; Bendevis et al., 2014), there were problems relating to photoperiod adaptation. The second phase of quinoa introduction in Italy involved the introduction of varieties established in Europe such as the 'Titicaca', 'Puno', 'Vikinga', 'Atlas', 'Pasto' and 'Rio Bamba'. Nonetheless, the biggest problems facing cultivation included the lack of adaptability to photoperiod, maturation difficulties, and a decrease in quality (Casini and Fabbrini, 2017). The introduction of quinoa in Italy could have had interesting prospects for farmers from the economic point of view. Farmers, due to the international quotations of common cereals, which are presently at minimum levels, are currently looking for valid alternatives.

Since the 1980s, various European countries have been conducting research on the cultivation of quinoa by exploiting the existing genetic variability (Jacobsen 1997, 2015). However, research in Italy has been limited (Casini, 1997, 2002; Casini and Proietti, 2002; Pulvento et al., 2010, 2012; De Santis et al., 2011, 2014, 2016; Lavini et al., 2014).

The first research project conducted in Central Italy (Tuscany) dates back to 1999, with the University of Florence as the national coordinator of the FAO-UNA-PERU project entitled "American and European test of quinoa (*Chenopodium quinoa*)" (Mujica et al., 2001). The research stressed how photoperiod sensitivity rendered the genotypes derived from northern areas of the Andean Altiplano (mostly from Bolivia and Peru), unsuitable for introduction in the Mediterranean environments. Moreover, only few of the twenty five accessions reached physiological maturation, with the highest grain production attained by 'E-DK-4, BAER II' and '02-Embrapa' (2.8, 0.9 and 1.1 t ha^{-1}, respectively). However, the results of the study were incomplete, and it was still necessary to address the problems facing the cultivation of quinoa. In fact, the identification of the most suitable sowing date is one of the most important agronomic aspects that needs to be taken into consideration for the successful cultivation of quinoa. The potential adaptation of this species to photoperiods, differing from that existing in the areas of origin, depends largely on an ecotype classification of varieties within the species. For example, the varieties of Chilean origin classified as "sea-level-type" are more easily adaptable to temperate environments, such as that of the Mediterranean areas (Wilson, 1990).

The only results published to date were those carried out in Italy (province of Caserta), whereby the period March-May was shown to be the most suitable sowing period (Pulvento et al., 2010; Lavini et al., 2014; De Santis et al., 2014). The only existing comparison between different sowing dates (Lavini et al., 2014), showed a considerable yield reduction of approximately 55 %, when the sowing date was delayed by one month in the period April-May. Therefore, it is evident that the potential for successful cultivation of quinoa in Italy necessitates further research.

The aim of the present study was to identify the most suitable sowing period for quinoa in the lowland areas of Central Italy. Moreover, the aim was also to assess whether two new varieties, selected on-site were suitable for cultivation and how this suitability may have been affected by sowing date. Suitability for cultivation was assessed, not only by examining effect of line and sowing period on the yield, but also on two biochemical parameters, namely protein and saponin content. Increased protein content is an important nutritional characteristic of quinoa, whereas reduced saponin content is a required technological aspect.

2 MATERIALS AND METHODS

The field experiment was carried out in Tuscany, Central Italy, in 2017 at the "Centro per il Collaudo ed il Trasferimento dell'Innovazione di Cesa (Arezzo)", 43° 18' N; 11° 47' E; 242 m a.s.l. The cultivation environment was comprised of a neutral, loamy-sandy soil. The principle physical and chemical characteristics of the soil were as follows: sand 36.0 %, loam 38.1 %, and clay 25.9 % respectively. The soil pH was 7.0. Total N was 0.110 % and P (Olsen) 13 ppm. Exchangeable Ca, Mg and K were 4123, 595 and 141 ppm,

respectively. Two new breeding lines, obtained by the University of Florence, in the same area of the experiment during 2010-2017, were used in the present research, and coded, ‘’DISPAA-Q42’ and ‘DISPAA-Q47-CB’. The lines were derived from two series of poly-crosses between Chilean "sea-level-type" genotypes that were selected based on photo-period adaptability, early-ripening and plant architecture according to the following ideotype defined by Donini (1997): maximum plant height of approximately 1.3 m, with no ramifications; early-ripening, and > 2.0 g mass of 1000 seeds. Based on previous observations (unpublished work), the autumn-winter sowing periods were not included due to serious damage induced by low temperatures. As a result, the sowing dates ranged from late winter to spring. Plots were arranged, according to a RCB split-plot design with three replicates. The size of the overall plot was 15.0 x 4.0 m, which constituted the main factor comprising line (‘DISPAA-Q42’ and ‘DISPAA-Q47-CB’), while the subplots constituted three different sowing dates (SD) as follows: February 23; March 17 and April 27 (hereon referred to as first, second and third SD). Each subplot had a width of 2.0 m (four rows wide with 0.5 m row spacing) and a length of 5.0 m. The sampling area was comprised of the two central rows only. A seed quantity of 30 kg ha^{-1} was used. In order to attain the correct planting density of 15 plants m^{-2}, seedlings were thinned at the two-true leaf stage. Fertilizer treatment before seeding was as follows: 76 kg ha^{-1} of N as ammonium nitrate, and 100 kg ha^{-1} of P_2O_5 as superphosphate. Plots were hand-weeded twice (35 and 55 Days After Emergence [DAE]) during the growth cycle. Due to the early onset of flea beetle (*Chaetocnema tibialis* (Illiger, 1807)), 10-15 DAE at all sowing dates, the seedlings were treated with the insecticide, deltamethrine (50 ml 100 l $water^{-1}$). The following field measurements were recorded: emergence of the 2-, 4-, 6- and 10- true-leaf stages; early panicle appearance; full panicle appearance; early flowering; waxy maturation and maturation at 75 %. For the maturation stage, both total leaf loss and seed consistency were taken in consideration together with complete filling (non-translucent endosperm).

Plant height was measured for each phenological stage, using a total of 10 plants per sample plot. Corresponding to the 10-true leaf stage, before the appearance and formation of the panicle, downy mildew (*Peronospora farinose* f. sp. *chenopodii* Fr.) was observed on the basal leaves of the plant. Sensitivity to the pathogen was estimated according the scale proposed by Inguilàn and Pantoja (2007). This scale takes into consideration the surface area percentage of the leaf showing disease symptoms. No specific treatment was applied.

The harvest was performed manually starting from July 7 to September 7, 2017. The duration of maturation was dependent on both the date of sowing and the line. As a result, the different plots of all replicates were harvested accordingly.

After drying the seeds to a standard humidity of 12 %, (airflow at 35 °C for 48 h), the yield calculations were performed. A sample from a seed batch was used to determine the mass of 1000 seeds. The saponin content was measured according to Koziol (1991). Total protein was determined from the N content (N x 6.25) using an Elemental Analyser EA FLASH 1112 of Therma Fisher Scientific. Climatic data was obtained from the meteorological station near the experimental site. Day length records were provided by “Centro Interdipartimentale di Bioclimatologia-CIBIC” (University of Florence). Cumulative Growing Degree Days (GDD) were recorded from the date of the first sowing period (February 23) to the last harvest period (September 9) with a T_z equal to 3 °C (Jacobsen and Bach, 1998) as follows:

T_m is the daily mean temperature:

$$GDD = \sum_{days} (Tm - Tz)$$

Cumulative Total Solar Radiation (TSR) recorded during the trial was provided by the “Centro Funzionale Regione Toscana” which uses an ETG Agrometeotological Station. Differences between response variables were assessed with COSTAT 6.45 software. Statistical differences were tested at $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$. The Tukey’s HSD test was used to evidence significant differences between means and homogenous groups.

3 RESULTS AND DISCUSSION

Given that photoperiod and climatic conditions are imperative to the potential success of quinoa cultivation in Central Italy, it was important to consider this information during the experimental trial. The climatic data shown in Figure 1, indicated high temperatures recorded throughout the crop cycle. In particular, maximum temperatures exceeding 30 °C were recorded during mid-June to mid-September. Another noteworthy characteristic was the thermal variability, especially between June and August, where temperatures oscillated between 15 and 20 °C.

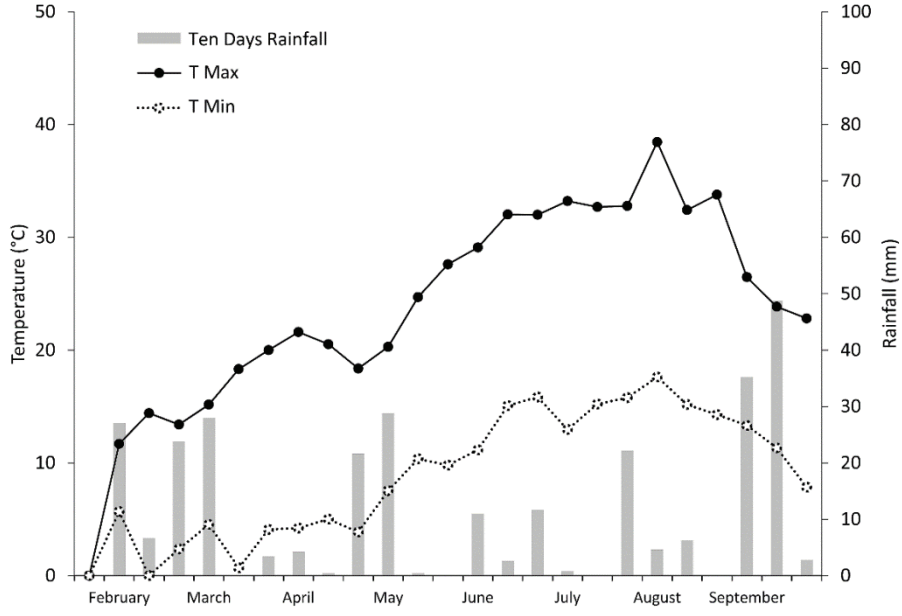

Figure 1: Temperature and rainfall recorded during the field experiment

The photoperiod and GDD trend are shown in Figure 2. From the first sowing date up until 200 DAE, approximately 2700 °C were accumulated and photoperiod increased until 110 DAE.

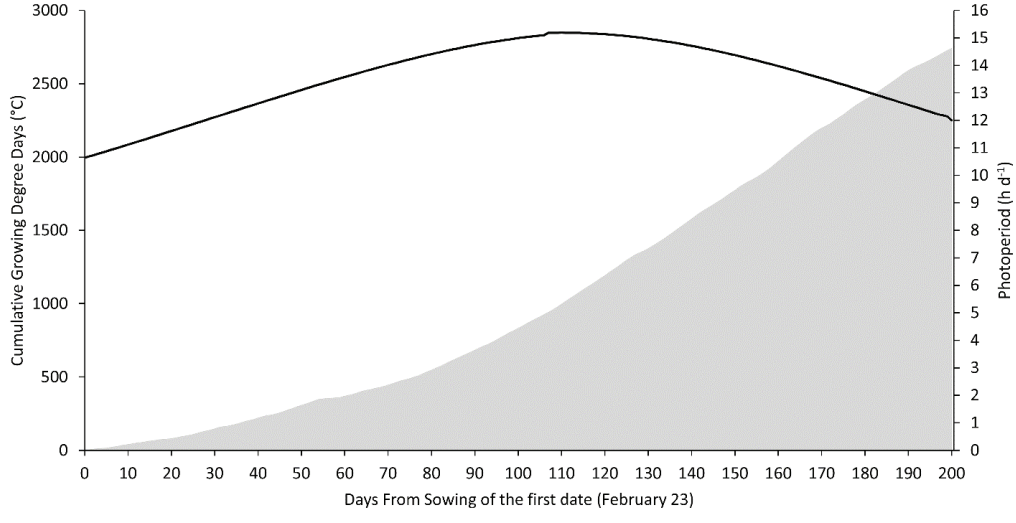

Figure 2: Cumulative Growing Degree Days (GDD) and day-length recorded during the field experiment

The analysis of variance was conducted to verify whether line, sowing date and "line x sowing date" were factors influencing yield, as well as various phenological and biochemical parameters of relevance to this crop. Results (Table 1) highlight the significant effect of line on yield, true-leaf stage development, flower development and maturity. In contrast, no effect was shown for emergence date, waxy maturation, saponins and proteins content. The effect of sowing date was significant for all parameters analyzed with the exception of the emergence date (Table 1). Excluding the 10-true leaf stage and saponin content, the interaction "line x sowing date" produced significant effects for all variables considered.

Table 1: Analysis of variance.

Source of variation	DF	Yield	Plant height	Emergence	Two true leaves	Four true leaves	Six true leaves	Ten true leaves	
Blocks	2	7.011	70.333	0.777	30.333*	7.444	8.444*	3.000	
Variety (V)	1	3.591**	953.388*	12.500	37.555*	144.512**	470.220***	107.556*	
Error	2	0.001	53.444	4.333	0.777	2.333	0.444	10.778	
Date of sowing (DS)	2	4.453**	11023.000**	80.111	312.333***	266.788***	843.111***	3710.333***	
DS x V	2	1.641**	1946.777**	3.000***	14.777**	110.334***	123.111***	5.444	
Error	8	0.055	147.555	15.555	4.222	15.566	20.445	24.889	
Source of variation	DF	First panicle appearance	Panicle	Early flowering	Waxy maturation	Maturation	Saponins	Proteins	1000 seeds weight
Blocks	2	2.111	1.444	4.777	9.000	11.444	0.072	0.204	0.088
Variety (V)	1	72.000**	102.722*	186.889**	5.555	401.388*	0.049	1.069	0.103*
Error	2	1.000	5.444	0.777	1.444	8.777	0.050	0.308	0.007
Date of sowing (DS)	2	3468.122***	2268.778***	2277.445***	764.333***	5633.777***	0.687***	23.207***	0.275***
DS x V	2	22.334***	24.777**	25.444**	38.111**	43.111**	0.059	5.643***	0.217
Error	8	3.555	9.767	9.112	15.556	16.444	0.099	0.183	0.015

*: significant at $p \leq 0.05$; **: significant at $p \leq 0.01$; ***: significant at $p \leq 0.001$.

Table 2 shows the number of days elapsing from the emergence date until the first appearance of the panicle, flowering, maturation and the respective duration of the photoperiod, besides GDD and the cumulative TSR. Generally, the number of days required for the appearance of the panicle and flowering date decreased significantly from the first to the third SD. In contrast, as regards maturation, the inverse trend was recorded.

Table 2: Main growth stages, day lenght, Growing Degree Days (GDD) and Total Solar Radiation (TSR) from emergence to flowering and from flowering to maturity.

Variety	Sowing date	First panicle appearance (DAE)[1]	Flowering date (DAE)	Maturation date (DAE)	Day length from emergence to flowering (h)	Cumulative GDD[2] from emergence to flowering (°C)	Cumulative TSR[3] from emergence to flowering (Mj m^{-2})	Day length from flowering to maturation (h)	Cumulative GDD from flowering to maturation (°C)	Cumulative TSR from flowering to maturation (Mj m^{-2})
DISPAA-Q42	February 23	79 b	94 ab	148 cd	11.3 - 15.2	907	1743.40	14.9 - 14.4	995	1679.78
	March 17	63 c	84 c	170 bc	12.6 - 15.1	1054	2058.35	14.6 - 13.5	1597	2275.38
	April 27	50 d	70 cd	196 a	14.1 - 14.6	1166	2095.12	14.0 - 12.2	2232	3554.80
DISPAA-Q47-CB	February 23	89 a	100 a	142 cd	11.3 - 15.2	1023	1934.64	15.0 -14.6	788	1289.33
	March 17	77 bc	94 ab	162 c	12.6 - 14.9	1231	2335.99	14.9 - 13.8	1282	1700.89
	April 27	60 cd	72 cd	180 b	14.1 - 14.6	1200	2162.61	14.1 - 12.6	1926	3119.12

Means followed by the same letter(s) are not different for $p \leq 0.05$ according to Tukey test.
[1] DAE: Days After Emergence.
[2] GDD: Growing Degree Days.
[3] TSR: Total Solar Radiadion.

Of note, for 'DISPAA-Q42', a significant difference in the number of DAE, culminating in the appearance of the panicle, was detected for each of the three respective sowing dates (ranging from 79 to 50 DAE). For 'DISPAA-Q47-CB', a significance difference was observed only for the first sowing date. Similarly, for both varieties, the number of DAE until the flowering date decreased significantly from the first to third SD, respectively. For 'DISPAA-Q47-CB', an increased number of days until flowering were required and differences in both temperature and solar radiation were also required. The same conditions of increasing photoperiod (11.3-15.2 h) for the first and third sowing periods, higher values of both GDD and cumulative TSR were required by 'DISPAA-Q47-CB' in comparison to that for 'DISPAA-Q42' (Table 2). The TSR requirement for the first and third SD was approximately 200-300 Mj m^{-2} higher for 'DISPAA-Q47-CB'. These results confirmed those obtained in previous research (Bertero et al.,1999; Bertero, 2003; Hirich et al., 2014), showing that the response of quinoa to photoperiod is significantly affected by temperature. The current work corroborates the necessity of this type of preliminary research to identify both suitable agronomic varieties and cultivation techniques, which are lacking for the successful cultivation of quinoa in Central Italy.

Given that the two varieties vary in the level of precocity, maturation was attained under different photoperiod as well as GDD and TSR (Table 2). Corresponding to the first SD, plants were subjected to a constant photoperiod from flowering until maturation: 14.9-14.4 h for 'DISPAA-Q42' and 15.0-14.6 h for

'DISPAA-Q47-CB'. A decreasing photoperiod with a maximum difference of 1.5-1.8 h was evident for the first and third SD.

An increase in both GDD and TSR was necessary for the maturation of plants sown in March and April compared to plants sown in February. Varietal differences were also noted. For 'DISPAA-Q42', differences of 1237 °C and 1875 MJ m^{-2} between SD1 and SD3 were required. In contrast, for 'DISPAA-Q47-CB', differences of 1138 °C and 1829 MJ m^{-2} were required.

The total duration of the crop growth, expressed as days to ripening, is of utmost importance in attaining satisfactory seed yields. Delayed sowings can excessively prolong the life cycle of the plants, thereby either resulting in seed maturation after 150-180 DAE (Jacobsen, 1997) or by generating unripe seeds.

For the third SD, maturation occurred at 196 and 180 DAE for 'DISPAA-Q42' and 'DISPAA-Q47-CB', respectively, in comparison to 148 and 142 DAE at the first SD for 'DISPAA-Q42' and 'DISPAA-Q47-CB', respectively. This clearly shows the wastage in days associated with delaying the sowing date. Additionally, all phenological phases were strongly influenced by the sowing dates for both varieties (Figure 3).

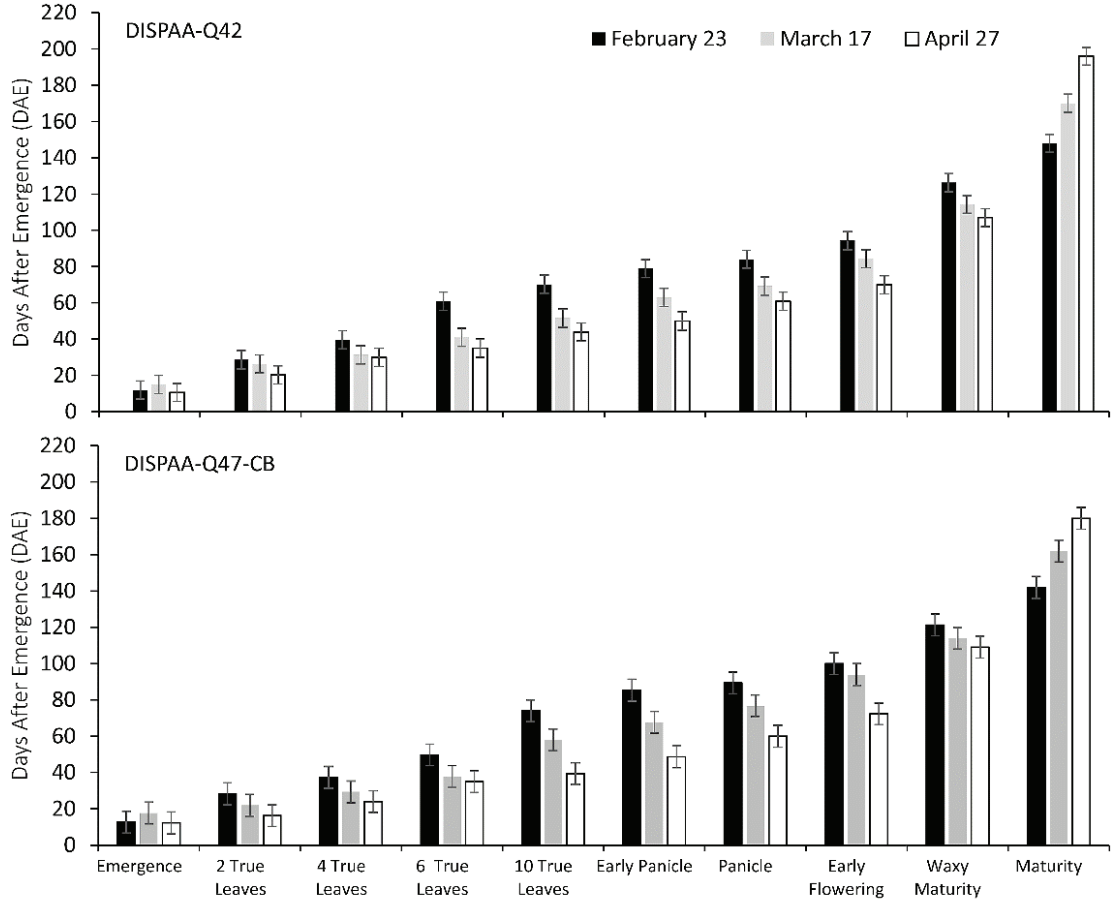

Figure 3: Date of the main phenological phases according to lines and sowing date. Error bars represent the interval of the variability of the Tukey test ($SD.q_{.95,2,8}$). If the bars do not overlap, the difference between averages is significant at $P \leq 0.05$.

When comparing the first SD and remaining two dates, differences became significant at the 10-true leaf stage. Particularly evident was the wastage of days for 'DISPAA-Q42', (18-20 d) that tended to decrease progressively proceeding towards waxy maturation. From this stage, the attainment of full maturation was

rapid for the first SD plants (54 d) and significantly longer for the third SD plants (124 d).
A similar response was observed in a different environment by de Vasconcelos et al. (2012). In the present experiment, plants of the late sowing date were exposed to long periods of high temperatures (> 30 °C) and marked drought (37 mm in the period June-August). If these climatic conditions reduced the time intervals of the main phenological stages proceeding from the first to the third sowing age, then the delay in maturation could be attributed to the reduced growth of the plants, more specifically, of the leaves. This response of quinoa contributes to maintaining a water balance that allow plants to survive water deficit conditions (Claeys and Inze, 2013). A smaller foliar, or assimilatory surface, may have resulted in a decreased seed-filling rate, and consequently a delay in full maturation.

For 'DISPAA-Q47-CB', a similar trend was observed. However, evident differences were found between the first and third SD, for the developmental phases between the 10-true leaf stage and the beginning of flowering. This amounted to a wastage of 20 d.

Risi and Galwey (1989) reported that time differences from emergence to panicle formation constitutes the first response of the plants to change in photoperiod. In the present study, from emergence until panicle formation, significant time differences were evident for the different sowing dates. Passing from increasing photoperiod (11.3-15.2 h), at the time of the first SD, to a stationary photoperiod (14.1-14.6 h), at the time of the third SD, the appearance of the panicle was delayed by 29 days for both varieties. Similar trends were reported for Chilean sea-level-type accessions cultivated in temperate environments in Argentina with photoperiods similar to that of the present experiment (Bertero and Ruiz, 2008). Of interest, even within the period between flowering and the very first anthesis, these varieties were shown to be very sensitive to photoperiod and GDD.

The developmental trend in plant height, shown in Figure 4, was significantly different for both lines and sowing date. Plant height was not different for the first and second SD until the 6-true leaf stage (attaining a height of 40 cm). From this phase onwards, plant growth of the second SD underwent a progressive reduction, which was maintained until maturation, quantifiable in 10 cm and 28 cm for 'DISPAA-Q42' and 'DISPAA-Q47-CB', respectively. The latter line was shown to be more susceptible to the delayed sowing. Plant height development in plants sown in April was significantly stunted (Figure 4), attaining just 30 cm at maturation. The present results corroborate those of other authors (Risi and Galwey, 1991; Vasconcelos et al., 2012). Moreover, those authors also showed that an improved plant development was positively correlated to seed yield. This was also evident in the present study. The same figure shows that both varieties were affected by downy mildew from the 10-true leaf stage. Only the basal leaves were affected by the disease. According to the classification of Inguilàn and Pantojia (2007), corresponding to the state of resistance-tolerance to the pathogen, results of the present study showed a gradation of symptoms that ranged between 1 and 2 (1-25 % of basal leaves affected).

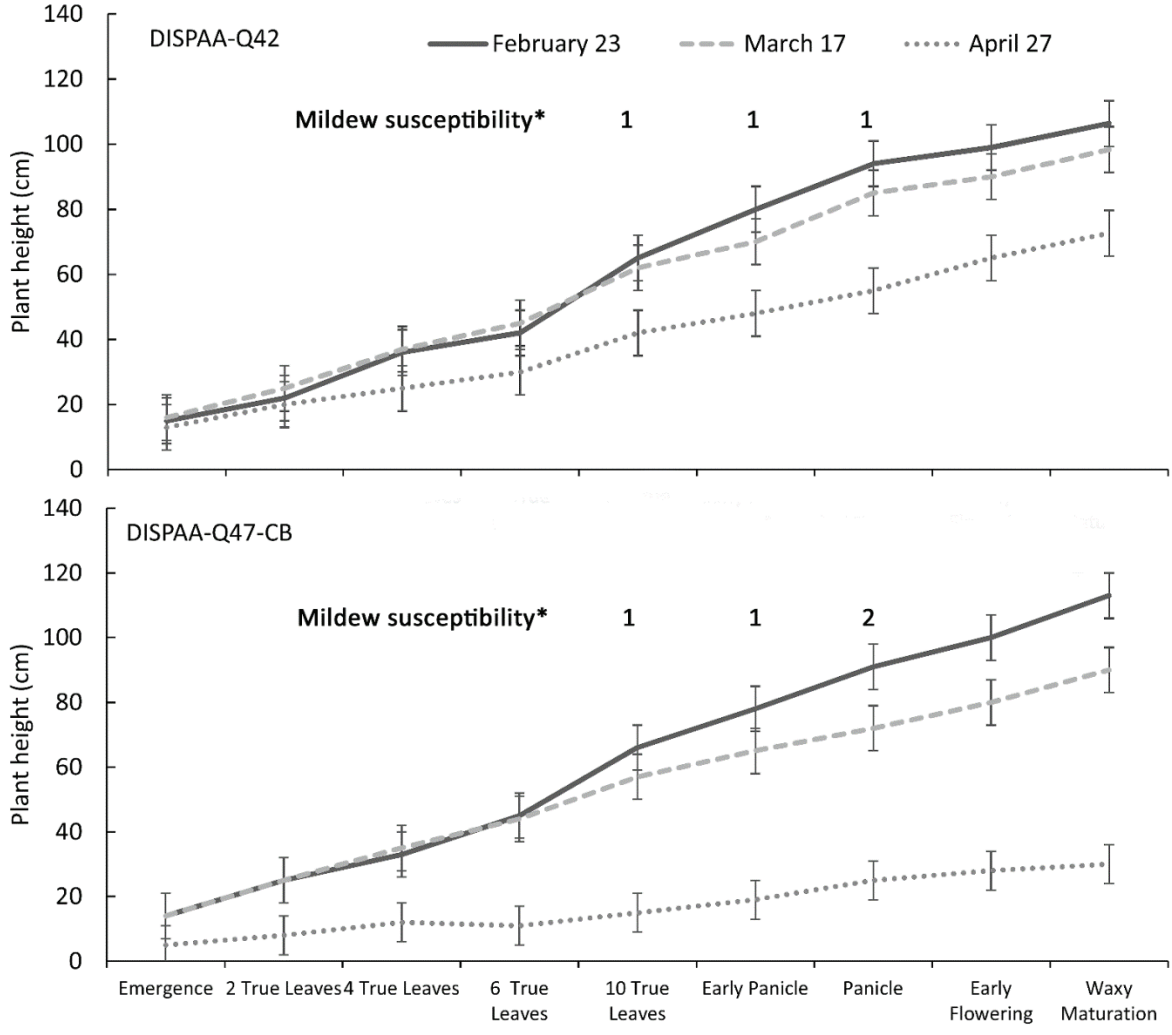

Figure 4: Trend of plant height according to line and sowing date. . Error bars represent the interval of the variability of the Tukey test ($SD.q_{.95,2,8}$). If the bars do not overlap, the difference between averages is significant at $P \leq 0.05$. *: numbers refers to the mildew susceptibility estimation according to Inguilàn and Pontoja (2007).

Figure 5 shows the seed yield of the two breeding lines. It is apparent that 'DISPAA-Q47-CB' is significantly less productive than 'DISPAA-Q42', with a maximum yield of 0.5 t ha^{-1} recorded for plants sown in February. However, of interest, this line was less sensitive to the delay in sowing of 22 d (March) with a limited reduction in the yield, equivalent to 10 %. 'DISPAA-Q42' was clearly the more productive line. Yields amounted to 2.0 t ha^{-1} for plants sown in February. However, seed yield was reduced by 25 % with the delay in sowing of 22 d (March). Both breeding lines produced negligible yields for the third SD, in which maturation occurred over 180 DAE. The yields of the first two SD of 'DISPAA-Q42' can be considered to be of a good standard compared to other varieties obtained after spring sowing in Italy (Pulvento et al., 2010; Lavini et al., 2014). In the latter studies, using a slightly higher sowing density (20 plants m^{-2}) and with cover nitrogen fertilization, the varieties, 'Titicaca' and 'Regalona', in addition to various genotypes of different origins, attained excellent yields of 2.3-3.6 t ha^{-1}.

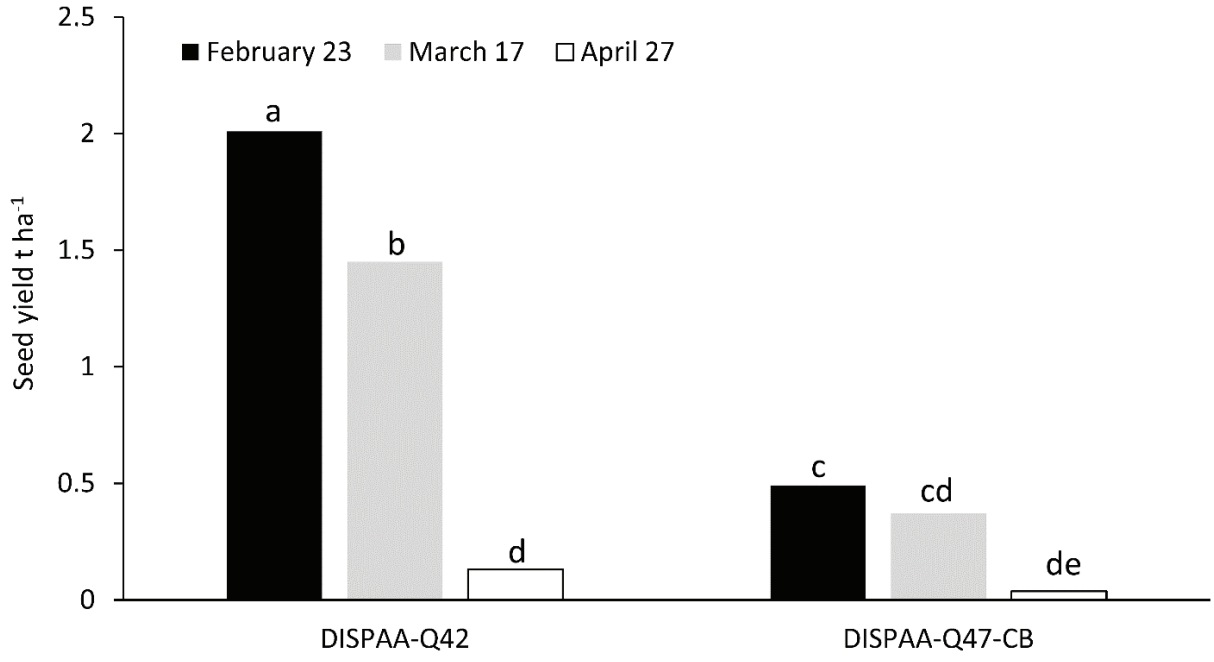

Figure 5: Seed yield of the varieties according to sowing date. Means within columns followed by same letter(s) are not different for $P \leq 0.05$ for the Tukey test

The significant decrease in production, associated with the sequential delay in sowing, can be ascribed to different factors. Above all, two factors appear relevant. Firstly, the growth of the plants (from emergence to flowering) sown in February and March occurred under conditions of increasing photoperiod 11.3-15.2 h and 12.6-14.9 h, respectively. Secondly, from flowering to the very first seed development in plants sown in March, high temperatures accompanied by low rainfall were registered. Negative effects on seed production attributable to climatic events were also found by Bertero (2003). The yield and plant height data at harvest confirmed the positive correlation highlighted by Vasconcelos et al. (2012). In our experiment, the correlation was significant (R^2 = 0,624**; Y = 49,81+106,87x- 40,49x^2).

Among the qualitative aspects of the seeds reported in Table 3, significant differences between the varieties were recorded for the mass of 1000 seeds. With the exception of saponins, the interaction "line x sowing date" generated significant differences at $P \leq 0.001$. The mass of 1000 seeds was on average below 2.0 g and decreased by 17 % from the first to second SD.

Table 3: Some seed quality characteristics as affected by sowing dates

Source of variation	Saponin (mg g^{-1} fresh mass)	Protein (%)	1000 seeds mass (g)
Variety (V)			
DISPAA-Q42	0.249	18.2	1.67
DISPAA-Q47-CB	0.353	17.7	1.52
f	*ns*	*ns*	*
Date of sowing (DS)			
February 23	0.166 b	16.1 c	1.72
March 17	0.577 a	17.8 b	1.64
April 17	0.1660 b	20.0 a	1.43
V x DS	*ns*	*ns*	***

Means followed by the same letter(s) are not different for P≤0.05 according to Tukey test.
*: significant at $p \leq 0.05$; **: significant at $p \leq 0.01$; ***: significant at $p \leq 0.001$;

The data of the present study was similar to that reported by Isobe et al. (2016), providing confirmation that Chilean varieties classified as "sea-level-type" are extremely sensitive to planting delay, leading to a general decrease in seed yield and a significant reduction in the ¨mass of 1000 seeds.

The protein content is an important characteristic of quinoa from an alimentary perspective. In addition, saponin content is an important technological aspect and it is essential that the saponins are either completely removed or significantly reduced before commercialization of the product. A significant reduction in saponin content (-34.7 %) was only found in both varieties for the second SD. The saponin content was shown by De Santis et al. (2012), to be strongly influenced by environmental conditions. It could be hypothesized that this result was attributable to the high temperatures and low rainfall that occurred in the period immediately after flowering, similar to that observed by De Santis et al. (2011) for Italian environments. The average seed protein content increased significantly with the delay of sowing from 16.1 % to 20.0 %, and was significantly and positively correlated (R^2 = 0.928**) with the age of maturation.

As previously mentioned, saponin and protein content were unaffected by breeding line. However, given that only two varieties were utilized, more research is required in order to determine whether the selection for specific biochemical characteristics can be made from the best yielding varieties.

4 CONCLUSIONS

At the end of a seven-year genetic improvement process, these results permitted us to evaluate the adaptability of two new quinoa lines to the environment of Central Italy at different sowing dates, spanning a period from the end of winter to early spring. Although the experimentation was conducted over the course of a single year, results showed that of the two varieties were obtained from poly-crosses between Chilean "sea-level-type" lines, only one line 'DISPAA-Q42' can be considered suitable to the Tuscan environment with satisfactory yields. This study, therefore, highlights the importance of assessing varietal performance. Moreover, as anticipated photoperiod and radiation were important determinants of plant growth and yield.

The shortening of the phenological phases until the flowering in relation to photoperiod and increasing solar radiation, confirmed the research of Hirich et al. (2014). However, the present results are also in contrast with those of Hirich et al. (2014) and Jacobsen (1997), who claimed that the early maturation or early genotypes (bloom to anthesis) maintained the same trend throughout the reproductive cycle. The lack of adaptability of 'DISPAA-Q47-CB', as well as the reduced production of seed, also manifested itself in terms of a strong reduction in the growth of plants. The significant yield reduction, corresponding to the March sowing period can be ascribed to the high temperatures and to the dry conditions occurring coinciding with bloom and anthesis. The spread of mildew was not evident, due to the hot and dry environmental conditions. The plants reacted to the presence of the mildew with an early filloptosis of the basal leaves affected. The potential repercussions of the fungus on the yield were not assessed by the present work. It was noted that at full formation of the panicle, 'DISPAA-Q47-CB' appeared more sensitive to the mildew compared to 'DISPAA-Q42'.

The accomplishment of the poly-crosses resulted in the production of at least one line, that appears to be well adapted to the environment of Central Italy notwithstanding the elevated average temperatures and prolonged drought that occurred between the complete emergence of the panicle and the milky maturation. Additionally, February was shown to be the most suitable sowing date.

Before reaching a definitive decision on the suitability of 'DISPAA-Q42', further experimentation is required to determine the performance in different environments and sowing densities. Based on small-scale experiments conducted this year (unpublished results) and from the literature (Risi and Galaway, 1991; Nurse et al., 2016), the above mentioned agronomic aspects significantly influence the date of maturation and seed production.

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Evaluating the effect of sowing date and drought stress on morphological and functional characteristics of three genotypes of winter oilseed rape (*Brassica napus* L.)

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ABSTRACT

To assess the effects of drought stress and sowing date on phenological, morphological, and yield traits of three different cultivars of winter oilseed rape (*Brassica napus* L.), this study was conducted in research farm of Sarayan agricultural college- University of Birjand in 2016-2017 growing season. Experiment was conducted in a split-factorial based on the randomized complete block design with drought stress in the main plots and three sowing date (September 22, October 6, and October 22) along with three cultivars of canola ('Homolious', 'Hayola50', and 'DK7070CL') in the subplots in three replications. The results of analysis of variance and means comparison analysis showed significant and negative effect of drought stress on seed yield and biological yield traits of investigated cultivars of canola. The interaction effect of drought stress × sowing date × cultivar was only significant on leaf twisting trait at 1 % probability level. 'Homolious' was assigned as the most drought tolerance cultivar, based on SI, SSI, RDI, TOL, MP, STI, GMP, YI, YSI, and HARM drought tolerance indexes, whereas 'Hayola50' was assigned as most drought sensitive cultivar of oilseed rape.

Key words: canola; drought; sowing date; selection criteria; tolerance index

IZVLEČEK

OVREDNOTENJE UČINKA DATUMA SETVE IN SUŠNEGA STRESA NA MORFOLOŠKE IN FUNKCIONALNE LASTNOSTI TREH GENOTIPOV OZIMNE OLJNE OGRŠČICE (*Brassica napus* L.)

Za ovrednotenje učinka datuma setve in sušnega stresa na fenološke in morfološke lastnosti ter lastnosti pridelka treh sort ozimne oljne ogrščice (*Brassica napus* L.) je bila v rastni sezoni 2016-2017 izvedena raziskava na kmetijski šoli Sarayan, Univerze v Birjandu, Iran. Poskus je bil izveden kot popolni naključni bločni poskus z deljenkami, s sušnim stresom na glavnih ploskvah in tremi datumi setve na podploskvah (September 22, Oktober 6, and Oktober 22), s tremi sortami oljne ogrščice ('Homolious', 'Hayola50', and 'DK7070CL') s tremi ponovitvami. Rezultati analize variance in primerjava poprečij so pokazali značilne negativne učinke sušnega stresa na pridelek semen in parametre biološkega pridelka vseh preučevanih sort oljne ogrščice. Medsebojni učinek sušnega stres, datuma setve in sorte je bil značilen samo za lastnost zvijanja listov pri 1 % verjetnosti. Sorta 'Homolious' je bila prepoznana kot na sušni stres najbolj odporna na osnovi parametrov kot so SI, SSI, RDI, TOL, MP, STI, GMP, YI, YSI in HARM-ov indeks tolerance na sušo, sorta 'Hayola50' je bila prepoznana kot na sušni stres najbolj občutljiva sorta oljne ogrščice.

Ključne besede: oljna ogrščica; suša; datum setve; selekcijski kriteriji; indeks tolerance

1 INTRODUCTION

The growing populationof the world along withmore requests for vegetable oils leads to more cultivation of oil seed crops. Winter oilseed rape (*Brassica napus* L.) is one of the most important oil seed crops that economically compete with cereal crops (Diepenbrock, 2000). Oilseed rape is the third source of vegetable oil in the world (USDA, 2016a). In the last decades, the yield stability of oilseed rape has not improved, beside its increased seed yield (Weymann et al., 2015). One of the main barriers to plant growth and yield is abiotic stress, especially the drought stress. Prolonged water deficit is a major abiotic stresses (Farooqet al., 2009).

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Breeding for a quantitative trait, such as drought resistance, with low heritability, is complicated using certain criterions that quantify the level of the desired quantitative trait and is more suitable than a direct selection (Farshadfar and Sutka, 2002). In this situation, plant breeders prefer to use drought indices that provide a measure of drought based on yieldloss under drought condition in comparison to normal condition (Mitra, 2001). Several selection indices have been suggested by various researchers based on a mathematical relationship between favorable and stress conditions (Clarke et al., 1984; Huang, 2000). Indices such as tolerance (TOL) (McCaig and Clarke, 1982; Clarke et al., 1992), mean productivity (MP) (McCaig and Clarke, 1982), stress susceptibility index (SSI)(Fischer and Maurer, 1978), geometric meanproductivity (GMP) (Fernandez, 1992), harmonic mean (HARM) (Schneider et al., 1997), relative drought index (RDI) (Fischer and Wood, 1979) and stress tolerance index (STI) (Fernandez, 1992) have been used by researchers under different conditions.

One of the wide-spread problems that seriously influence the production and quality of rapeseed is drought stress. However, the lack of effective selection criteria is hampering the development of resistant cultivars (Shiranirad and Abbasian, 2011). Sowing date is another important factor that plays a major role in determining the seed yield and the quality of rapeseed (Ozer et al., 2003). The objective of the present study was to investigate the interaction effect of drought stress and sowing date on yield and yield components traits of three cultivars of rapeseed and find the drought resistance genotype based on different drought indices.

2 MATERIALS AND METHODS

2.1 Plant materials

Three new and superior genotypes of winter oilseed rape, including 'Homolious', 'Hayola50' and 'DK7070CL' were cultivated in the experimental field of Sarayan agricultural college-Birjand University, in South Khorasan province-Iran in 2016-2017 growing season.

2.2 Fields Experimental conditions

The experiment was carried out in the form of split-factorial based on randomized complete block design (RCBD) with drought stress in the main plots and three sowing date (September 22, October 6 and October 22) along with three mentioned cultivars of oilseed rape in the subplots in three replications. All investigated genotypes were cultivated in their allocated subplots. Each subplot was consisted of four rows with 2 m length and with 20 cm distance between lines. In the normal experimental field, normal irrigation (one time per 10 days) of oilseed rape was applied but in drought stress environment, irrigation was interrupted in the flowering stage of genotypes.The volume of irrigated water for each subplot was calculated according to below equation (Moghadam and Fanay, 2016):

$$D = (Fc\ \theta) \times \rho_b \times D/100$$

Which d is the depth of irrigation water (mm), FC is the percentage of soil moisture content at from the field capacity, θ is the percentage of soil moisture content before irrigation, ρ_b is the bulk density of farm soil (g cm^{-3}), and D is the maximum depth of root development (m). A precise meter was used to enter the calculated amount of water in subplots (4800 m^3).

The drought stress was applied using water holding method (Bao et al., 2009). The irrigation of each flowering genotype was delayed for five days (one time irrigation per 15 days). The amount of irrigation water for each subplot was same to normal irrigation (4800 m^3).

Phenological assessments including days to flowering (DF) and days to maturity (DM) were collected during the growing season. Morphological characteristics of plant height (PH) and chlorophyll content (CC) along with number of siliques per plant (SP), number of seeds per silique (SS), 1000-seed mass (TW), biological yield (BY), seed yield (SY), harvest index (HI), plant height (PH), number of branches (NB), stem diameter (SD), silique length (SL), root length (RL), first branch height (FBH), and leaf twisting (LT) were recorded for each combination of treatments at the end of growing season, separately.Ten plants per plot were randomly selected to measure the mentioned characteristics. The chlorophyll content was measured using a SPAD 502 chlorophyll meter apparatus before the yellowing and drying of plants. The total dry matter of harvested plants (root + shoot) was considered as biological yield. To measure the seed yield, two middle rows of each plot was select and their plants were harvest. The seeds were left in fresh air to reach their moisture to 12 % and then the seed yield was measured.

2.3 Drought tolerance indices

To calculate the drought tolerance indicators, potential yield of each genotype in normal (the calculated seed yield in normal irrigated plots) (Y_p) and drought stress environment (Y_s), the average performance of all

investigated genotypes in normal ($\overline{Y}_p$) and drought stress environment ($\overline{Y}_s$), were measured and then TOL, MP, GMP, SSI, HARM, RDI, and STI were calculated according to below equations, respectively:

$$TOL = Yp - Ys$$ (1) (McCaig & Clarke, 1982; Clarke et al., 1992),

$$Mp = \frac{Yp + Ys}{2}$$ (2) (McCaig & Clarke, 1982),

$$GMP = \sqrt{Yp \times Ys}$$ (3) (Fernandez, 1992),

$$SSI = \frac{1 - (Ys / Yp)}{1 - (\overline{Ys} / \overline{Yp})}$$ (4) (Fischer & Maurer, 1978),

$$HARM = \frac{2(Yp \times \overline{Ys})}{(Yp + Ys)}$$ (5) (Schneider et al., 1997),

$$RDI = \frac{(Ys / Yp)}{(\overline{Ys} / \overline{Yp})}$$ (6) (Fischer & Wood, 1979),

$$STI = \frac{Ys \times Yp}{\overline{Y}_p^2}$$ (7) (Fernandez, 1992),

Yield Index (YI) and Yield Stability Index (YSI) were calculated using below equations:

$$YI = \frac{Ys}{\overline{Ys}}$$ (8) (Gavuzzi et al., 1997),

$$YSI = \frac{Ys}{Yp}$$ (9) (Bouslama & Schapaugh, 1984).

2.4 Statistical analysis

Statistical analyses including the analysis of variance (ANOVA) and means comparison analysis were carried out using the SAS software (Ver. 9.2). Means comparison analysis was conducted using Duncan's multiple range tests at 5 % probability level. All drought tolerance indices were calculated using Excel 2010 software. Simple Pearson correlation analysis was carried out to calculate the correlation of investigated plant characteristics and estimated drought tolerance indexes using SAS software.

3 RESULTS AND DISCUSSION

3.1 Analysis of variance and means comparison

The results of analysis of variance of split-factorial design (Table 1) showed the significant effect of drought stress on seed yield and leaf twisting traits at 5 % and on biological yield trait at 1 % probability level, respectively. The main effect of drought stress was not significant on other investigated traits. The genetic analysis of grain yield of twenty one F_2 progenies of oilseed rape using diallel cross analysis revealed that the additive gene effects are more effective than non-additive effects (Amiri-Oghanet al., 2009) and a S_1 recurrent selection program can help improve the seed yield of investigated cultivars of oilseed rape under drought stress.

The main effect of sowing date was only significant for the day to flowering and day to maturity traits at 1 % probability level. It is obvious that these two important phenological traits can be affected by sowing date. Amiri-Oghan et al (2009) reported 81.99 % heritability for days to maturity in 21 F_2 progenies of oilseed rape. Therefore, it is a trait with high heritability and direct or recurrent selection can be helpful to select the desired genotypes for this trait.

The interaction effect of drought stress × sowing date had significant effect on1000-seed mass, stem diameter, first branch height, chlorophyll content, and days to maturity at 5 % probability level. The results of analysis of variance revealed significant differences among three investigated cultivars of oilseed rape for 1000-seed mass, biological yield, seed yield, plant height, stem diameter, silique length, first branch height, chlorophyll content, day to flowering, and day to maturity at 1 % probability level and for root length at 5 % probability level. The number of seeds and their average mass are the parameters that affect the seed yield, therefore the improvement of1000-seed mass can improve the seed yield of investigated genotypes of oilseed rape (Labra et al., 2017).There was no significant difference between investigated cultivars of oilseed rape for harvest index, anyway it is obvious that a greater harvest index can leads to higher seed yield in canola (Diepenbrock, 2000). Shiranirad and Zandi (2012) also reported non-significant effect of drought stress on harvest index of twenty cultivars of spring rapeseed.

Based on the results of analysis of variance, the interaction effect of drought stress × cultivar was only significant for number of branches at 1 % probability level (Table 1).

The interaction effect of sowing date × cultivar was significant for leaf twisting at 1 % probability level and

fornumber of branches, first branch height, and seed yield at 5 % probability level (Table 1). Azizi (2015) also reported the different response of two investigated cultivars of oilseed rape ('Hyola401' and 'RGS003') in different sowing date for number of branches per plant and number of siliqua per plant. Based on the results of analysis of variance, three ways interaction effect of drought stress × sowing date × cultivar was only significant for leaf twisting trait at 1 % probability level (Table 1).

The results of means comparison analysis using Duncan's multiple range tests at 5 % probability level showed the adverse effect of drought stress on seed yield and biological yield of three investigated cultivars of oilseed rape under three different sowing date (Table 2).

Table 1: Analysis of variance of investigated traits of oilseed rape under drought stress and different sowing dates

S. O. V	df	Means of squares					
		Number of siliques per plant	Number of seeds per silique	1000-seed weight	Seed yield	Biological yield	Harvest Index
Block	2	92166.50^{ns}	47453985.7^{ns}	0.19^{ns}	8284.04^{ns}	224788.85*	10.68^{ns}
Drought Stress	1	33908.18^{ns}	9170609.7^{ns}	1.80^{ns}	92865.43*	1457781.33**	70.99^{ns}
Error a	2	46033.68	42062174.3	0.60	3583.32	6281.41	12.98
Sowing date	2	51151.31^{ns}	11216812.6^{ns}	0.10^{ns}	3635.54^{ns}	111737.25^{ns}	3.05^{ns}
Drought Stress × Sowing date	2	61056.82^{ns}	12628299.4^{ns}	0.54*	5486.26^{ns}	481462.13^{ns}	2.25^{ns}
Error b	8	38015.90	31571885.3	0.06^{s}	1575.62	151174.58	5.00
Cultivar	2	13219.55^{ns}	37133130.6^{ns}	1.68**	14493.77**	1352266.10**	9.44^{ns}
Drought Stress × Cultivar	2	10305.26^{ns}	6420690.9^{ns}	0.22^{ns}	599.38^{ns}	50258.78^{ns}	8.37^{ns}
Sowing date × Cultivar	4	32689.63^{ns}	11086189.0^{ns}	0.03^{ns}	6288.07*	181704.66^{ns}	10.55^{ns}
Drought Stress × Sowing date × Cultivar	4	8478.08^{ns}	4357525.4^{ns}	0.09^{ns}	1840.96^{ns}	107095.99^{ns}	8.34^{ns}
Error c	24	31652.40	24389046.4	0.10	2138.55	95242.40	6.75
Total	53	34155.28	23069582.1	0.22	4933.52	199279.01	8.34
C.V (%)		18.3	27.45	10.07	18.23	17.21	18.25

** ,*: significant at 1% and 5% probability level, respectively.

Table1: continued

S. O. V	df	Mean of Squares									
		Plant height	No. of branches	Stem Diameter	Silique length	Root length	First branch height	Chlorophyll content	Days to flowering	Days to maturity	Leaf twisting
Block	2	21.35 ^{ns}	189.0 ^{ns}	0.01 ^{ns}	0.10 ^{ns}	2.48 ^{ns}	8.1 ^{ns}	9.33 ^{ns}	6.80 ^{ns}	0.80 ^{ns}	0.07 ^{ns}
Drought Stress	1	90.74 ^{ns}	9.0 ^{ns}	0.02 ^{ns}	3.65 ^{ns}	0.67 ^{ns}	41.6 ^{ns}	35.85 ^{ns}	1.50 ^{ns}	6.00 ^{ns}	3.13 *
Error a	2	208.13	17.3	0.06	0.35	12.77	220.3	2.08	5.06	3.17	0.07
Sowing Date	2	316.69 ^{ns}	510.8 ^{ns}	0.16 ^{ns}	0.16 ^{ns}	5.59 ^{ns}	61.5 ^{ns}	15.67 ^{ns}	401.46 **	550.13 **	0.02 ^{ns}
Drought Stress × Sowing Date	2	384.57 ^{ns}	316.7 ^{ns}	0.23*	0.12 ^{ns}	3.13 ^{ns}	69.1 *	36.38*	3.72 ^{ns}	3.39*	0.02 ^{ns}
Error b	8	306.91	219.2	0.05	0.27	5.85	14.7	8.13	2.29	0.45	0.13
Cultivar	2	6355.0 **	150.7 ^{ns}	0.60 **	10.19 **	24.55 *	572.2 **	144.04 **	244.13 **	80.57**	0.02 ^{ns}
Drought Stress × Cultivar	2	21.35 ^{ns}	655.5 **	0.08 ^{ns}	0.06 ^{ns}	0.80 ^{ns}	22.1 ^{ns}	4.71 ^{ns}	2.17 ^{ns}	0.72 ^{ns}	0.02 ^{ns}
Sowing Date × Cultivar	4	182.19 ^{ns}	315.6 *	0.08 ^{ns}	0.44 ^{ns}	7.55 ^{ns}	56.5 *	22.42 ^{ns}	2.13 ^{ns}	0.30 ^{ns}	0.57 **
Drought Stress × Sowing Date × Cultivar	4	30.19 ^{ns}	190.6 ^{ns}	0.04 ^{ns}	0.13 ^{ns}	4.08 ^{ns}	27.1 ^{ns}	28.32 ^{ns}	0.56 ^{ns}	3.11 ^{ns}	1.24 **
Error c	24	85.44	100.9	0.03	0.28	6.52	14.3	22.19	2.19	2.66	0.06
Total	53	378.49	186.6	0.08	0.70	6.59	51.8	23.79	26.60	25.75	0.25
CV		9.6	13.31	16.45	12.18	14.63	22.01	11.69	1.02	0.79	14.97

** ,*: significant at 1 % and 5 % probability level, respectively.

Table 2: Means comparison analysis of investigated characteristics of oilseed rape cultivars under drought stress condition.

Drought Stress	Seed Yield (g m^{-2})	Biological Yield (g m^{-2})	Leaf twisting
Normal irrigation	295.08 a	1957.39 a	1.815 a
Drought stress	212.15 b	1628.78 b	1.333 b

The means with the same letter(s) at each column had no significant difference at 5 % level

The adverse effect of drought stress in seed yield is obvious, in addition, it is reported that late plantings can lead to reduced seed yield in oilseed rape because of shortening the vegetative stage (Azizi, 2015).

Another surprising result of means comparison analysis was that leaf twisting in normal irrigation was higher than in drought stress condition (Table 2). These results indicate that leaf twisting is not a drought tolerance strategy in investigated cultivars of oilseed rape. The results of means comparison analysis for the main effect of sowing date using Duncan's multiple range test at 5 % probability level revealed that the highest means of days to flowering was achieved in September 22 cultivation date, and later sowing dates lead to significant decrease in day to flowering of investigated cultivars of oilseed rape (Table 3). It is reported that both additive and non-additive genetic effects involved in controlling flowering and maturity time of oilseed rape, however additive effects are more important than non-additive genetic effects (Amiri-Oghanet al., 2009). Therefore, the direct selection for shorter flowering period under drought stress condition can be effective.Though our results are obtained in winter oilseed rape, however, in spring rapeseed it is reported that early sowing dates gave higher yields than late sowings and yield differences in this situation can related to the changes in branch numbers, silique numbers per plant, and 1000 seed mass (Ozer, 2003). The results of means comparison analysis for the main effect of cultivar using Duncan's multiple range test at 5 % probability level revealed that the highest means of number of seed per silique, 1000-seed mass, biological yield, plant height, stem diameter, root length, chlorophyll content, days to flowering, and days to maturity were related to 'Homolious' of oilseed rape, whereas the highest means of silique length was related to 'Hayola50' cultivar (Table 4).The growth rate and duration of the growing period are the two main factors that affect the biological yield of oilseed rape (Diepenbrock, 2000). These results were also evident in the present study, as the 'Homolious' with the highest

days to maturity showed the highest mean of biological yield.

The means comparison analysis using Duncan's multiple range test at 5 % probability level showed that the highest mean of 1000-seed mass was obtained in normal irrigation condition and in September 22 sowing date, anyway there was no significant difference between this interaction effect and interaction effect of normal irrigation × October 6 sowing date for 1000-seed mass trait at 5 % probability level (Table 5).These results indicate to it that normal irrigation in early sowing date can improve 1000-seed mass of oilseed rape but in delayed sowing date providing sufficient soil moisture is not very helpful to improve this trait. Andersen et al (1996) reported that drought stress during flowering did not affect seed mass of oilseed rape, whereas the earlier drought stress adversely affects the seed mass of oilseed rape.

The lowest mean of 1000-seed mass was achieved from drought stress condition and latest sowing date (October 22) (Table 5). These results indicate that drought stress and delayed sowing date can significantly reduce 1000-seed mass of investigated cultivars of oilseed rape in South-Khorasan province of Iran. These results are completely agreed with the findings of Andersen et al (1996). The growth rate and duration of the growing period are the main factors that involved in biological yield, both of which indicate the potential for improvement in yield (Diepenbrock, 2000).

The highest and lowest means of first branch height were achieved from normal irrigation × Septamber 22 and drought stress × October 22 treatments, respectively (Table 5).

Table 3: Means comparison analysis of phonological characteristics of three investigated cultivars of oilseed rape under different sowing data

Sowing Date	Days to flowering
September 22	150.44a
October 6	145.83b
October 22	141.00c

The means with the same letter(s) at each column had no significant difference at 5 % level

Table 4: Means comparison analysis of phonological, morphological, and yield traits in three investigated cultivars of oilseed rape.

Cultivar	1000-seed mass	Biological Yield (g m^{-2})	Plant Height (cm)	Stem Diameter (cm)	Silique length (cm)	Root length (cm)	Chlorophyll content	Days to flowering	Days to maturity
Homolious	3.40 a	2109.54 a	117.28 a	1.32 a	3.86 b	18.78 a	42.88 a	149.83 a	207.06 a
Hayola50	3.15 a	1638.58 b	82.33 b	1.00 b	5.22 a	17.02 ab	37.27 b	144.78 b	203.17 b
DK7170CL	2.79 b	1631.12 c	87.83 b	1.01 b	3.99 b	16.57 b	40.74 b	142.67 b	203.67 b

The means with the same letter(s) at each column had no significant difference at 5 % level

Table 5: Means comparison analysis of interaction effect of drought stress and sowing date on investigated traits of three oilseed rape cultivars

Drought stress × Sowing Date	1000-seed mass(g)	First branch height(cm)	Chlorophyll content	Days to maturity
Normal irrigation ×September22	3.52 a	23.20 a	43.41 a	207.44 a
Normal irrigation × October6	3.46 a	13.80 b	37.78ab	203.67 b
Normal irrigation ×October22	2.91bc	16.89ab	42.14ab	203.78 b
Drought stress ×September22	3.28ab	23.76 a	42.34ab	206.67 a
Drought stress × October6	2.84bc	11.84 b	36.77 b	202.67 b
Drought stress ×October22	2.68 c	13.02 b	39.33ab	203.56 b

The means with the same letter(s) at each column had no significant difference at 5 % level

The identification of the primary and secondary yield components can help to analyze seed yield and improve it under different condition (Diepenbrock, 2000).

The highest and lowest means of chlorophyll content were achieved from normal irrigation × September 22, and drought stress × October 6, respectively (Table 5). The small photo synthetically active area can limit the source and affects source and sink relation and therefore affects the seed yield of oilseed rape (Diepenbrock, 2000).

The earliest sowing date (September 22) lead to the highest number of days to maturity in both normal and drought stress condition (Table 5), whereas there were no significant differences among other interaction effects of drought stress and sowing date for this trait (Table 5). In short-season areas and also in stressful conditions determination of optimum sowing date is likely to be of critical importance because delayed sowing limits the size to which the crop grows before the change from vegetative to reproductive development which in turn controls yield potential (Gross, 1963; Ozer, 2003). Our results indicate that earlier sowing date can lead to longer vegetative period in both normal and drought environments; therefore the earlier sowing date is not a perfect strategy to escape from terminal drought stress.

The results of means comparison analysis for interaction effect of drought stress × cultivar showed that the highest and the lowest means for number of secondary branches were achieved from drought stress × 'Hayola50' and drought stress × 'Homolius', respectively (Table 6). There was no significant difference between number of branches in 'Homolius' under normal condition and in 'Hayola50' cultivar under drought stress condition at 5 % probability level (Table 6).

The results of means comparison analysis for interaction effect of sowing date × genotype using Duncan's multiple range tests at 5 % probability level revealed that the highest and the lowest means of seed yield were achieved from September 22 × 'Homolious' and September 22 × 'Hayola50' (Table 7). Except for September 22 × 'Hayola50', there were no significant differences among cultivars for this trait in different sowing dates at 5 % probability level (Table 7).

The results of means comparison on analysis showed that the highest and the lowest means of number of branches were achieved from October 22 × 'DK7170CL' and September 22 × 'Hayola50', respectively (Table 7). Although it is reported that delayed sowing led to the lowest effective branching in winter oilseed rape (Momoh and Zhou, 2001), however in the present study the number of branches in delayed sowing date can be related to the effect of drought stress in field capacity condition, whereas in transplanting method the plantlets are not faced with drought stress.

For first branch height trait, the highest and the lowest means were corresponded to October 6 × 'Homolious' and September 22 × 'Hayola50', respectively (Table 7).

For leaf twisting trait, the highest and the lowest means were achieved from October 6 × 'Hayola50' and September 22 × 'Hayola50', respectively (Table 7). These results indicated that the sowing date can significantly affect leaf twisting of Hayola50 cultivar of oilseed rape and delayed cultivation can significantly increase its leaf twisting properties.

As it shown in Table 1, the interaction effect of drought stress × sowing date × cultivar was only significant for leaf twisting trait, therefore in this step means comparison analysis was conducted for this trait.

Table 6: Means comparison analysis of interaction effect of drought stress and cultivar on number of secondary branch trait of oilseed rape

Drought stress × Cultivar	No. of branches
Normal irrigation × Hayola50	71.33 b
Normal irrigation × DK7170CL	72.81 b
Normal irrigation × Homolius	80.92 a
Drought stress × Hayola50	84.96 a
Drought stress × DK7170CL	71.96 b
Drought stress × Homolius	70.59 b

The means with the same letter(s) at each column had no significant difference at 5 % level

Table 7: Means comparison analysis of interaction effect of sowing date and genotype on investigated traits of oilseed rape cultivars under drought stress condition

Sowing Date × Cultivar	Seed Yield ($g\ m^{-2}$)	No. of branches	First branch height (cm)	Leaf twisting
Sept. 22 × Homolious	299.23 a	76.88 ab	24.00 ab	1.83 ab
Sept. 22 × Hayola50	181.95 b	65.67 b	11.03 d	1.33 c
Sept. 22 × DK7170CL	233.33 ab	69.55 ab	11.43 d	1.50 bc
October 6 × Homolious	279.68 a	74.22 ab	27.20 a	1.33 c
October 6 × Hayola50	264.88 ab	79.61 ab	14.37 cd	2.00 a
October 6 × DK7170CL	225.03 ab	69.33 ab	15.77 cd	1.50 bc
October 22 × Homolious	277.99 a	83.33 ab	19.23 bc	1.50 bc
October 22 × Hayola50	247.93 ab	71.89 ab	13.07 cd	1.50 bc
October 22 × DK7170CL	272.50 a	88.39 a	17.67 bcd	1.67 abc

The means with the same letter(s) at each column had no significant difference at 5 % level

Ozer (2003) also reported not significant effect of sowing date × cultivar × nitrogen on yield and yield component traits of two cultivars of spring rapeseed. The results of means comparison analysis using Duncan's multiple range test revealed that there were no significant differences among most of the investigated three ways interaction effects at 5 % probability level for leaf twisting, anyway the lowest means of this trait were achieved from drought stress × October 6 × 'DK7170CL', drought stress × October 22 × 'Homolious', and drought stress × October 22× 'Hayola50' (Table 8). It is obvious that delayed sowing date can decrease this trait in three investigated cultivars of oilseed rape under drought stress condition.

3.2 Estimation of drought tolerance indices

The comparison of estimated seed yield under drought stress conditions (Y_s), and yield under normal conditions (Y_p), for all investigated cultivars revealed that the highest yield was achieved from 'Homolious' under drought stress condition, whereas the lowest seed yield was related to 'Hayola50' under drought stress condition (Table 9). At all, Y_s of all investigated cultivars of oilseed rape was less than Y_p, except for 'Homolious' that its Y_s were higher than Y_p (Table 9). These results indicate to it that the severity of applied drought stress was not enough; therefore, in addition to the longer period of irrigation, the amounts of entered water to each plot should also be reduced. Stress intensity [$SI = 1 - (Y_s / Y_p)$] that shows the ratio of yield under drought stress conditions to yield under normal condition was also calculated. The highest SI was related to 'Hayola50' but this index was negative in 'Homolious' (-0.090) (Table 9), which indicate to higher Y_s than Y_p in this cultivar. The stress susceptibility index (SSI) was also negative estimated for 'Homolious' of oilseed rape, whereas the highest level of this index was corresponded to 'Hayola50' (Table 9). Genotypes that have SSI less than a unit are drought resistant, because their yield reduction under drought condition is smaller than the mean yield reduction of all genotypes (Fischer and Maurer, 1978). SSI is a suitable selection index to identify resistant cultivars against susceptible genotypes (Kutlu and Kinaci, 2010).

Based on the calculated drought tolerance indices, the highest values of RDI, MP, STI, GMP, YI, YSI, and HARM were related to 'Homolious', whereas the lowest values of these indices were achieved from 'Hayola50' of oilseed rape (Table 9). The highest value of TOL index was related to Hayola50 cultivar, whereas this index was negative in Homolious cultivar of oilseed rape (-241.733) (Table 9). Shiranirad and Abbasian (2011) used different drought tolerance indices including SSI, TOL, MP, GMP, and STI to find drought tolerance genotypes among six winter rapeseed cultivars and reported GMP, STI, and MP as the most suitable recognizing indexes.

Table 8: Means comparison analysis of interaction effect of drought stress, sowing date, and cultivar on leaf twisting trait of oilseed rape cultivars

Drought Stress × Planting Date × Genotype	Leaf twisting
Normal irrigation × September 22 × Homolious	1.67 ab
Normal irrigation × September 22 × Hayola50	1.67 ab
Normal irrigation × September 22 × DK7170CL	2.00 a
Normal irrigation × October 6 × Homolious	1.67 ab
Normal irrigation × October 6 × Hayola50	2.00 a
Normal irrigation × October 6 × DK7170CL	2.00 a
Normal irrigation × October 22 × Homolious	2.00 a
Normal irrigation × October 22× Hayola50	2.00 a
Normal irrigation × October22 × DK7170CL	1.33 bc
Drought stress × September 22 ×Homolious	2.00 a
Drought stress × September 22 × Hayola50	1.00 c
Drought stress × September 22 × DK7170CL	1.00 c
Drought stress × October 6 × Homolious	1.00 c
Drought stress × October 6 × Hayola50	2.00 a
Drought stress × October 6 × DK7170CL	1.00 c
Drought stress × October 22 × Homolious	1.00 c
Drought stress × October 22 × Hayola50	1.00 c
Drought stress × October 22 × DK7170CL	2.00 a

The means with the same letter(s) at each column had no significant difference at 5 % level

According to RDI, genotypes that show the highest value of this index can be select as drought resistant genotypes (Fernandez, 1992). Higher values of TOL indicate more sensitivity to stress. MP is the mean production under both stress and non-stress conditions (Rosielle and Hamblin,1981), so this index is based on arithmetic means and therefore it has an upward bias due to a relatively larger difference between Y_s and Y_p, but GMP is less sensitive to large extreme values (Fernandez, 1992). Anyway, based on MP and GMP, 'Homolious' of oilseed rape had more uniform performance in both stress and non-stress conditions than other investigated genotypes in the present study. STI is able to identify cultivars producing high yield under both stress and non-stress conditions (Kutlu and Kinaci, 2010), therefore this index can help to selection of drought resistance genotypes with acceptable level of seed yield in both irrigated and non-irrigated environments. YI index refers to the rate of seed yield in stress and mean stress, therefore this index ranks investigated genotypes only based on their yield under stress, but YSI is the rate of stress and non-stress a genotype, therefore genotypes that show higher YSI are expected to have high yield under both irrigated and drought stress conditions.

The ranking of investigated cultivars of oilseed rape based on their calculated drought tolerance indices is presented in Table 10. The highest values of RDI, MP, STI, GMP, YI, YSI, and HARM indexes were related to Homolious cultivar, whereas the highest values of SI, SSI, and TOL indexes were related to Hayola50 cultivar of oilseed rape. Based on all calculated drought tolerance indices, 'DK7170CL' had interstitial situation of Homolious and Hayola50 cultivars of oilseed rape (Table 10). SSI and SI can help to select drought tolerance genotypes in severe drought stress environments, whereas MP, GMP, and STI can help to distinguish drought tolerance genotypes in less severe drought stress environments. Using of MP, GMP, HARM, YI, and YSI can help to select genotypes with uniform performance in both stress and non-stress environments. Khaliliet al (2012) used eleven different drought tolerance indices and finally reported 'Hyola 308', 'Heros' and 'SW5001' as the most droughts tolerant cultivars of rapeseed by ranking the drought tolerance indices.

4 CONCLUSIONS

Significant differences were observed among investigated cultivars of oilseed rape for studied phenological, morphological, and yield properties. The main effect of drought stress was only significant on seed yield, biological yield, and leaf twisting traits. These results indicate that the applied drought stress was not severe enough. Sowing date had significant effect on days to flowering and days to maturity. There were significant differences among investigated cultivars of oilseed rape for most of the studied traits. The interaction effect of drought stress and sowing date had significant effect for stem diameter, first branch height, chlorophyll content and days to maturity. The interaction effect of drought stress and cultivar was only significant for number of branches. Number of branches, first branch height, and leaf twisting were significantly affected by the interaction effect of sowing date × cultivar. The three ways interaction effect of drought stress, sowing data, and cultivar was only significant for leaf twisting. Drought stress led to the significant decrease of seed yield and biological yield in investigated cultivars of oilseed rape. Later sowing dates led to significant reduction in days to maturity and days to flowering of three investigated cultivars of oilseed rape. The highest 1000-seed mass was achieved in normal irrigation and earlier sowing data. However drought stress and later sowing date led to significant reduction in 1000-seed mass of oilseed rape cultivars. The highest seed yield was related to earlier cultivation of 'Homolious' of oilseed rape. The seed yield of genotype in earlier sowing date was lower than in later sowing dates. Based on the drought stress indices, Homolious cultivar of oilseed rape was recognized as the most drought tolerance genotype that can keep its performance in severe drought stress environments, whereas 'Hayola50' was found as the most drought susceptible cultivar. However, the bigger Ys than Yp for 'Homolious' r can be related to mild applied drought stress, so that more severe drought stress can lead to rational results for this cultivar.Based on calculated MP, GMP, HARM, YI, and YSI indices, 'Homolious' lead to uniform performance in both stress and non-stress environments. The results of the present study can be used in future studies for seed yield improvement of oilseed rape in drought stress condition. Regarding to significant effect of sowing data × cultivar for seed yield trait, the obtained results can help to find the best sowing date for different cultivars of winter oilseed rape.

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Table 9: Drought tolerance indices and seed yield under normal and drought stress conditions measured in the three oilseed rape cultivars

Genotype	Ys	Yp	SI	SSI	RDI	TOL	MP	STI	GMP	YI	YSI	HARM
Homolious	292.404	268.231	-0.090	-0.486	1.338	-241.733	2803.178	1.090	2800.571	1.396	1.137	2797.966
Hayola50	106.850	254.844	0.581	3.134	0.515	1479.944	1808.472	0.419	1650.155	0.510	0.416	1505.698
DK7170CL	229.137	248.253	0.077	0.416	1.133	191.156	2386.956	0.923	2385.041	1.094	0.891	2383.128
Average	2094.641	2571.096	0.189	1.021	0.995	476.456	2332.869	0.811	2278.589	1.000	0.815	2228.931

Ys: Yield under drought stress conditions (g m^{-2}); Yp: Yield under normal conditions (g m^{-2}); SI: Stress Intensity; SSI: Stress Susceptibility Index; RDI: Relative drought index; TOL: Tolerance; MP: Mean Productivity; STI: Stress Tolerance Index; GMP: Geometric Mean Productivity; YI: Yield Index; YSI: Yield Stability Index; HARM: Harmonic Mean Productivity.

Table 10: Ranking of three oilseed rape genotypes in respect to different drought tolerance indices

Genotype	SI	SSI	RDI	TOL	MP	STI	GMP	YI	YSI	HARM	Mean
Homolious	3	3	1	3	1	1	1	1	1	1	1.6
Hayola50	1	1	3	1	3	3	3	3	3	3	2.4
DK7170CL	2	2	2	2	2	2	2	2	2	2	2.0

SI: Stress Intensity; SSI: Stress Susceptibility Index; RDI: Relative drought index; TOL: Tolerance; MP: Mean Productivity; STI: Stress Tolerance Index; GMP: Geometric Mean Productivity; YI: Yield Index; YSI: Yield Stability Index; HARM: Harmonic Mean Productivity.

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Bionomics of *Chilocorus infernalis* Mulsant, 1853 (Coleoptera: Coccinellidae), a predator of San Jose scale, *Diaspidiotus perniciosus* (Comstock, 1881) under laboratory conditions

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ABSTRACT

The bionomics of *Chilocorus infernalis* Mulsant, 1853, a natural enemy of San Jose scale, was studied under laboratory conditions (26 ± 2˚C, and 65 ± 5% relative humidity). The eggs were deposited in groups and on average 45.68 ± 24.70 eggs were laid by female. Mean observed incubation period was 6.33 ± 1.52 days. Four instar grubs were observed, and mean duration of all four grubs was found to be 19.98 days. The pupal stage lasted for 8.00 ± 0.50 days and after adults emerged out.

Key words: bionomics; natural enemies; San Jose scale; incubation period; larval instars

IZVLEČEK

BIONOMIJA VRSTE *Chilocorus infernalis* Mulsant, 1853 (Coleoptera: Coccinellidae), PLENILCA AMERIŠKEGA KAPARJA (*Diaspidiotus perniciosus* (Comstock, 1881)) V LABORATORIJSKIH RAZMERAH

Bionomija vrste *Chilocorus infernalis* Mulsant, 1853, naravnega sovražnika ameriškega kaparja, je bila preučevana v laboratorijskih razmerah (26 ± 2˚C in 65 ± 5 % relativne zračne vlažnosti). Samice plenilca so jajčeca odlagale v skupinah, v poprečju 45,68 ± 24,70 jajčec na samico. V povprečju so se ličinke razvile iz jajčec v 6,33 ± 1,52 dneh. Ugotovljene so bile štiri larvalne stopnje, katerih povprečna življenska doba je bila 19,98 dni. Razvojni štadij bube je trajal 8,00 ± 0,50 dni, nakar so se izlegli imagi.

Ključne besede: bionomija; naravni sovražniki; ameriški kapar; inkubacijska doba; stopnje ličink

1 INTRODUCTION

Coccinellidae is the largest family of order Coleoptera commonly known as ladybird beetles or lady bugs which are recognized for their predacious nature. They are important group of beetles from both economic point of view as their use in biological control and in their diversity and adaptation to a number of differing habitats. They play important role in regulating insect pests, especially aphids, leafhoppers, scale insects, mealy bugs, mites and softbodied insects (Slipinski, 2007). Among the six sub families of Coccinellidae, sub family Chilocorinae is one of the most important as it is the predator of scale insects. The latter are sap feeding insects named for the scale or shell like waxy covering their bodies. They possess piercing-sucking type of mouth parts. Depending upon species, scale insects may be found on plant stems, twigs, trunks or fruits. Sap feeding by scale insects cause yellowing or wilting of leaves, stunting or unthrifty appearance of the plants, and eventually death of all or part of the plant when infestations are heavy.

San Jose scale, *Diaspidiotus perniciosus* (Comstock, 1881) is one of the recognized pests of fruit crop in Kashmir. The reddish round spots appear on fruit as a result of infestation due to scales; this not only gives it bad shape but also reduces its market value. They also affect general vigour of plant and terminal twigs usually die (Masoodi & Trali, 1987). The incidences of the pest vary from year to year and from area to area because of changes in the factors influencing their population dynamics and dispersal (Sofi, 2006). *Chilocorus infernalis* Mulsant, 1853 was introduced in swat for the

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control of *D. perniciosus* on apples, resulting in reduction of pest populations (Mohyuddin, 1982). Both adults and larvae of *C. infernalis* were found feeding on San Jose scale (Rahman et al., 1961). It is the most beneficial beetle against scale pests as its mature and immature stages are voracious feeders. Thus this beetle plays important role as biocontrol for those crops that are especially susceptible to scales. The present study was therefore, carried out to gather relevant information with particular reference to biology of *C. infernalis,* the predator of scales.

2 MATERIALS AND METHODS

The experiment was conducted in the Entomology laboratory of department of Zoology, University of Kashmir under controlled conditions (26 ± 2˚C and 65 ± 5% relative humidity). The present study was done during two years (2014 to 2015). Adults of *C. infernalis* were collected from apple orchards and brought to the laboratory. Three mating pairs were kept in glass jars (15 × 5 cm) covered with muslin cloth. They were provided with abundant supply of food in the form of infested twigs of San Jose scale, *Diaspidiotus perniciosus* until oviposition. Dry twigs were replaced with fresh ones after every 24 hours in order to avoid contamination. The glass jars were also provided with crumpled paper to act as oviposition site. The eggs laid on crumpled paper and on walls of glass jars were removed using camel hair brush. They were counted and transferred in Petri dishes (12 cm in diameter). In order to maintain humidity moist filter paper was placed at bottom of Petri dishes. The filter paper was replaced daily to avoid contamination untill hatching. Observations were recorded carefully. The newly hatched first instar grubs were placed gently with the help of camel hair brush and transferred individually in Petri dishes. They were also provided with food (scales). Larval duration of each instar was recorded after moulting and different larval instars were separated from each other by head capsule measurement (Dyar, 1890). Measurement of head capsule was done with the help of digital vernier calliper. Duration of each larval instar was also observed and recorded carefully.

Newly emerged adults were placed in separate glass jars in pairs to observe the mating behaviour, duration of mating, oviposition and adult's longevity. The whole experiment was replicated 3 times.

Arithmetic mean, range, standard error (SE) and standard deviation (SD) were used to present the obtained data. Head width of different larval instars was used to calculate total larval instars by Dyar's ratio. Statistical analysis was done by using SPSS (Version 16.00).

3 RESULTS AND DISCUSSION

The life history of *C. infernalis* includes following life stages:

3.1 Egg stage

The eggs are evenly rounded; yellow in colour and cylindrical in shape at both ends. Eggs are laid in groups on the surface of leaf. On average, 42 eggs are laid in batches. The eggs are about 1.22 mm in length and 0.25 mm in breadth (Table 1).

Table 1: Measurement of egg of *Chilocorus infernalis*

Variable	N	Mean ± SD	Minimum	Maximum
Egg Length (mm)	10	1.22 ± 0.17	1.00	1.44
Egg Breadth (mm)	10	0.25 ± 0.06	0.19	0.35

N = Number of observations

3.2 Larval stages

Before hatching, the grub is visible through the egg shell as a coiled mass. Body covered with prominent senti on dorsum. Prothorax possesses five pairs of senti while mesothorax possesses four pairs. Larval instars are black in colour. 1st, 2nd, 3rd and 4th larval instars are differentiated on the basis of head width (Dyar's rule). Table 2 gives us mean length of 1st, 2nd, 3rd and 4th instars. Mean duration of 1st instar was 4.00 days, 4.3 days for 2nd instar, 5.00 days for 3rd instar and 6.00 for 4th instar (Table 3).

Table 2: Comparison of observed (mean) and expected values of head capsule widths (mm) of the grub of *Chilocorus infernalis*

Larval Instars	Head capsule width (mm)			Difference (mm)
	Observed (Mean ± SE)	Range	Expected[a]	
I	0.30 ± 0.33	0.10-0.35	0.30	0.00
II	0.50 ± 0.34	0.35-0.63	0.48	0.02
III	0.78 ± 0.01	0.63-0.85	0.8	0.02
IV	0.86 ± 0.00	0.85-0.88	1.2	0. 34

[a]Expected head capsule width established by Dyar's ratio (1.6 mm). Multiplying Dyar's ratio with the observed head capsule width of 1st instar grub gives the expected head capsule width of 2nd instar which when multiplied again with Dyar's ratio gives expected head capsule width of 3rd instar and so on.

Mean observed head capsule width of 1st instar grub (N = 10) = 0.30 mm
Mean observed head capsule width of 2nd instar grub (N = 10) = 0.50 mm

$$\text{Growth ratio (Dyar's ratio)} = \frac{\text{Head capsule width of 2nd instar grub}}{\text{Head capsule width of 1st instar grub}}$$

= 0.50/0.30
= 1.6 mm

Table 3: Duration of immature stages of *Chilocorus infernalis*

Developmental stage	Observations			Mean ± SD
	1	2	3	
1st instar grub	3.5 days	4 days	4.5 days	4.00 ± 0.50
2nd instar grub	5 days	4.5 days	3.5 days	4.33 ± 0.77
3rd instar grub	6 days	5.5 days	4 days	5.00 ± 1.32
4th instar grub	7.5 days	5.0 days	5.5 days	6.00 ± 1.32
Total grub period				19.33

3.3 Pupa

The pupa is formed within the shed larval skin. Pupa is somewhat triangular in shape with light brown in colour just after pupation and gradually changes into deep brown and black. Only extreme posterior parts are visible from upper side and eight abdominal segments are also visible dorsally. Wing pads are meeting on ventral side. Pronotum is emarginated and laterally prolonged for reception of head. Lateral marginals are rounded. The pupal period was observed to take 6.0 - 8 days. Pupal length ranged between 3.97 and 5.01 mm, whereas breadth ranged between 2.72 –and 3.02 mm.

3.4 Adult

Adults are sub hemispherical and very moderately compressed. Head is black deeply inserted and not visible from above. Pronotum is deeply black. Scutellum is clearly visible, black and having shiny lusture. Elytra were black in color with a pair of reddish spots on each. Both spots are present in a transverse line. Females are generally larger in size as compared to males. Mean length of adult was 5.15 mm and breadth was 4.32 mm (Table 4).

Table 4: Measurement of adult of *Chilocorus infernalis*

Variable	N	Mean ± SD	Minimum	Maximum
Adult Length (mm)	10	5.15 ± 0.25	4.72	5.50
Adult Breadth (mm)	10	4.32 ± 0.08	4.20	4.43

N = Number of observations

Table 5 and Fig.1 depicts the mean of three observations of different developmental stages of *C. infernalis*.

Table 5: Developmental duration (in days) of different life stages of *Chilocorus infernalis*

Parameter	Observations			Mean ± SD
	A	B	C	
Mating period (in minutes)	65	40	30	45.00 ± 18.02
Oviposition (in days)	22	20	14	18.68 ± 4.16
Fecundity (eggs in batch)	72	42	23	45.68 ± 24.70
Incubation period (in days)	8	6	5	6.33 ± 1.52
1st instar (in days)	4.5	4	3.5	4.00 ± 0.50
2nd instar (in days)	5	4.5	3.5	4.33 ± 0.77
3rd instar (in days)	6	5.5	4	5.00 ± 1.32
4th instar (in days)	7.5	5	5.5	6.00 ± 1.32
Prepupal period (in days)	3	2	2	2.33 ± 0.57
Pupal period (in days)	8.5	8	7.5	8.00 ± 0.50
Male longevity (in days)	45	43	40	42.68 ± 2.51
Female longevity (in days)	80	76	53	69.68 ±14.58

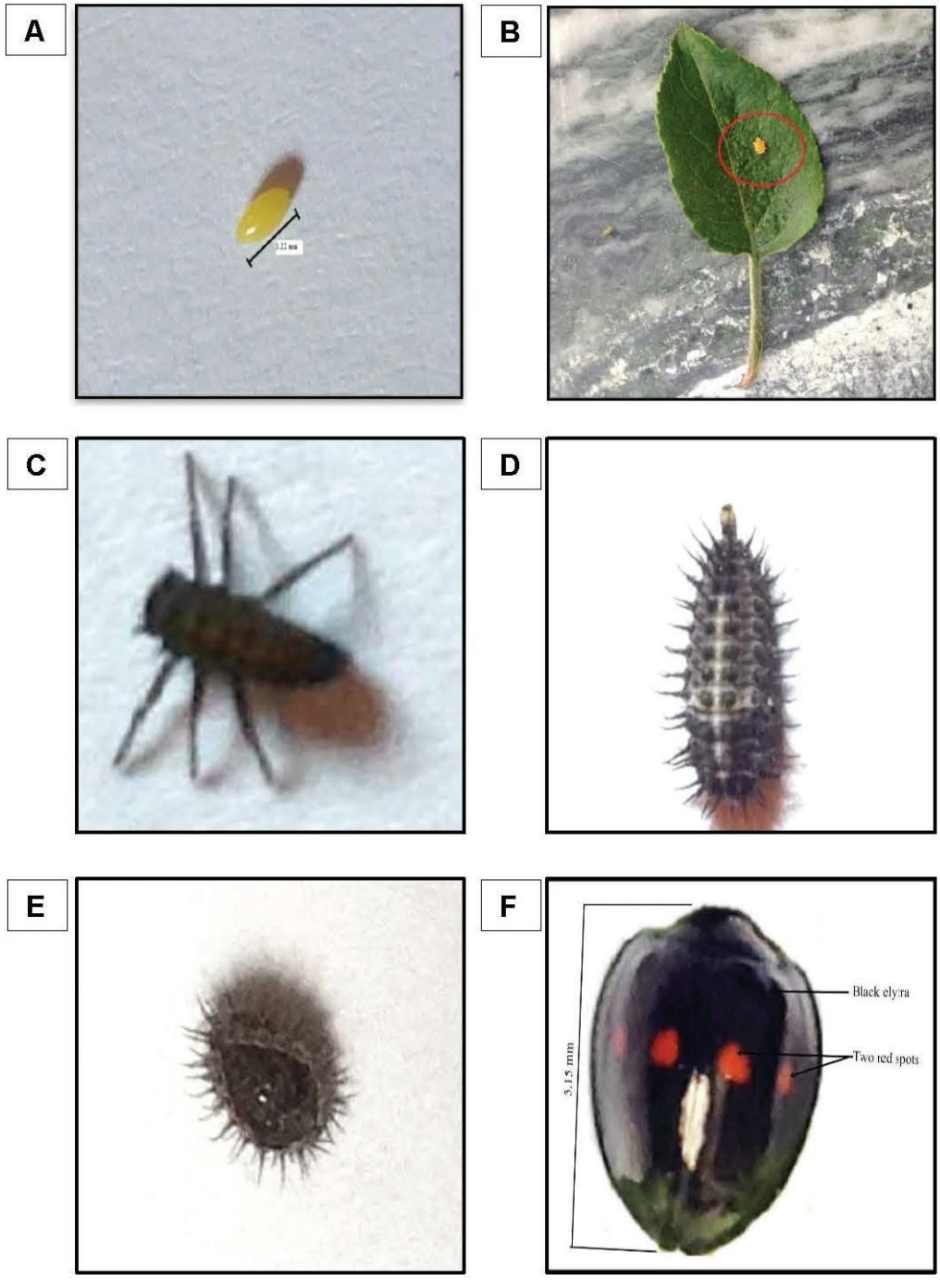

Figure 1: Different developmental stages of *Chilocorus infernalis*. (A) Egg of *C. infernalis*. (B) Eggs laid on leaf surface. (C) First instar grub. (D) Fourth instar grub. (E) Pupa. (F) Adult.

During the present study *C. infernalis* was found as a dominating species in fruit ecosystem and wild vegetation due to availability of prey, but it was found absent in vegetable ecosystem in a previous study conducted by Rasheed & Buhroo (2018). Buhroo et al. (2000) found that the common ladybird beetle, *C. bijugus* was effective and predominant predator of San Jose scale which passed three generations along with its host. It was also observed that the development of this predator corresponded to the development of San Jose scale and has got well established in the orchard ecosystem. Furthermore, Thakur et al. (1989) conducted extensive field surveys in India and their study revealed that four species of parasitoids and three species of

predators, *Chilocorus bijugus,* Mulsant 1856, *Coccinella septempunctata* (Linnaeus, 1758), *Sticholotis marginalis* Kapur, 1956 were among the effective natural enemies of *D. perniciosus* Comstock, 1881 in Jammu and Kashmir. Ahmad et al. (1999) studied the spatial distribution and phenology of adult coccinellid *C. infernalis* at two localities on 8-10 year-old apple orchards at high altitudes (1500-1700 meter above sea level) of Kashmir. The results showed that adult *C. infernalis* was the most common species than the *Coccinella septempunctata*; *Exochomus flavipes* (Thunberg, 1781) and three unidentified species which were very few in numbers. Khan (2010) studied exploitation of *C. infernalis* for suppression of the San Jose scale in apple orchards at five locations of Kashmir. The results revealed that the release of 35 individuals of *C. infernalis* / plant reduced significantly the infestation of the San Jose scale in all locations of Kashmir.

Rawat et al. (1992) studied the biology of *C. bijugus* Mulsant, a predator of San Jose scale. The results revealed that mean length of egg is 0.93 mm and mean breadth 0.46 mm. Whereas incubation period ranged from 3 to 6 days (Mean = 4.8). However these findings are not in agreement with present study. Kapur (1954) reported incubation period as 3.44 days without mentioning the temperature at which the study was made, whereas Jalali and Singh (1989) reported it as 6.0 to 6.8 days on different host insects at 27 ± 1.8 °C and 55 ± 2.3 % relative humidity. These are close observations with present study. However, these findings are not in agreement with those reported by Chanyuvadze (1976) (8 to 9 days) and Murashevskaya (1969) (8-9 days) for *Chilocorus renipustulatus*. These differences in the incubation periods may be attributed to variations in ambient temperature and relative humidity.

Ahmad and Ghani (1966) reported total grub period of 21.0 days which are in fair agreement to our findings, but differ from Rawat et al. (1992) and Gupta and Inderjit (2007) which showed total grub period of 25 to 40 days (average 31.9 days) and 12 to 16 days respectively.

Rawat et al. (1992) showed mean length of pupa was 5.75 mm (range 5.10 to 6.50) and the average breadth 3.70 mm (range 3.50 to 4.20). The pupal duration varied from 11 to 16 days with an average of 12.62 days. These results however do not agree with the findings of present study. Our observations corroborate with those of Chanyuvadze (1976) who reported 11 days in case of *C. bijugus*, Kapur (1954), Ahmad and Ghani (1966) and Jalali and Singh (1989) who reported the pupal duration as 7.2 days, 8.0 days for *C. infernalis* and 6.1 to 8.0 days in case of *C. bijugus* respectively.

Rawat et al. (1992) reported the oviposition period varied from 8 to 16 (average 11.6 ± 0.48) days under laboratory conditions. The female on an average produced 100.7 ± 1.44 (range 60 to 135) eggs. However during present study oviposition period was found varied between 14 to 22 (average 18.68 ± 4.16) days. The females on an average produced 45.68 ± 24.70 eggs (range 23 to 72). These results differ from findings of Jalali and Singh (1989) who reported that *C. bijugus* had a high fecundity of 92 on *D. perniciosus* and Ahmad and Ghani (1966), Ahmad (1970) and Greathead and Pope (1977). These workers have reported very high fecundity (228 to 858 eggs) in case of *Chilocorus nigritus*Fabricius, 1798.

4 CONCLUSIONS

It can be concluded that the biology of *C. infernalis* under laboratory conditions showed better longevity. The eggs were deposited in groups and on average 45.68 ± 24.70 eggs were laid by female. Incubation period was 6.33 ± 1.52 days. Four instar grubs were observed and mean duration of four instars was found to be 19.98 days. The pupal stage lasted for 8.00 ± 0.50 days and after that, adults emerged out. Due to its short life cycle, it can be successfully used for mass rearing and then its establishment in pest prevalent regions. This suggests the possible role of this beetle as an efficient biological control agent. This will also decrease the application of harmful pesticides and allow these natural enemies to do their function successfully in the field.

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Hybridization potential *Aegilops* sp. / durum wheat: which interest for the genetic breeding of the drought tolerance?

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ABSTRACT

To study their hybridization potential, two species of the genus *Aegilops* (*Aegilops geniculata* Roth; *Aegilops triuncialis* L.) and two durum wheat (*Triticum durum* Desf.) varieties (‘Oued Zenati’ and ‘Hoggar’) were crossed, where *Aegilops* was the female parent. The four cross combinations were tested during five years in order to release the genitors having the most affinity for obtaining interspecific hybrids. The parents were also characterized for their drought stress tolerance during the crossing period. The results confirm the tolerance of *Aegilops* sp. and adaptation of the durum wheat varieties to climatic conditions governing the Algerian cereal zones. 81 hybrids F1 were obtained. Differences in hybridization affinity between the parents were very remarkable. The combination of parents *Aegilops geniculata*/’ Oued Zenati’ has produced the highest number of hybrids (54 or a rate of 5.23 %), followed by *Aegilops triuncialis*/’Oued Zenati’ (18 hybrids or a rate of 3.88 %). The crossing of the two *Aegilops* species with the Hoggar variety produced a small number of hybrids. Among the advantages of this crossing, the obtaining of hybrids in caryopsis without resorting to the embryos rescue. Hybrid seedlings expressed a maternal cytoplasmic heredity. However, no adult plant could have been regenerated.

Key words: *Aegilops*; durum wheat; drought tolerance; interspecific hybridization; genetic breeding

IZVLEČEK

HIBRIDIZACIJSKI POTENCIAL KRIŽANCEV OSTIKE (*Aegilops* sp.) IN TRDE PŠENICE (*Triticum durum* Desf.) PRI VZGOJI KRIŽANCEV ODPORNIH NA SUŠO

Za preučevanje hibridizacijskega potenciala sta bili križani dve vrsti iz rodu ostike (*Aegilops geniculata* Roth.; *Aegilops triuncialis* L.) in dve sorti trde pšenice (‘Oued 'Zenati' in 'Hoggar'), pri čemer je bila ostika ženska starševska vrsta. Križanci teh štirih kombinacij so bili preiskušani v obdobju petih let z namenom vzgoje potomcev s čim večjim deležem medvrstnih lastnosti. Starševske vrste so bile v obdobju križanja preučevane glede njihove odpornosti na sušni stres. Rezultati so potrdili odpornost vrst ostike na sušo in prilagoditev sort trde pšenice na podnebne razmere v žitnih območjih Alžirije. Vzgojenih je bilo 81 F1 križancev. Razlike v sposobnosti križanja med starševskimi vrstami so bile opazne. Kombinacija staršev *Aegilops geniculata*/’Oued Zenati’je dala največje število križancev (54 ali 5,23 %), tej je sledila kombinacija *Aegilops triuncialis*/’Oued Zenati’ (18 križancev ali 3,88 %). Križanje obeh vrst ostike s sorto Hoggar je dalo le malo križancev. Med prednostimi tega križanja je pridobitev križancev v kariopsi, brez uporabe metode reševanja zarodkov. Hibridne sejanke so pokazale znake maternalnega citoplazmatskega dedovanja. Kljub naporom ni uspelo vzgojiti nobene odrasle hibridne rastline.

Ključne besede: *Aegilops*; trda pšenica; toleranca na sušo; medvrstno križanje; žlahtnenje

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1 INTRODUCTION

The cultivation of durum wheat in Algeria represents an economic and social importance (Attab & Brinis, 2012; Chahbar & Belkodja, 2016). The major constraint of this culture in the Mediterranean region is drought; the fluctuations of the rain combined to an intense heat, especially at the end of the cycle, affect sensibly the productivity (Ashraf, 2010; Kosová et al., 2014). The capacity of plants to be acclimatized to the water deficit is associated to their adaptability to the photosynthesis reduction which involves disturbances in multiple biochemical and physiological processes (rate of transpiration, stomatal conductance, effectiveness of water use) and a negative impact on growth (Anjum, 2011; Aissa & Redouane, 2014). The genus *Aegilops*, related to the genus *Triticum*, represents an important source of genes with potential interest for the wheat genetic amelioration (Ashraf, 2010). Indeed, many *Aegilops* species are adapted to various bioclimatic levels, notably arid and semi- arid, and therefore present a tolerance to drought (Molnár et al., 2004; Dulai et al., 2006) and to salinity (Colmer et al., 2006). The interspecific hybrids were significantly exploited in the amelioration of traits presenting simple genetic determinism (Jahier et al., 2006). Addition or substitution lines were developed from interspecific hybrids between wheat and *Aegilops* species (Schneider et al., 2005) allowing the successful introgression of many genes with disease resistance from *Aegilops* species (Schneider et al., 2008; Mujeeb-Kazi et al., 2013). Like the introduction into wheat of an eyespot resistance gene from *Aegilops ventricosa* (Jahier et al., 2006). Recently, similar lines have been created in order to introduce genes that code for: efficient utilization of phosphor by the plant (Wang et al., 2010); high values of zinc and iron of the seeds (Tiwari et al., 2010; Neelam et al., 2011) and amelioration of pastes and breadmaking quality (Wang et al., 2013).Thus, the interspecific hybrids offer remarkable genetic variability for use in wheat genetic breeding programs (Rolland et al., 2014). Many studies report the natural occurrence of interspecific hybrids between wheat and *Aegilops* species which are considered as the female parent (Morrison, et al., 2002; Cifuentes et al., 2006). Nevertheless, these works intended to study the incorporation of transgenes into *Aegilops* species from cultivated wheat varieties. Unfortunately, works dedicated to the introgression of tolerance traits for abiotic stress from *Aegilops* species remain rare (Mujeeb-Kazi et al., 2013). Thus, the objective of this work is the study of the hybridization potential between species of the genus *Aegilops* and durum wheat varieties and the influence of the crossing direction on obtaining interspecific hybrids *Aegilops*/durum wheat. As this study is a part of wheat breeding program to drought tolerance, by wild species as *Aegilops*, the genitors were also characterized for their tolerance to water stress during the crossing period.

2 MATERIALS AND METHODS

The plant material in this study consists of two durum wheat varieties and two tetraploid species of the genus *Aegilops* (Table 1). The seeds were provided by ITGC, El Khroub (Technical Institute of Great Cultivation, Constantine, Algeria), except for *Aegilops geniculata* which is a local natural collection (Constantine).

Table 1: Characteristics of wheat varieties and *Aegilops* species studied (Kellou, 2003 ; Van Slageren, 1994)

Species	Characteristics
Triticum durum Desf.	
Variety Oued Zenati 368 (O.Z)	Selected from the 'Oued Zenati' local population, it is a late-variety, adapted to the anterior plains, characterized by a black, long beard spike and high straw.
Variety Hoggar (Hog)	Introduced from Spain, ITGC / Tiaret Selection, 1986. It is adapted to the Highlands and Saharan areas.
Species of the genus *Aegilops*	
Aegilops geniculata Roth*(Ae.gen)* (syn. *Ae.ovata* L.)	Annual species, allo-tetraploid (2n = 4x = 28), its genomic formula is UUMM. It grows in the Mediterranean region, the Middle East and the southern parts of Russia and Ukraine.
Aegilops triuncialis (*Ae.tri*)	Annual species, amphi-tetraploid, (2n = 4x = 28). Its genomic formula is UUCC. It grows in the Mediterranean region.

Experiments on drought tolerance and interspecific hybridization were assured in a greenhouse at the Genetics, Biochemistry and Biotechnology Laboratory of Mentouri Brothers University 1, Constantine, Algeria. For both experiments, the seeds were previously disinfected and pre-germinated in Petri dishes. They were transplanted into pots of 5 kg containing a mixture of ground and sand (2: 1 v / v) at the rate of three seedlings per pot and periodically watered to their field capacity.

2.1 Drought tolerance

At the heading stage, plants were divided in three lots: Control lot (**C)**: whose plants were periodically irrigated to saturation; First level stressed lot **(L1)**: abstention of watering for one week (7days); Second level stressed lot **(L2)**: abstention of watering for two weeks (15 days). The treatment of L2 was applied a week before that of the L1 in order to synchronize the samples and the measurements. Three replications per variety and per treatment were applied.

2.1.1 Physiological parameters

The physiological parameters measured are: the relative water content (RWC [%]) calculated from the formula of Clarck & McCaing (1982). Stomatal resistance (SR, [m^2.s mol^{-1}]) is measured using a Porometer (Delta Devices® MK3). The total chlorophyll content (TCC [unit of SPAD "Soil Plant Analysis Development"]) is measured with a chlorophyll SPAD meter (502 of Minolta®).

2.1.2 Biochemical parameters

They concerned the determination of soluble sugars content ([SSC µMol 100 mg^{-1} of fresh material] saccharose, glucose, fructose, their methyl derivatives and polysaccharides) by the phenol method of Dubois et al., 1956. The antioxidant activity of peroxidase and catalase is measured on enzymatic extracts, obtained after grinding 0.500 mg of fresh leaves in a phosphate buffer (50 mmol l^{-1} at pH7), centrifugation and filtration of the supernatant. The activity of peroxidase "POX" (EC 1.11.1.7.) is determined at 470 nm using guaiacol as a substrate. The reactional mixture contained 1 ml of hydrogen peroxide H_2O_2 (0.01 N), 1 ml of guaiacol and 1 ml of enzymatic extract. Data was recorded every 20 sec for 2 min. The catalase activity "CAT" (EC 1.11.1.6.) is determined in a reactional mixture containing 1 ml of hydrogen peroxide (0.01N), 1 ml of 50 mmol l^{-1} phosphate buffer and 1 ml of enzymatic extract. The decomposition of H_2O_2 was followed at 240nm (Cakmak & Marschner, 1992), the data were recorded every 15sec for 2min. The enzymatic activity is expressed in µkat mg^{-1} of proteins contained in the plant extract used) (Micro-Katal [µKat]: disappearance of one µmole of substrate per second). The statistical treatment of the results was carried out by the software Minitab version 2017.

2.2 Interspecific hybridization

The interspecific crosses of *Aegilops* sp. / durum wheat varieties were conducted over five years. Three sowing dates were completed for each parent to synchronize their flowering time. Before anthesis, *Aegilops* sp. spikes have been emasculated and wrapped to avoid cross-pollination. They were pollinated with fresh pollen from the durum wheat varieties, without any growth hormones uses after emasculation and pollinisation. The hybrids were harvested in caryopsis.

2.3 *In-vitro* culture

A mature embryo culture of hybrid caryopsis was undertaken to break the dormancy of these seeds. They were disinfected under a laminar flow hood in a 70 % C_2H_5OH solution for 30 sec, rinsed with sterile distilled water and then placed in a dilute 12 % sodium hypochloride solution for 15 min. They were rinsed 5 times with sterile distilled water. The disinfected caryopses were placed in Petri dishes with absorbent paper previously sterilized and they were soaked with sterile distilled water. After 24 h, the mature embryos were removed under binocular and under sterile conditions. They were transferred to MS regeneration medium (Muraschige & Skoog, 1962) supplied with Kinetin (0.25 mg l^{-1}), AIB (Indole butyric acid) (1 mg l^{-1}). The cultures were incubated in a culture chamber in the dark, at a temperature of 25 ± 2 ° C for one week. After the release of coleoptiles, a photoperiod of 16 h day / 8h night was applied. Mature embryos were transplanted to a new medium every four weeks.

3 RESULTS

3.1 Drought tolerance

Tolerance to water stress was evaluated for two *Aegilops* species and two durum wheat varieties.

3.1.1 Physiological parameters

3.1.1.1. Relative water content

The relative water content decreases with the intensity of the stress (Fig. 1a) comparing to the controls (C)

whose value varies from 87.90 to 98.08 %, the non-watered plants during one week (L1 of stress) have displayed WRC from 81.81 to 94.11 % and the unsprayed for two weeks (L2 of stress) from 60.90 to 87.15 %. The variance analysis showed very highly significant differences between the genotypes as well as for the treatments at the threshold α = 0.001, the genotype x treatment interaction, proved not significant.

3.1.1.2 Stomatal Resistance

For all the studied genotypes, the SR increases markedly with the severity of the stress (Fig. 1b). The means values range from 1.89 to 27.97 m^2.s mol^{-1} for C plants; from 36.73 to 131.00 m^2.s mol^{-1} for L1; from 81.00 to 175.00 m^2.s mol^{-1} for L2. The *Aegilops* have been remarked by high resistance means and a fast response from L1 (especially for *Aegilops geniculata* "*Ae.gen*") compared to wheat varieties. As a result, the variance analysis of the genotypes as well as the treatments was very highly significant at the threshold $\alpha = 0.001$, the genotype x treatment interaction is significant at the threshold $\alpha = 0.05$.

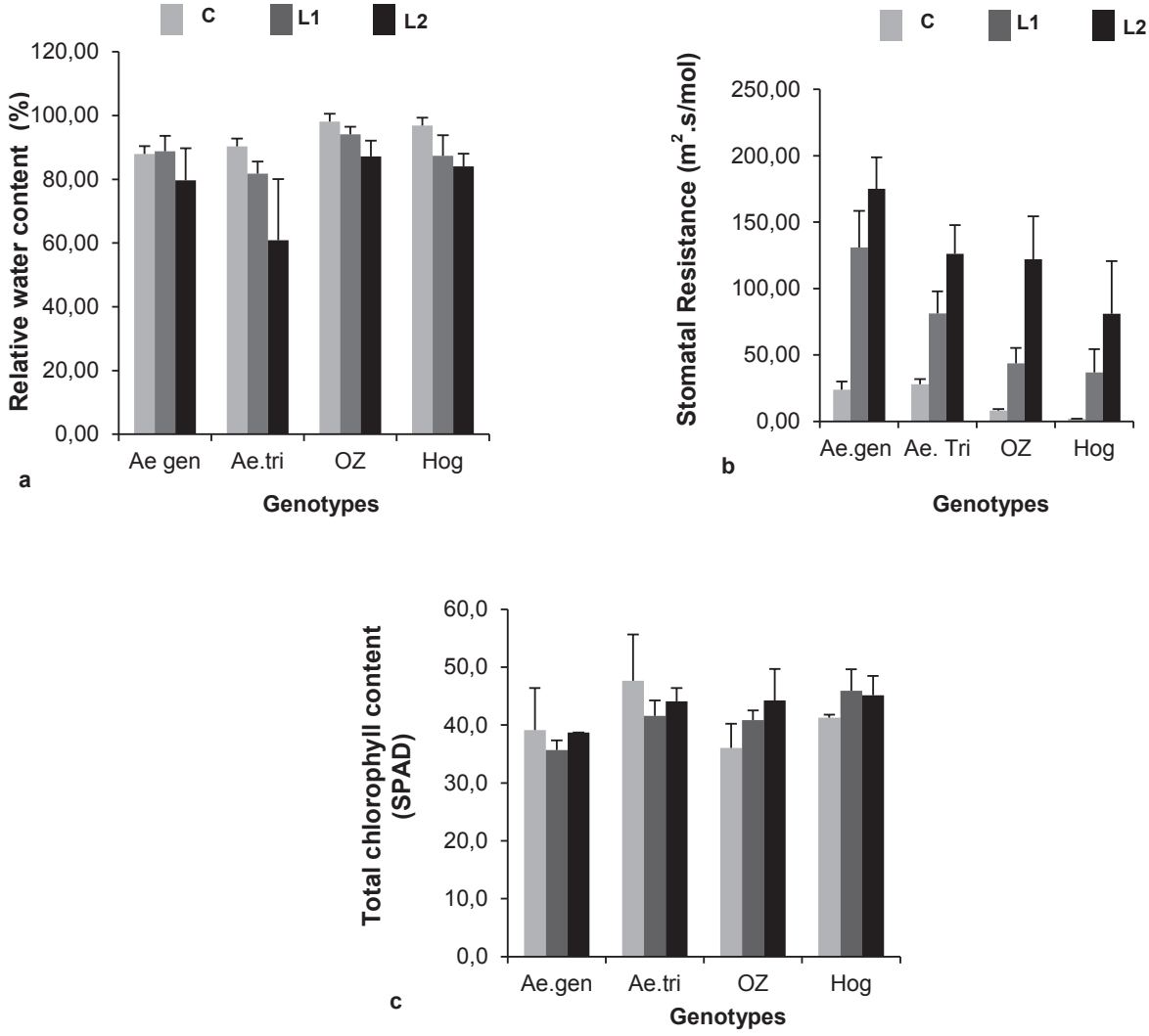


Figure 1: Water stress effect on physiological parameters: a. Relative water content; b. Stomatal resistance; c. Total chlorophyll content.

3.1.1.3 Total chlorophyll content

Ae.gen and *Ae.Tri*, present values of 39.1 and 47.7 SPAD for C that decrease at L1 to 35.7 and 41.6 SPAD but increase at L2 to 38.7 and 44.1 SPAD, respectively (Fig. 1c). O.Z has an increase in TCC with stress levels, compared to C whose value is 36.1 SPAD, TCC reaches 40.9 SPAD at L1, then 44.3 at L2. For Hog, the value of C is 41.3SPAD, the TCC increases to 45.9 SPAD at L1 and then decreases slightly at 45.1 SPAD at L2. As a result, the analysis of variance revealed a significant

difference between the genotypes studied at $\alpha = 0.05$ threshold, whereas the treatments as well as the genotype x treatment interaction proved to be insignificant.

3.1.2 Biochemical parameters

3.1.2.1 Soluble sugar content

The recorded SSC revealed different stress behaviours (Fig. 2a). For *Ae.gen* and *Ae.tri*, respectively, the SSC is 0.765 and 0.813 µmol for C, it reaches 0.793 and 0.864 µmol at L1, but decreases at 0.790 and 0.756 µmol at L2. For O.Z, a decrease in SSC is observed at L1 (0.839 µmol) compared with C (0.863 µmol), then an increase at L2 (0.883 µmol). Hog, has a SSC of 0.980 µmol for C which decreases for L1 to 0,799 µmol and remains constant at this value at L2. The variance analysis revealed highly significant differences between the genotypes as well as the genotype x treatment interaction at $\alpha = 0.01$ threshold, whereas the treatments proved to be insignificant.

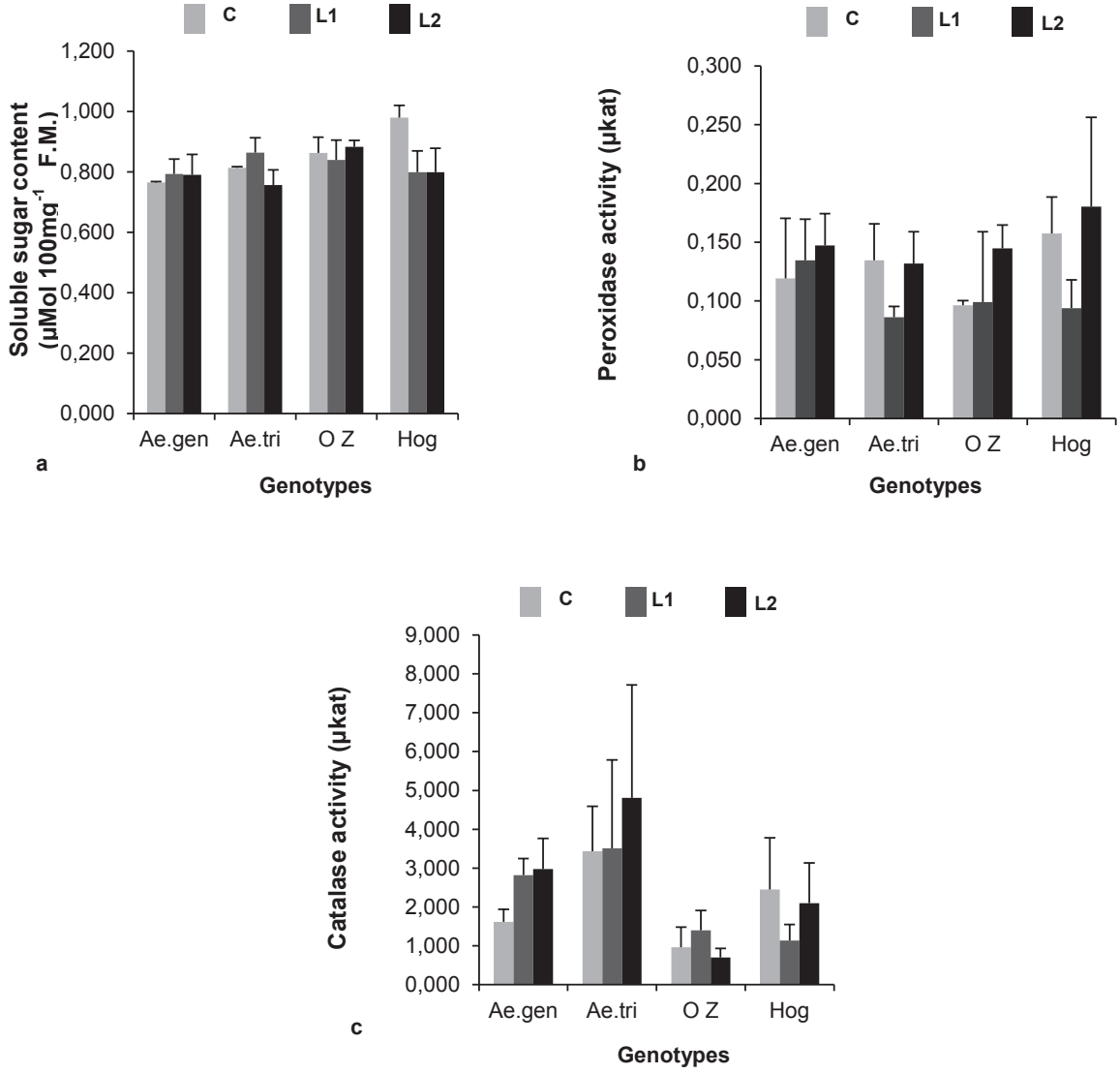


Figure 2: Water stress effect on biochemical parameters: a. Soluble sugar content; b. Peroxidase activity; c. Catalase activity

3.1.2.2 Peroxidase activity

Aegilops sp. show an increase in this activity in stressed plants compared to control plants which have a value of 1.617 and 3.436 µKat mg^{-1}, at L1 the value reaches 2.820 and 3.511 µKat mg^{-1}, at L2 it is 2.977 and 4.808 µKat mg^{-1} respectively, for *Ae.gen* and *Ae.tri*, this latter presents the most important values of POX activity (Fig. 2b). The O.Z control present a POX

activity of 0.959 µKat mg^{-1}, which increases for L1 to 1.398 µKat mg^{-1} but decreases at 0.699 µKat mg^{-1} for L2. Whereas in the Hog variety, the POX activity decreases for L1 at 1.132 µKat mg^{-1} compared to C whose activity is 2.447 µKat mg^{-1} and then increases at L2 to 2.098 µKat mg^{-1}. The analysis of variance was very highly significant between the different genotypes (α = 0.001), whereas the treatments as well as the genotype x treatment interaction proved to be insignificant.

3.1.2.3 Catalase activity

The CAT activity (Fig. 2c) shows, for *Ae.tri* and Hog variety, respectively, a decrease at L1 (0.086 and 0.094 µKat mg^{-1}) in comparison with the C plants (0.135 and 0.157 µKat mg^{-1}), then an increase at L2 (0.132 and 0.180 µKat mg^{-1}). For O.Z, the activity increases slightly at L1 (0.099 µKat mg^{-1}) compared to C (0.096 µKat mg^{-1}), it increases considerably at L2 (0,145 µKat mg^{-1}). For *Ae.gen*, the increase in enzymatic activity with stress levels is more remarkable than in O.Z, the CAT is 0.119 µKat mg^{-1} for C and it reaches 0.135 µKat mg^{-1} at L1 then 0.147 µKat mg^{-1} at L2. For this parameter, the treatments appeared significant at α = 0.05 threshold, while the genotypes as well as the genotype x treatment interaction proved to be insignificant. The comparison between the two enzymes activities reports higher values of POX than those of CAT (Fig. 2b and c). The correlations between physiological and biochemical parameters are shown in Table 2, where we recorded two significant positive correlations (α = 0.05) between SSC and SR, and between WRC and SSC. A highly significant negative correlation (α = 0.01) between WRC and SR is also observed.

Table 2: Linear correlations matrix of physiological and biochemical parameters

	TCC	SSC	CAT	POX	SR
SSC	0.027				
CAT	0.242	0.092			
POX	0.159	-0.082	0.158		
SR	-0.070	-0.352*	0.166	0.317	
RWC	-0.169	0.350*	-0.145	-0.280	-0.481**

$p \leq \alpha = 0.05$: (*)significant differences. $p \leq \alpha = 0.01$: (**)highly significant differences

The grouping of the four genotypes, with a dendrogram using the single linkage and the Squared Pearson distance and for a minimum similarity level of 50 % (Fig. 3), enabled to distinguish four homogeneous groups: the first is represented by *Ae.gen*, the second by *Ae.tri*, the third consists of O.Z and the fourth group of Hog.

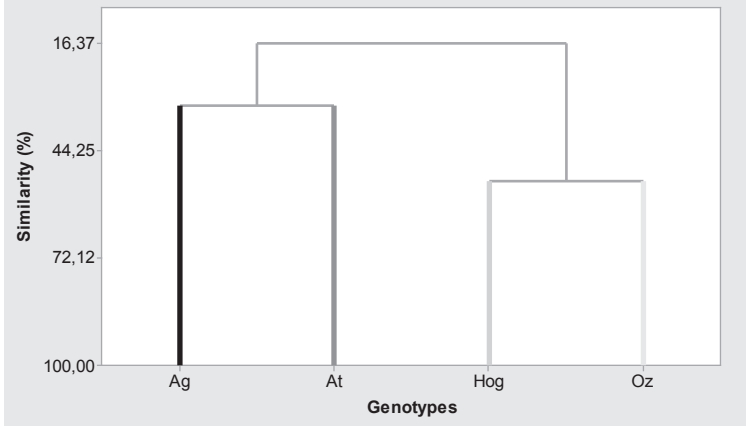


Figure 3: Grouping dendrogram of studied genotypes. **Ag:** *Ae. geniculata;***At:** *Ae. triuncialis*; **Hog:** Hoggar; OZ: Oued Zenati

3.2 Interspecific hybrids obtaining

The interspecific hybridization between two species of the genus *Aegilops* as the female parent, with the two durum wheat varieties, allowed us to obtain 81 hybrids. Table 3, summarizing the five-year results for the four possible combinations of crossing, represents the cross-ability between the genitors, expressed as a percentage of the number of hybrids obtained reported to the number of pollinated flowers.

Table 3: Five-year hybridizations results according to genitors combinations

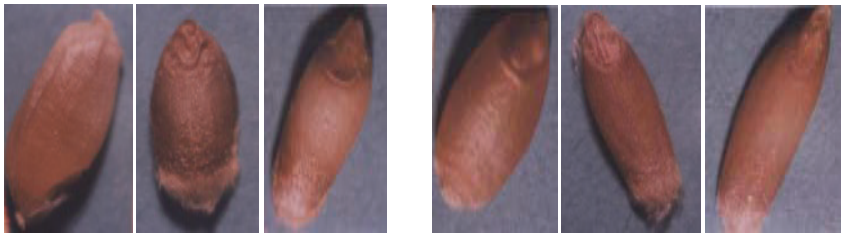
Crosses	NS	NSK	NPF	NFS	NHS	Cross-ability%
Ae.gen /OZ	192	524	1032	714	54	**5. 23**
Ae.gen/ H	38	97	194	157	4	**2. 06**
Ae.tri /OZ	66	237	464	361	18	**3.88**
Ae.tri/H	47	171	336	269	5	**1.49**

NS: Number of pollinated spikes. NSK: Number of pollinated spikelets. NFP: Number of pollinated flowers. NFS: Number of fruit set. NHS: Number of hybrids seeds.

The results show differences in hybridization affinity between parents. The combination of *Ae.gen* and O.Z produced the largest hybrids number (54 a rate of 5.23 %). The crossing between *Ae.tri* and O.Z, comes second in hybrids production (18 a rate of 3.88 %). Combinations of *Ae.gen* and Hog as well as *Ae.tri* and Hog gave a small number of hybrids.

3.3 Mature embryo culture and plantlet regeneration

All the hybrids were collected in caryopsis resembling the female parent *Aegilops* sp. (Fig. 4), of different sizes (very noteworthy for hybrids whose parent is *Ae.tri* characterised by long caryopsis), mostly with a normal endosperm, only a few were scalded.



a b c d e f

Figure 4: Harvested hybrid caryopsis photographs in comparison with those of respective parents. a-Hog, b- Hybrid Ae.gen/Hog, c-Ae.gen, d- O.Z, e- Hybrid Ae.tri/OZ, f- Ae.tri.

Several cold stratifications as well as the scarification of the seeds did not allow the break dormancy of hybrids, observed under natural conditions. Only mature embryos culture allowed germination and regeneration of hybrid seedlings (Table 4). The embryos collected were of different sizes, some very small not exceeding one millimeter in diameter.

Table 4: Germination rate and number of hybrid seedlings regenerated by mature embryo culture

Hybrids	Cultured embryos	Germination %	Regerenated seedlings	Adult plants
Ae gen/.O.Z	7	100%	2	0
Ae gen/Hog	5	100%	2	0
Ae tri/Hog	2	100%	1	0
Ae tri/O.Z	1	100%	1	0
Total	15	100%	6	0

From fifteen embryos, six seedlings were regenerated in a relatively average rate of regeneration of 40 %, however, no adult plant is obtained. After successful germination (100 %) of all hybrids, those with normal growth (Fig. 5a) regenerated the seedlings. While for others, anomalies have been detected leading to precocious death causes by lack of root system edification (Fig. 5b); lack of the coleoptile development (Fig. 5c); and albino coleoptiles regeneration (Fig. 5d).

Figure 5: Hybrid mature embryo photographs of MS medium germination. **a-***Ae.tri*/OZ, two weeks of culture. **b-***Ae.gen*/OZ, two weeks of culture. **c-***Ae.tri*/Hog, one week of culture. **d-** Another hybrid *Ae.gen/*OZ, four weeks of culture

The death of seedlings, at the acclimation stage, occurring at different stages of growth (the most advanced is that of tillering for the hybrid *Ae.gen* / Hog) is due to the weak growth of the seedlings (stunted plantlets, leaves with very small surface, weak root system) (Fig. 6). However, hybrid seedlings also exhibited morphological features of female parents *Ae.gen* and *Ae.tri* in the early stages of development, similar to twisted pre-foliation and leaf color.

Figure 6: Regenerated hybrid plantlets. **a:** *Ae.gen*/O.Z. **b**: *Ae.tri*/Hog. **c:** *Ae.gen*/Hog (six weeks of culture). **d-** *Ae.gen*/Hog (twelve weeks of culture).

4 DISCUSSION

Obtaining interspecific hybrids offers significant variability. It is the crucial step in any program of genes introgression from wild species. However, its success depends not only on the choice of genitors that suits the objectives of the program, but also on crossing affinity. Thus, the study of drought tolerance confirms the high potential of *Aegilops* species to tolerate water stress, particularly that of *Ae.geniculata*, which corroborates with the works of (Rekika et al., 1998; Zaharieva et al., 2001; Baalbaki et al., 2006) and the adaptation of the OZ variety to Mediterranean stress type (Ali Dib et al., 1992; Meziani et al., 1993). The RWC and the SR had a significant impact on the other physiological and biochemical parameters of this study, SR - RWC (r = -0.481 **); SSC - RWC (r = 0.350 *); SR and SSC (r = -352 *) (Table 2). In fact, the ability to maintain elevated

RWCs in a situation of water stress is related to the osmotic adjustment capacity or to the high elasticity of the plant tissues (Bousbaa et al., 2013). The opening or closing of stomata, sensitive to the concentrations of abscisic acid produced by the roots, is the most element affected by the water stress of all those in relation to the water of the plant (Anjum et al., 2011; Shang et al., 2016). By closing its stomata, the plant saves the available water and preserves cell integrity, thus constituting one of the best strategies for water stress tolerance (Djekoun & Ykhlef, 1996; Ykhlef et al., 2000; Bousbaa et al., 2013; Shang et al., 2016). Consequently, the closure of the stomata leads to a decrease of the photosynthesis (Maurino & Peterhansel, 2010; Gallais, 2015) and an increase of the reactive oxygen species where peroxidase plays an important role in their elimination, especially in conditions of water stress (Anjum et al., 2011).

The few works dedicated to interspecific crosses where species of the genus *Aegilops* are taken as female parent, report the weakness of obtaining such hybrids, which seems, more favourable in field conditions (Guadagnuolo et al., 2001). Many criteria influence the acquisition of fertile hybrids and backcross progenitor for introgression between two genera, including genetic relationships, ploidy level, and hybridization direction (Waines & Hegde, 2003). The success of obtaining hybrids depends largely on the parental genotypes involved in the crossing. The differences in hybridization affinity of *Aegilops* species and wheat varieties are highly observed (Guadagnuolo et al., 2001; Waines & Hegde, 2003; Stone & Peeper, 2004; Hadzhiivanova et al., 2012; Ykhlef et al., 2007). In many studies, the common sharing of the D genome between the bread wheat and the wild parent allowed the pairing of homeologous chromosomes and obtaining fertile hybrids (Snyder et al., 2000; Schoenenberger et al., 2005; Martins et al., 2015). The effect of the Ph1 locus is only suppressed in some diploid *Aegilops* species (Al-Kaff et *al*, 2007), so allowing the pairing of homeologous chromosomes in the hybrid (Waines & Hegde, 2003). The lethality of hybrids, manifested by meristem tissues anomalies from germination to a weak growth at advanced stages of development, are often reported in interspecific hybridization, resulting from incompatibilities between the nuclear and cytoplasmic genomes, due to complementarities or epistasis interactions between genes (Tikhenko et al., 2008; Matsuoka et al., 2007; Mizuno et al., 2010). In the case of *Poaceae*, a paternal heredity of chloroplast DNA has never been observed (Guadagnuolo et al., 2001), so in our study, the maternal cytoplasmic heredity explains the morphological characters of resemblance between hybrids and the female *Aegilops* parent. Differences in establishment of pre-or post-zygotic hybridization barriers between parents manifest according to the direction of hybridization, which make easier the obtaining of hybrids in one of the directions (Riesberg & Carney, 1998). Following our results, interspecific hybridization where *Aegilops* is the female parent have the advantage of obtaining caryopsis hybrids (Guadagnuolo et al., 2001; Cifuentes et al., 2006) compared to the reciprocal hybridization durum wheat /*Aegilops* where interspecific hybrids were obtained only by embryos rescue (Hadzhiivanova et al., 2012; Ykhlef et al., 2007). Our study is a contribution to the identification of genitors and mechanisms that facilitate interspecific hybrids obtaining. We have focused on the cross-ability of the O.Z variety, widely used and adapted to the Mediterranean stress type, with the *Ae.gen* and *Ae.tri* species, and the quality of hybrids obtained in the *Aegilops* /durum wheat direction. Thus, research within these two species of accessions, that are more favourable for obtaining hybrids, is promising success and less expensive hybridization.

5 CONCLUSION

We have undertaken in this study, the hybridization of two species of the genus *Aegilops* and two durum wheat varieties. The characterization of the genitors for their drought tolerance during the hybridization period, confirms our choice of the genitors, where we noticed the superiority of the *Aegilops* for water stress tolerance comparing to the wheat and the adaptation of durum wheat varieties to the climate of Algerian cereal zones. The duration of the stress applied seems average. Therefore, in a short term the plants reacted by a fast closing of the stomata which remedied the loss of water by transpiration and consequently maintained a high RWC favourable to the good cellular functioning. Obtaining hybrids, even with a low rate of 3.9 %, indicates the possibility of interspecific hybridization between *Aegilops* species and durum wheat, taking *Aegilops* as the female parent. Their success is affected by several parameters where the genotype of the involved parents and the degree of relationship are important criteria, because of the existence of genes that inhibit homeologous pairings between parental genomes as well as the establishment of genes that cause lethality and the sterility of hybrids in some species during speciation. The obtaining of caryopsis in very good condition and without recourse to the embryos rescue, confirms that genetic mechanisms of post zygotic isolation have been expressed in the hybridization direction where *Aegilops* is considered as the female parent. The study and understanding of these mechanisms and the identification of their responsible

genes will overcome these barriers and facilitate the acquisition of hybrids in order to succeed the introgression programs of interesting genes from wild species. We lead cytogenetic and molecular studies to characterize hybrids and to elucidate potential problems that led to the loss of hybrid seedlings during the acclimation phase.

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Chromium-induced alkaloid production in *Catharanthus roseus* (L.) G.Don in vitro cultured shoots and related gene expression patterns particularly for the novel gene *GS*

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ABSTRACT

This study aimed to determine the effects of methyl jasmonate (Mj) combined with chromium (Cr) as elicitor on production of medicinal alkaloids, its antioxidant potential, and its effects on the expression of signaling and biosynthetic enzymes. Combined treatment had positive effects on secondary metabolism and changed genes expression levels of mitogen-activated protein kinase 3 (*MAPK3*), a transcription factor (TF) known as octadecanoid-responsive *Catharanthus* AP2-domain 3 (*ORCA3*) upstream of plant alkaloids biosynthetic pathway. Maximum expression levels of peroxidase1 (*PRX1*), geissoschizine synthase (*GS*) (24 h-treatment), *MAPK3* and *ORCA3* (8 h-treatment), were 6.25−, 4.87-, 7.67-, and 5.38-fold higher than control, respectively, in response to 100 µM Mj + 50 µM Cr. This value was 5.92-fold for strictosidine synthase (*STR*) in response to 100 µM Mj + 100 µM Cr after 24 h. The maximum total yield of vincristine was 1.52-fold more than control in response to 100 µM Mj after one week. This increase was 2.16, 4.01, 2.39 and 1.97-fold for ajmalicine, vinblastine, vindoline and catharanthine respectively, in response to 100 µM Mj + 50 µM Cr. Mj + Cr can elevate alkaloid production by induction of *MAPK3* and *ORCA3* signaling pathway, which induces expression of downstream terpenoid indole alkaloids (TIAs) biosynthetic enzymes.

Key words: antioxidative responses; chromium; *GS*; *MAPK3*; *ORCA3;* real time PCR

IZVLEČEK

S KROMOM VZPODBUJENA PRODUKCIJA ALKALOIDOV PRI VRSTI *Catharanthus roseus* (L.) G.Don V *IN VITRO* GOJENIH POGANJKIH IN Z NJO POVEZANI VZORCI IZRAŽANJA GENOV, ŠE POSEBEJ NOVEGA GENA *GS*

Namen raziskave je bil določiti učinke metil jasmonata (Mj) v povezavi s kromom (Cr) kot elicitorjev v produkciji medicinskih alakaloidov, njun antioksidacijski potencial in njune učinke na ekspresijo signalizacije in biosinteze encimov. Kombinirano obravnavanje je imelo pozitivne učinke na sekundarni metabolizem in spremenilo ravni izražanja genov mitogen-aktivirane protein kinase 3 (*MAPK3*), transkripcijskega faktorja (TF) poznanega kot oktadekanoid-odzivne *Catharanthus* AP2-domene 3 (*ORCA3*), ki vzpodbuja biosintezo rastlinskih alkaloidov. Največje ravni izražanja peroksidaze1 (*PRX1*), geisošizin sintaze (*GS*) (24 h-obravnavanja), *MAPK3* in *ORCA3* (8 h-obravnavanja) so bile 6,25−, 4,87-, 7,67-, in 5,38-krat večje kot pri kontroli kot odziv na hkratno obravnavanje s 100 µM Mj + 50 µM Cr. Ta vrednost je bila za striktozidin sintazo (*STR*) 5,92-kratna kot odziv na 100 µM Mj + 100 µM Cr po 24 h. Največji celokupni pridelek vinkristina je bil za 1,52-krat večji kot pri kontroli kot odziv na 100 µM Mj po enem tednu. Enako povečanje je bilo 2,16, 4,01, 2,39 in 1,97-kratno za ajmalicin, vinblastin, vindolin in katarantin, kot odziv na 100 µM Mj + 50 µM Cr. Mj + Cr lahko povečata produkcijo alkaloidov z indukcijo *MAPK3* in *ORCA3* signalne poti, ki inducira izražanje encimov za biosintezo terpenoid indolnih alkaloidov (TIAs).

Ključne besede: antioksidacijski odziv; krom; *GS*; *MAPK3*; *ORCA3;* realni čas PCR

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1 INTRODUCTION

Catharanthus roseus L. (Apocynaceae) is a significant pharmaceutical plant that contains more than 130 alkaloids named terpenoid indole alkaloids (TIAs), 25 of which are found in nature in dimeric form and have antidiabetic, bactericide, antihypertensive, and anticancerous activities. Vincristine and vinblastine are two dimeric alkaloids that are potent antineoplastic factors and indispensable elements in most cancer chemotherapies. Additionally, two precursors of them, catharanthine and vindoline, are also of great importance. Furthermore, *C. roseus* is the source of ajmalicine which has been identified as an antihypertensive agent (Ncube & Van Staden, 2015).

The pharmacological significance of TIAs and their low amounts in the plant which is the unique source of them (around 0.0005 % of dry mass) have motivated broad research on the TIA pathway for manipulating plant metabolism to enhance alkaloid amounts. One important technique for raising secondary metabolite contents is the utilization of heavy metals in plant cell, tissue, and organ cultures not only to enhance the generation of secondary metabolites, but also to promote the *de novo* biosynthesis of them (Wójciak-Kosior et al., 2016). Chromium (Cr) is a special example of a heavy metal that, in hexavalent (Cr^{+6}) form, is soluble and highly mobile (Eleftheriou et al., 2015). Methyl jasmonate (Mj) is a key element of plant's immune system that regulates the protective reactions against stresses, also participates in signal transduction chain and results in high production of TIAs (Van Moerkercke et al., 2015). The effects of Cr combined with Mj on the expression levels of key elements of the biosynthetic pathway, such as strictosidine synthase (STR), deacetylvindoline-4-O-acetyltransferase (DAT), geissoschizine synthase (GS) and a peroxidase1 (PRX1), has not yet been determined. STR, DAT, and PRX1 are three main bottleneck steps. STR condenses tryptamine and secologanine to form strictosidine, the first monoterpene indole alkaloid. DAT acetylates deacetylvindoline to form vindoline, and then PRX1 dimerizes the vindoline and catharanthine to make dimeric TIAs. GS is a novel gene that forms 19E-geissoschizine from 4, 21-dehydrogeissoschizine, one of the intermediate steps in stemmadenine biosynthesis. GS was identified by Qu et al. (2018), and to date, the expression of this gene in response to any treatment has not been studied.

The critical enzymes of the pathway and important transcription factors (TFs) affecting their activity and regulatory pathways have always been considered in elicitation studies. A manifest example of this is the octadecanoid-responsive *Catharanthus* AP2-domain *3 (ORCA3),* a jasmonate-inducible TF that regulates many important jasmonate and elicitor responsive genes in the TIA pathway by attaching to an area in their promoter (Raina et al., 2012). In addition to TFs, mitogen-activated protein kinase (MAPK) cascades, upstream of these factors, are important in the regulation of the pathway.

Little information is available on how the plant responds to this kind of stress (Mj + Cr) at the gene expression level. Thus, the purpose of this assay was to determine the joint effects of Cr and Mj synchronically as a kind of elicitor on the production of the medicinal alkaloids vincristine, vinblastine, catharanthine, vindoline and ajmalicine; its antioxidant potential; its effects on the expression of *CrMAPK3*, the TF *ORCA3*, the biosynthetic enzymes *CrPRX1*, *STR*, *DAT*, *GS;* and finally, the relationship between alterations in gene expression and the production of TIA alkaloids.

2 MATERIAL AND METHODS

2.1 Plant growth conditions and elicitor preparation

Seeds of *C. roseus* var. *pacifica* 'XP Cherry Red Halo' procured by the Pan American Seed Company (U.S.A.) were germinated in MS medium in a growth chamber with a temperature of 25 ± 2 °C and a 16-h photoperiod with 400 µm m^{-2} s^{-1} photon flux density. After 5 weeks, shoot explants were moved to MS medium augmented with 100 µM Mj (Sigma–Aldrich) separately and in combination with 50 and 100 µM concentrations of Cr^{+6} as $K_2Cr_20_7$ (Merck). For the control group, explants were cultured in basal MS medium. Treated and control samples were then harvested after 0.5, 4, 8, 24 hours (h) and one week elicitation, chilled in liquid N_2, and then kept at −80 °C until analysis.

2.2 Lipid peroxidation

The malondialdehyde (MDA) content represents lipid peroxidation in plant tissue, and it was measured by thiobarbituric acid reaction using the method of Heath and Packer (1968).

2.3 Total phenolic and flavonoid contents

To evaluate total phenolic content, the Folin–Cio-calteu method was used (Dewanto et al., 2015). 0.5 ml of deionised water and 125 µl of the Folin–Cio-calteu reagent were added to 125 µl of the diluted sample extract. After standing for 6 min and then adding 1.25 ml of a 7 % aqueous Na_2CO_3 solution, the ultimate

volume was arranged to 3 ml with water. Consideration was performed after 90 min in 760 nm. The results were expressed as mg gallic acid equivalents per g fresh mass(mg GAE g^{-1} FM). Flavonoid content was determined using a colorimetric method that was explained by Dewanto et al. (2015). 0.05 ml of a 33 % aqueous acetic acid solution and 0.1 ml of a newly made 10 % $AlCl_3$ solution were added to 0.5 ml of the suitably diluted sample. By using ethanol, the ultimate volume was reached to 2.5 ml and after 30 min, absorption of samples was read at 414 nm. The outcomes were displayed as mg quercetin equivalents per g fresh mass(mg QE g^{-1} FM).

2.4 Alkaloid extraction and analysis

Alkaloids were extracted according to Miranda-Ham et al. (2007). To determine the content of vincristine, vinblastine, catharanthine, vindoline and ajmalicine, a quantitative HPLC by a Knauer GmbH HPLC system was used. A5 µm C18 vertex column (125 mm × 4 mm ID) was applied for the separation of samples. A volume of 20 µl was injected, and the column temperature was 25 °C. The mobile phase was made up of a blend of 5 mM Na_2HPO_4 (pH adjusted to 6 with H_3PO_4) (solvent A) and acetonitrile (solvent B). Flow-rate was 1.0 ml min^{-1}. The UV detector of the HPLC system was adjusted at 258 nm. Alkaloids were computed as µg g^{-1} DW. Total alkaloid content was counted at 280 nm using a UV-VIS spectrophotometer (Vario 2600).

2.5 Protein content and assays of antioxidant enzyme activity

Bradford's method was used to consider the protein content. Catalase (CAT; EC 1. 11.1.6), peroxidase (POD; EC 1. 11.1.7), and superoxide dismutase (SOD; EC 1. 15.1.1) activities were determined by standard methods as previously described in Sanchez-Rojo et al. (2015).

2.6 RNA extraction, cDNA synthesis, and gene expression

Total RNA was extracted from in vitro-cultured *C. roseus* plantlets (0.1 g) using RNX plus (Cinnaclon). The qualities and concentrations of the extracted RNA were checked with agarose gel electrophoresis and spectrophotometer, respectively. After DNaseI treatment, the first strand of cDNA was synthesized from 6 µg of total RNA using an oligo-d (T) primer. Reverse transcription was performed using the following program: 37 °C for 15 min, 85 °C for 5 s and 4 °C as a final hold. The sequence of oligonucleotide primers used for study was as follow: F: *MAPK3* (5′−CGAAAACATAATTGCCATAA-3′), R: *MAPK3* (5′−TGACAATGCTCCTCAGATAGA-3′), F: *ORCA3* (5′−CAGGAGGATTCTGTTGTGG-3′), R: *ORCA3* (5′−CTGGATCCTTTCTTTTTCG-3′), F: *PRX1* (5′−TCACTTCTGACCAAGATTTGTA-3′), R: *PRX1* (5′−CTTGATTCCCCGTTAACAC-3′), F: *RBCL* (5′−GCTGCTGAATCTTCTACTGG-3′), R: *RBCL* (5′−GTCTAAGGGGTAAGCTACATAAG-3′), F: *STR* (5′−GGTTCTACACTTCCGTCCA-3′), R: *STR* (5′−CAATGGTCTTTTCTCTGGATC-3′), F: *DAT* (5′−CCAAGCTATTAATTTACGTCC-3′), R: *DAT* (5′−CTTTCCTTAGCTCATTAATCACT-3′), F: *GS* (5′−GTGAACGGGATGTGAAGAT-3′), R: *GS* (5′−TCTCTACTTTGCTGCCAACT-3′). Real-time quantitative RT-PCR amplification was accomplished using PrimeScript ^{TM} RT Reagent Kit (Takara) according to the manufacturer's instructions. PCR conditions consisted of a 95 °C for 2.5 min, 40 cycles of 95 °C for 15 s, 78 °C for 15 s and 72 °C for 20 s. The abundance of targeted genes transcripts was normalized to *rbcl* mRNA and was determined by the standard $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001).

3 RESULTS AND DISCUSSION

3.1 Alkaloid contents

As it was considered before, the low levels of dimeric anticancer drugs, their costliness, and their difficult chemical biosynthesis have attracted the attention of many researchers and prompted them to find ways to optimize the production of these TIAs. Using Cr + Mj as an inducer and studying the responses of the plant to this abiotic stress and examining individual gene expression in the biosynthetic pathway and the relationship between genes and the construction of TIAs could enhance the comprehension of the whole interplay. Previous researches explained that heavy metals, when applied in low concentrations, in most cases causes positive effects and increased metabolite production (Wójciak-Kosior et al., 2016). Also Mj has been proved to be able to elicit the production of several compounds (alkaloids, terpenoids and phenolic phytoalexines) in many plant species (Van der Fits & Memelink, 2000). The present investigation found that after 0.5 and 8 h treatment there wasn't any significant difference between groups. After 4 h-treatment, only 100 µM Mj + 100 µM Cr and 100 µM Mj + 50 µM Cr caused a significant increase in ajmalicine and vinblastine respectively. After 24 h-treatment, Mj separately and in combination with two concentrations of Cr significantly elevated vincristine content compared to control but the content of vinblastine, ajmalicine and catharanthine significantly increased only in joint treatment. About vindoline, only 100 µM

Mj + 50 µM Cr significantly increased it compared to control. The maximum total yield of vincristine was 1.52-fold more than control in response to 100 µM Mj. This increase was 2.16, 4.01, 2.39 and 1.97-fold for ajmalicine, vinblastine, vindoline and catharanthine respectively, in response to 100 µM Mj + 50 µM Cr (Figures 1a, 1b, 1c, 2a, 2b). Mj alone and combined with 50 and 100 µM Cr significantly increased total alkaloids after 4, 8, 24 and one week of treatment (Figure 2c). So, this result confirmed earlier reporters on TIA biosynthesis under Copper treatment (Pan et al., 2015) or Mj application (Peebles et al., 2009). Also it can be deduced that the highest values for all alkaloids were observed after application of two treatments simultaneously that shows the additive effects of combined treatments on increasing alkaloid. Reduction in 100 µM Cr treatment is probably due to the effects of high concentration of metal and gradual degredation of the plant. This improvement in indole alkaloid production may also be explained by the activation of the transcription of their biosynthetic genes.

3.2 Gene expression analysis

In the current study, qRT-PCR was used to study *PRX1*, *DAT*, *STR*, *GS*, *ORCA3,* and *MAPK3* transcripts in response to Mj + Cr. As seen in Figure 3, when exposed to100 µM of Mj combined with 50 and 100 µM of Cr, the expression of *PRX1*, *DAT*, *STR*, and *GS* increased significantly compared to control. The maximum expression level of *PRX1* and *GS* were obtained after 24 h treatment and was 6.25 and 4.87-fold respectively in response to100 µM of Mj + 50 µM Cr. *DAT* expression levels didn't show any significant difference in response to100 µM of Mj + 50 and 100 µM Cr after 24 h. The maximum expression level of *STR* was obtained after 24 h of treatment and was 5.92-fold in response to to100 µM of Mj + 100 µM Cr. This result is in agreement with previous studies that showed vincristine and vinblastine were accumulated significantly in plants with PRX1, DAT, and STR overexpression, indicating that *PRX1*, *DAT,* and *STR* are actively involved in the biosynthesis of these alkaloids (Pan et al., 2016). GS expression under stress has never been investigated, but from these results, it seems to have a pattern similar to *PRX1*, *DAT,* and *STR.* Some investigations about the effects of exogenous Mj on the expression of the biosynthetic genes of TIA pathway has been done and all of them have shown that Mj cause an up regulation of many genes like G10H, TDC, STR, D4H, etc in this pathway (Zhang et al., 2011). Also, many abiotic stresses (like heavy metal Cr^{+6}) not only increased the TIA biosynthetic genes but also induced the genes related to biosynthesis and signaling of JA (Raina et al., 2012). From these studies, we infer that both of these two exogenous treatments, Mj and Cr are participating in signal transduction pathways that cause the accumulation of TIAs in stress conditions in *C. roseus*.

The molecular and signal transduction mechanisms involved in the plant's defense against Cr stress was partially known, but recent findings suggest that transcriptional regulation of TIA pathway is under a complex control containing many TFs, known as ORCAs, which are regulating the primary and secondary metabolism of *C. roseus* in response to jasmonate. The TFs directly regulate the expression of many downstream stress-related genes by making a connection with the cis-elements located in the promoter region and thus leading to abiotic stress tolerance (Trinh et al., 2014). There are many reports about the role of ORCAs against various abiotic stresses in recent researches (Singh et al., 2002). Also in *C. roseus,* the elevated expression of several genes from the biosynthetic pathway of TIAs, like *As*, *Cpr*, *Str*, *Sgd*, *Tdc*, *D4h,* and *Dxs*, due to overexpression of *ORCA3* can enhance the accumulation of TIAs. Some previous studies on DAT have shown that this gene is regulated by another TF named ORCA2 (Liu et al., 2007).

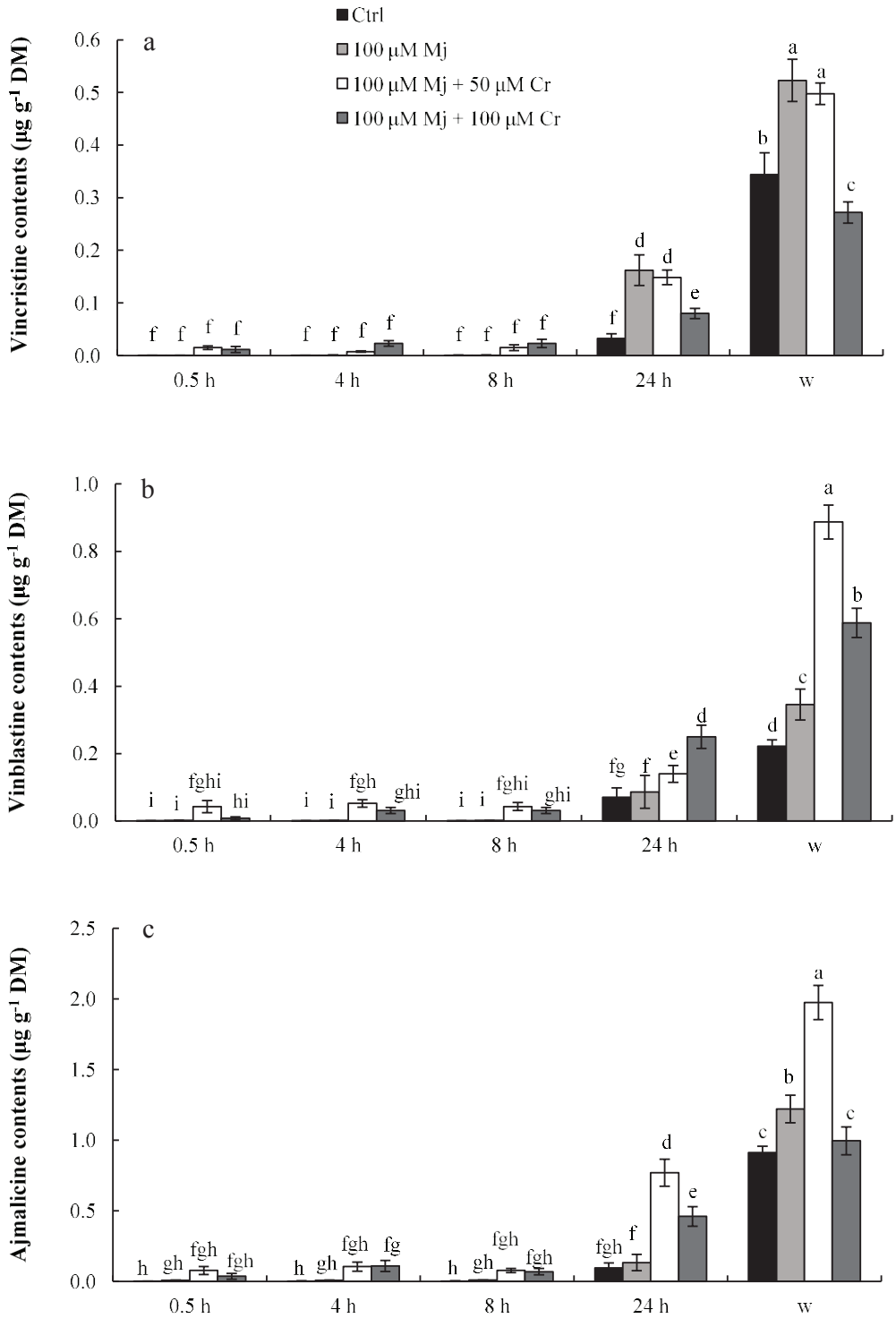

Figure 1: Effects of Cr + Mj treatments on vincristine (a), vinblastine (b), ajmalicine (c) contents on in vitro propagated *C. roseus* shoots after 0.5, 4, 8, 24 h and one week (w) treatment. The experiments were performed in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at $P < 0.05$ according to Duncan test.

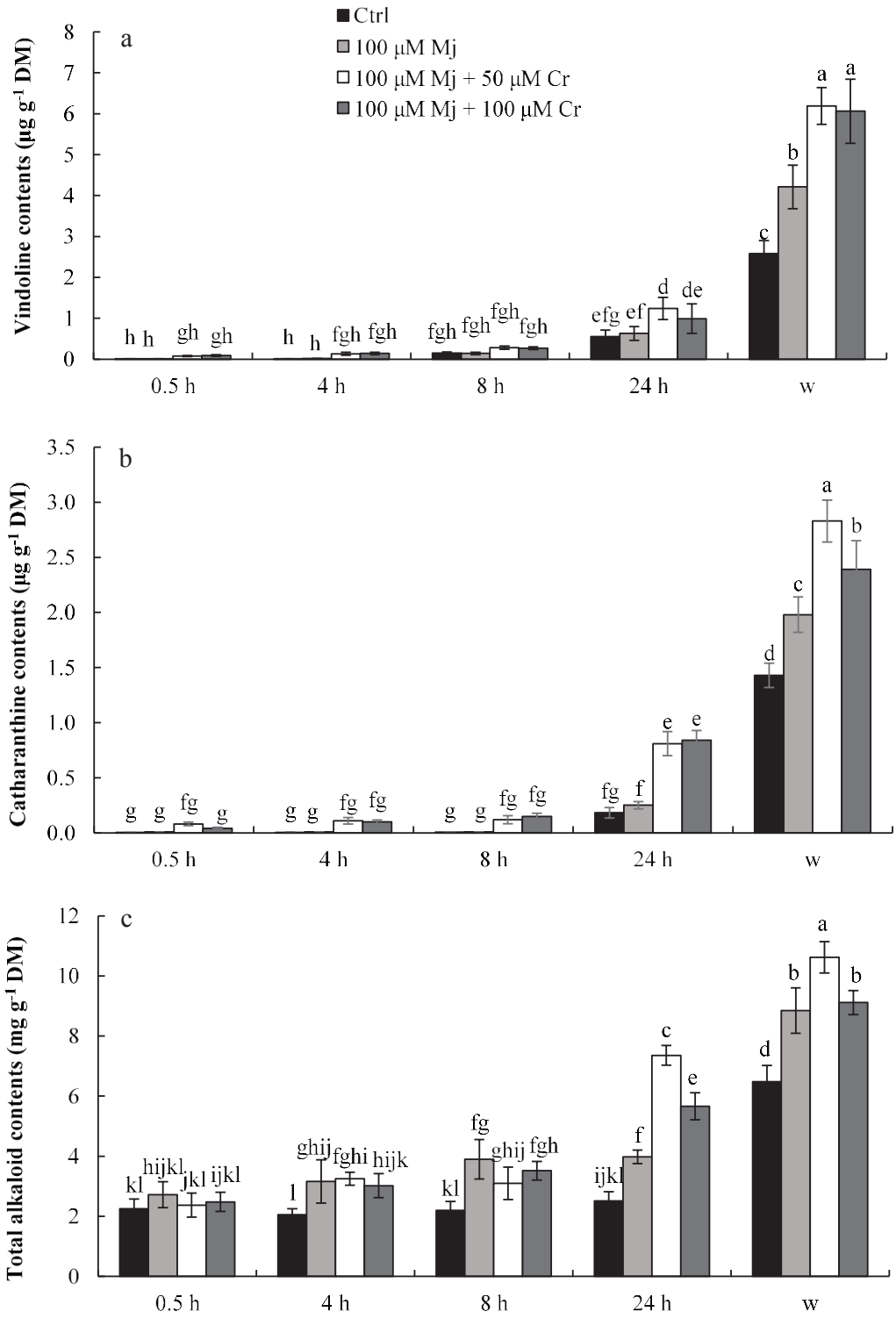

Figure 2: Effects of Cr + Mj treatments on vindoline (a), catharanthine (b), total alkaloid (c) contents on in vitro propagated *C. roseus* shoots after 0.5, 4, 8, 24 h and one week (w) treatment. The experiments were performed in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at $P < 0.05$ according to Duncan test.

Internal or external signals regulate the TFs and cause controlled responses (Pan et al., 2016). Protein kinases and protein phosphatases are two main parts of signal transduction and response to stresses in plants that work through the phosphorylation and de-phosphorylation of proteins. MAPK cascade is one of these protein kinases that have several critical tasks, such as activation in defense responses to many stresses jasmonates biosynthesis, expression of jasmonate-inducible genes, responding to hormones that include ROS signaling (Dubey et al., 2010, Gao et al., 2010). In all eukaryotic cells, the activation of MAPK cascade results in the activation of TFs which convert the extracellular stimulus to intracellular responses. This cascade includes three major kinases, one of which is the MAPK that is the terminal component of this signaling cascade. *MAPK3* is the most well characterized of these MAPKs. The current results showed that there was an upregulation of *ORCA3* and *MAPK3* in response to Mj alone and in combination with Cr treatments, in consistent with Gao et al. (2010) reporting that transcription of *ORCA3* and orthologs of *MAPK3* in other plants were upregulated by Mj treatment and different stresses like high salinity, cold, heat, wounding, chitin, UV, osmotic, and oxidative stresses. The highest level of *MAPK3* and *ORCA3* expression was 7.67 and 5.38-fold higher than control appear 8 h after 100 µM of Mj + 50 µM Cr treatment.

Based on our results, it takes a longer time to raise *STR*, *GS*, *DAT,* and *PRX1* transcription levels compared to *MAPK3* and *ORCA3*. This is evidence of the fact that *MAPK3* and *ORCA3* are at the beginning of the signaling pathway, activated at an early stage immediately after the induction of stress. Their expression is increased, but over time, they may influence the other defense responses and biosynthetic genes of secondary metabolites. One of the most important points that previous studies have detected about *ORCA* and *MAPK* is that these genes interact with each other. They may also have reciprocal regulation roles between them which elevate the expression of the TIA pathway genes to combat abiotic stress (Pan et al., 2016). In the current work, *ORCA3* and *MAPK3* reacted positively to the signals, signifying peculiar cognition and exhibited their maximal activity. They also promoted each other in the expression of TIA pathway genes to combat Mj and Cr stress. According to recent studies, abiotic stresses, for example dehydration, cold, H_2O_2 and salicylic acid (SA), initiate the signal transduction pathway which is similar for heavy metal responsive TFs (DalCorso et al., 2008). Therefore, following these findings, the mechanism of Mj and Cr may be done through two distinct ways and cross-talk across these two separate ways or stimulation of a third way by the joint attendance of Mj and Cr." This could explain the increasable effect perceived for the accumulation of vinblastine and vincristine.

3.3 Lipid peroxidation

Cr as a toxic heavy metal has several effects and mechanisms to induce ROS. This is the initiation of oxidative stress, because these free radicals might mutilate the membrane architecture, cause oxidative damage, and motivate lipid peroxidation as reported in other higher plants. Here, the results (Figure 4a) suggest that combined treatment elevated the amount of lipid peroxidation after 4 h and one week but more precisely, 100 µM Mj + 100 µM Cr causes significant increase in all time courses, indicates that extensive oxidative damages could have occurred to the cells under Cr stress especially in its higher concentrations. It was showed that Cr, like other metals recently studied such as aluminum, lead, and arsenic, has promoted production of ROS leading to a rise in lipid peroxidation and have similar toxic effects (Sharmin et al., 2012). Also, Kupper et al. (2009) demonstrated that Mj has a strong potential to stimulate ROS production and oxidative stress with the strongest response at 100 mM according to Kumari et al. (2015). In proceed to the previous studies, application of joint treatment in our work increased ROS production, mutilate the membrane architecture, create an oxidative damage and motivate lipid peroxidation.

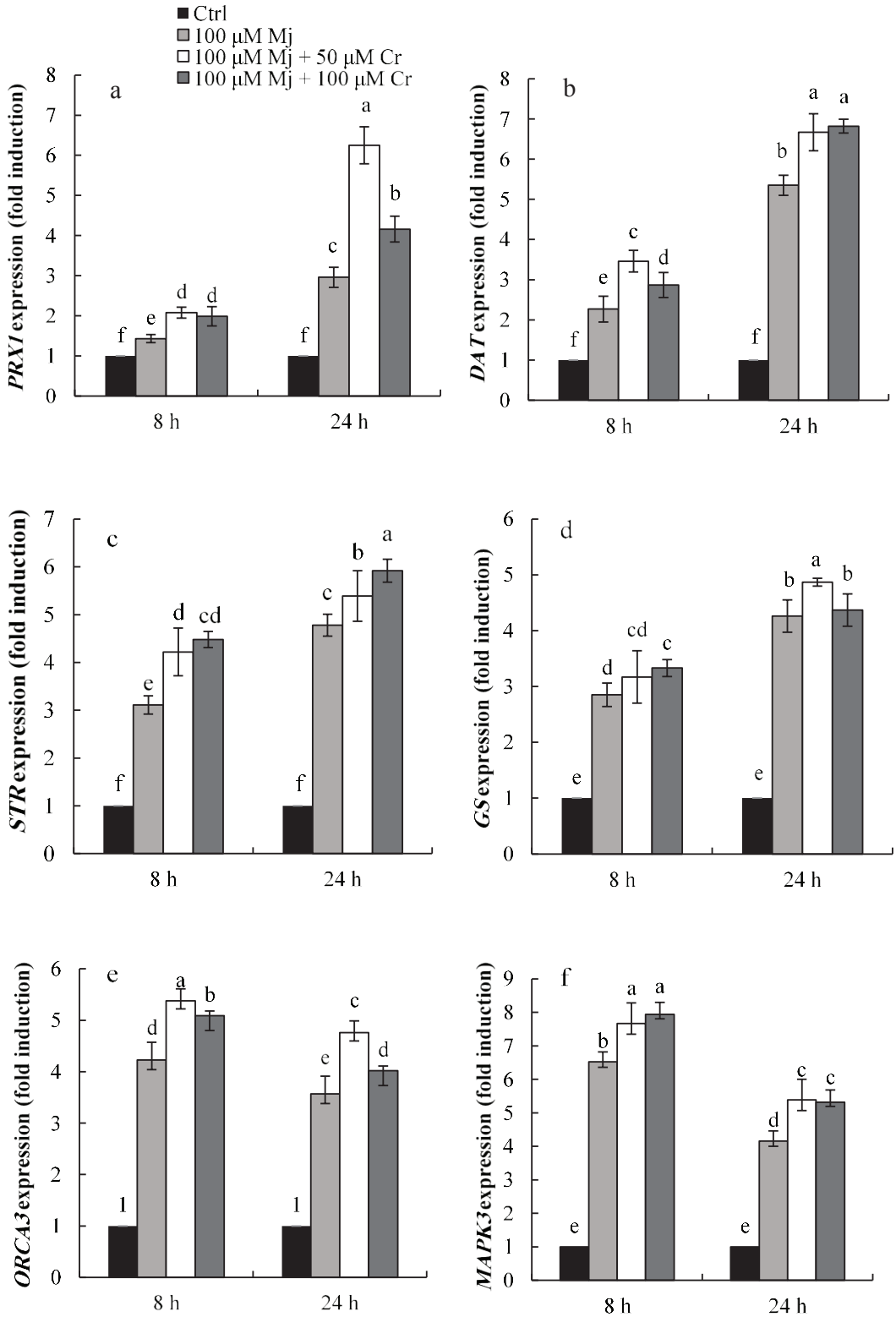

Figure 3: Effects of Cr + Mj treatments on expression patterns of *PRX1* (a), *DAT* (b), *STR* (c), *GS* (d), *ORCA3* (e), *MAPK3* (f) for 8 h and 24 h. The experiments were performed in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at $P < 0.05$ according to Duncan test.

3.4 Phenol, flavonoid and carotenoid contents

In addition to producing alkaloids as nonenzymatic antioxidants, carotenoids and phenolic compounds may also play a role during stress by preserving unstable macromolecules and inducing stability against metals. After 0.5 h treatment, there wasn't any significant difference in phenol and flavonoid contents compared to control but slowly over time, variations happened and Mj alone and combined with two concentration of Cr caused significant increases in total phenol and flavonoid contents after 8, 24 h and one week. The highest contents of total phenol and flavonoid (1.310 and 1.042 mg g^{-1} FM, respectively) were observed in the 100 µM Mj + 100 µM Cr treatments after one week (Figures 4b and 4c).

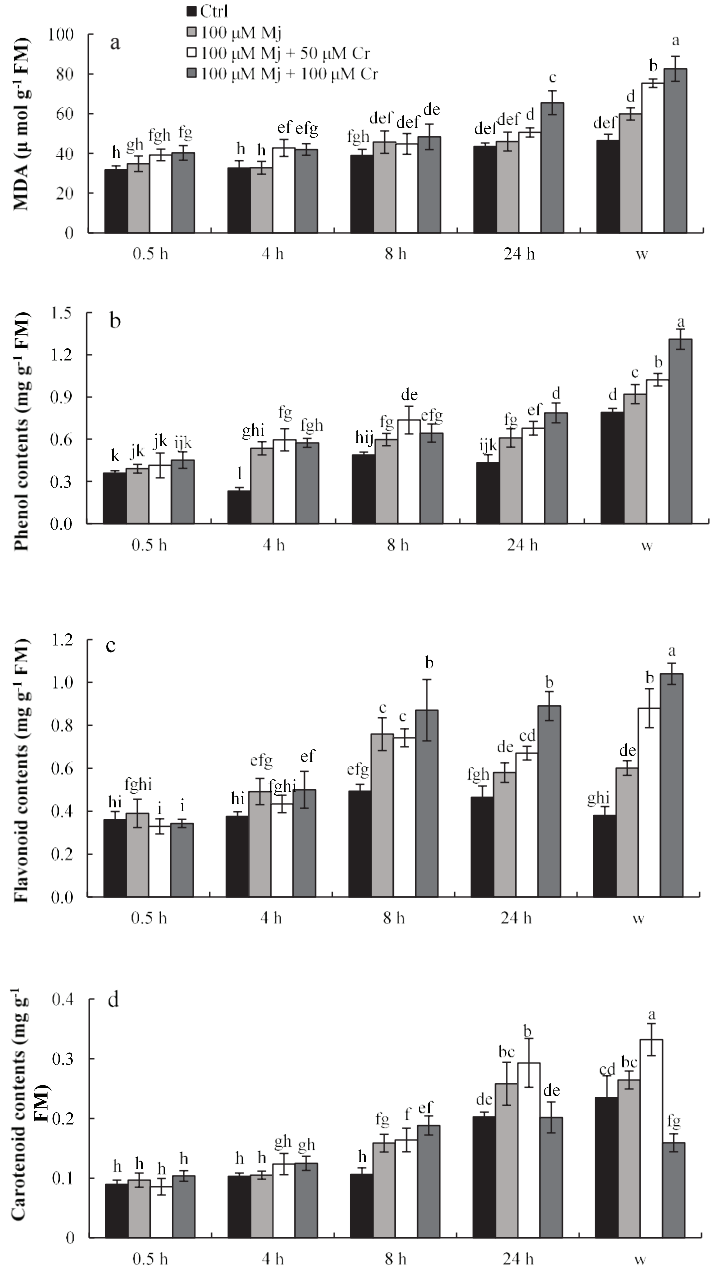

Figure 4: Effects of Cr + Mj treatments on MDA (a) total phenol (b), flavonoid (c), carotenoid (d) contents on in vitro propagated *C. roseus* shoots after 0.5, 4, 8, 24 h and one week (w) treatment. The experiments were performed in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at $P < 0.05$ according to Duncan test.

This increase offered elevated defense system because the hydroxyl and carboxyl groups of these metabolites have the ability to attach metal ions like Cr, chelate them, and thereby prevent Fenton's reaction, which is the main origin of ROS production. Flavonoids also regulate the polar transportation of auxin which controls the stomatal opening and manages the allocation of resources to help the plant overcome weak growth conditions under stress (Singh et al., 2016). The increase in total phenol and flavonoid contents in a separately heavy metal and Mj stress was proved in previous studies like Emamverdian et al. (2015) reviewed heavy metal effects on plants, and also the study of Ozturket al. (2015) worked on effects of pre-harvest Mj and reported matching results. İncreased contents of total phenolic and flavonoid compounds in the current work are in consonance with those studies, it also proves that the simultaneous effect of these two treatments has strengthened this antioxidant property.

C. roseus showed increased carotenoid contents after 8 h as a defense strategy when encountering metal stress, because these pigments protect the chlorophyll pigments and avoid the excited singlet oxygen biosynthesis. Furthermore, they suppress the photodynamic reactions and replace peroxidation. Meanwhile, it should be noted that 100 µM Cr significantly decreased the carotenoid contents after one week which may be again due to gradual degredation of the plant. The highest content was 0.33 mg g^{-1} FM and observed in the 100 µM Mj + 50 µM Cr treatments after one week (Figure 4d).

3.5 Enzymatic analysis

Plants possess another kind of defense known as enzymatic antioxidants (SOD, CAT, and POD) that act as the scavengers of free radicals. Antioxidative enzymes may behave variably in response to oxidative stresses. Antioxidative enzymes work in a contributive or synergistic manner to safeguard against oxidative stress. The current results showed after 0.5 h, Mj combined with 50 µM Cr increased CAT and combined with 100 µM Cr increased SOD activity. Combined treatment also increased POD activities after 4, 8 and 24 h. After one week, all treatments increased POD activity but for the two enzymes CAT and SOD, this happened only at the treatments of Mj alone and combined with 50 µM Cr and a decline at 100 µM compared to 50 µM Cr (Figure 5a, 5b and 5c) was observed. This decrease may be attributed to the high affinity of Cr ions to thiol compounds that interrupts protein synthesis and enzyme activity (Mourato et al., 2012). As can be seen in some conditions, lower concentrations of a metal may cause an increase in enzyme activities, but using higher concentrations breaks the defense system and decreases the activities. The activation of an enzyme itself or the upregulation of its gene expression may be reasons for the increase in amounts of an enzyme. Furthermore, metals can change enzyme structures, and therefore the enzymes activities are decreased. On the other hand, it was found that Mj (50 and 100 µM) elevated production of numerous antioxidative enzymes and their storage (Giri & Zaheer, 2016) so it can alleviate the oxidation by improving the ROS scavenging system stress (Jung, 2004, Aftab et al., 2011). Therefore, it is inferred that combined use of Mj + Cr induces a stronger activation of enzyme activities and these defense responses could act separately or be joined into one strategy to reduce membrane destruction and elevate cell growth or preserve cell maintenance in response to stress.

Our findings demonstrated that after 0.5 h, combined treatments increased the protein contents. After 4, 8 and 24 h, there wasn't any significant difference but after one week only Mj alone significantly increased it and a significantly decrease in protein contents occurred by 100 µM Cr compared to other treatment groups (Figure 5d). The protein rising in early hours is probably due to the plant's rapid response for launching defensive responses under stress conditions. The elevation by jasmonates may be because of induction of gene expression leading to biosynthesis of many proteins maybe proteins related to defense mechanisms, in agreement with Poonam et al. (2013) reported the accumulation of proteins induced by Mj in *Cajanus cajan* (L.) Millsp., but Cr induced decrease after one week might be a consequence of the elevation of catabolic enzymes like proteases, which were stimulated under Cr stress. Another reason is the protein denaturation and oxidation as a consequence of changes in thiol groups of proteins, which leads to increases in the production of carbonyl groups and in the rate of proteolysis that similar to our results was reported by Mourato et al. (2012).

The present study is a small-scale assay aimed at revealing the signal transduction mechanism of *C. roseus* and treatment with Cr and Mj to provide a way to produce significant values of these anticancer metabolites, the only precursors of anticancer drugs. Based on the findings, it is suggested that Mj responsive MAPK3 and ORCA3 are important components of the signal transduction pathway. However, full recognition of the regulatory mechanisms of this biosynthetic pathway requires further studies in this regard.

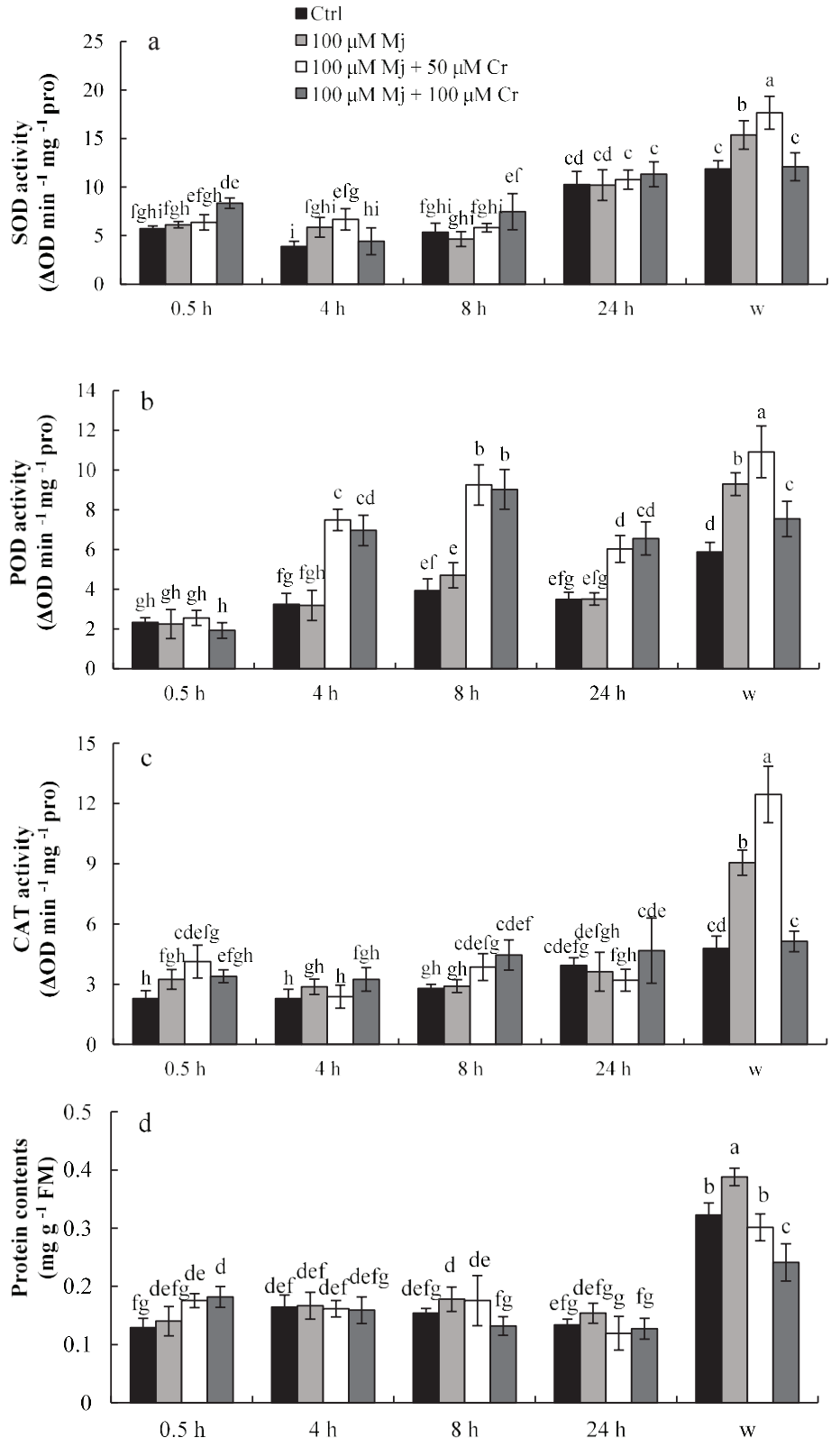

Figure 5: Effects of Cr + Mj treatments on activities of SOD (a), POD (b), CAT(c) and protein contents (d) on in vitro propagated *C. roseus* shoots after 0.5, 4, 8, 24 h and one week (w) treatment. The experiments were performed in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at $P < 0.05$ according to Duncan test.

4 CONCLUSIONS

The results of the current study have demonstrated that combined abiotic treatments such as Mj + Cr can influence the production of secondary metabolites and can be a commercial way to enhance the potential to overproduce medicinal valuable chemicals (here, alkaloids like vindoline, catharanthine, vincristine, vinblastine, and ajmalicine) with high pharmaceutical values. Both Mj and Cr are oxidizing agents and induce the formation of free radicals and hyperactivates the antioxidant defense system, like phenolics, flavonoids, carotenoids, and enzymes, as a part of the general stress response. However, 100 µM Mj + 100 µM Cr showed a toxic effect on samples and reduced the alkaloids. The results of the current study agreed with a recent model named "elicitor-based signaling model" for appended stimulation of gene expression in this plant, which explained the connection of elicitor to receptor turns on the signal transduction pathway of the MAPK cascade, leading to endogenous JA biosynthesis. Increase in endogenous in addition to exogenous JA as a signal messenger activates the synthesis of nuclear proteins ORCA3. These proteins cooperate with the promoter of the biosynthetic genes and motivate the biosynthesis of TIA alkaloids.

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Detection and characterization of endophytic bacteria causing knot in young olive trees

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ABSTRACT

Olive knot is an important disease in most countries where olives are commercially grown. In the spring of 2015, some galls were observed on the trunk and branches of 4-year-old olive trees in the north of Iran. The bacteria were isolated from galls and all isolates were gram-negative, aerobic, and capable of producing florescent pigment. Other phenotypic characteristics of the isolates were assessed. Pathogenicity tests were carried out on olive branches incubated with different isolates. Primary symptoms were observed after two weeks. Sequences of 16S rRNA and RNA polymerase beta subunit genes of pathogenic isolates were completely similar to *Pseudomonas savastanoi* pv. *savastanoi* (Smith 1908) Young et al. 1978 in GenBank. Based on the results from phenotypic analyses, pathogenicity tests and phylogenetic data, the isolates were identified as *P. savastanoi* pv. *savastanoi*. The host range of our isolates was specific to olive trees. None of the inoculated oleander (*Nerium oleander* L.), winter jasmine (*Jasminum nudiflorum* Lindl.), Japanese privet (*Ligustrum japonicum* Thunb.) and ash (*Fraxinus excelsior* L.) developed disease symptoms. No difference in disease resistance was observed between six studied olive cultivars. There was no olive tree or orchard around the studied orchard as far as more than one kilometer. As the disease agent listed in Iran's foreign quarantine pests and diseases list, appropriate quarantine and phytosanitary measures were undertaken to eradicate the disease.

Key words: *Pseudomonas savastanoi* pv. *savastanoi*; Phenotypic identification; 16S rRNA, *rpoB*; cultivar resistance

IZVLEČEK

DOLOČANJE IN OPIS ENDOFITSKIH BAKTERIJ, KI POVZROČAJO OLJKOVEGA RAKA NA MLADIH OLJKAH

Oljkov rak je pomembna bolezen v vseh deželah, kjer gojijo oljke. Spomladi leta 2015 so bili v severnem Iranu na deblih in vejah štiriletnih oljk opaženi tumorji. Iz njih so bile izolirane gram negativne bakterije, ki so bile sposobne tvoriti fluorescentni pigment. Ocenjene so bile tudi druge fenotipske lastnosti izolatov. Test patogenosti različnih izolatov je bil opravljen na oljčnih vejah. Prva bolezenska znamenja so se pojavila po dveh tednih. Zaporedja 16S rRNK in genov beta podenote RNK polimeraze iz patogenih izolatov so bila popolnoma podobna tistim iz bakterije *Pseudomonas savastanoi* pv. *savastanoi* (Smith 1908) Young et al. 1978, iz GenBank. Na podlagi rezultatov fenotipskih analiz, testov patogenosti in filogenetskih podatkov so bili izolati določeni kot vrsta *P. savastanoi* pv. *savastanoi*. Gostitelji izoliranih bakterij so bile samo oljke. Na nobeni od inokuliranih drugih vrst, kot so zimski jasmin (*Jasminum nudiflorum* Lindl.), navadni oleander (*Nerium oleander* L.), japonska kalina (*Ligustrum japonicum* Thunb.) in veliki jesen (*Fraxinus excelsior* L.*),* se niso razvila bolezenska znamenja. Med šestimi preučevanimi sortami oljk ni bilo razlik v odpornosti proti bolezeni. V okolici preučevanega oljčnika ni bilo v razdalji več kot kilometer nobene oljke, niti oljčnika. Povzročitelj bolezni je v Iranu na seznamu tujerodnih karantenskih škodljivcev in bolezni, zato so bili sprejeti ustrezni karantenski in fitosanitarni ukrepi za izkoreninjenje bolezni

Ključne besede: *Pseudomonas savastanoi* pv. *savastanoi*; fenotipska identifikacija; 16S rRNA, *rpoB*; sortna odpornost

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1 INTRODUCTION

Pseudomonas savastanoi Smith 1908) Young et al. 1978 includes four pathovars; *P. savastanoi* pv. *savastanoi*, pv. *nerii*, pv. *fraxini*, and pv. *retacarpa* that cause knot or excrescences in olive (*Olea europaea* L.), oleander (*Nerium oleander* L.), common ash (*Fraxinus excelsior* L.), and Spanish broom (*Retama sphaerocarpa* L.), respectively (Caballo-Ponce et al., 2017). In addition, bacterial agents of soybean brown spot and halo blight disease on bean are *P. savastanoi* pv. *glycinea* and *P. savastanoi* pv. *phaseolicola*, respectively (Addy & Wahyuni, 2016; Marques & Samson, 2016).

The gamma proteobacterium *P. savastanoi* pv. *savastanoi* (here after Psv) causes olive knot disease. The disease is one of the most economically relevant diseases of the olive trees and cause serious reduction in crop yields (Agrios, 2005; Campos et al., 2009). Olive knot represents a serious disease in many olive-producing areas, which can cause a progressive plant decline that leads to reduction in the number of fruit-bearing shoots and tree yield potential (Quesada et al., 2010). Disease symptoms are characterized by tumorous outgrowths, called knot or gall. The knots appear on different parts of the plant, mainly on twigs and young branches (Ramos et al., 2012). Olive knot disease seriously affects olive trees mainly in Mediterranean countries, where climatic conditions often favor spread of the disease. The development of these galls results from uncontrolled cell growth due to disruption in plant hormone balance. Gall appearance is dependent on auxin phytohormone, indole-3-acetic acid (IAA), produced by pathogenic bacteria (Kieffer et al., 2010). Produced IAA can interfere with plant development by disturbing the auxin balance in plants (Caballo-Ponce et al., 2017). Several auxin biosynthetic pathways in plant galls forming bacteria have been described, which are mostly dependent on L-tryptophan as a precursor (Spaepen et al., 2007).

It has been reported that olive knot formation is hrp/hrc dependent (Sisto et al., 2004) and biosynthesis of auxin has been described as a pathogenicity or virulence factor (Patten et al., 2013). The other phytohormones involved in gall production are cytokinins (CKs) such as zeatin, dihydrozeatin, 1-methyl-zeatin, ribosylzeatin, ribosyldihydrozeatin, and ribosyl-1-methylzeatin, as well as diverse methylated zeatin derivatives (Caballo-Ponce et al., 2017). Some of the functions that are attributed to these hormones are control of different processes in plant growth and development of plant defenses against stresses (O'Brien & Benkova, 2013).

In recent years, there has been an increasing interest in olive cultivation in many countries probably due to the olive oil benefits for human health. The bacterium lifestyle in olive knots has already been described in greater detail (Rodriguez-Moreno et al., 2009). In saprophytic phase, Psv can duplicate on phylloplane of the olive tree (Quesada et al., 2007) and spreads by windblown aerosols, splashing rain and cultural practices at short distances. Wounds caused by insects, pruning and harvest create entry sites through which infection can occur (Quesada et al., 2010). Secondary tumors develop with migration of the pathogen within the host (Penyalver et al., 2006; Marchi et al., 2009). The bacterium can also survive in side knots from one season to the next. Efficient control of olive knot disease is based on the preventive measures (Quesada et al., 2010; Ramos et al., 2012). Recently, schemes for the production of certified olive plants free from bacteria and other pathogens, including Psv have been published (EPPO, 2006).

Olive tree cultivation has expanded in recent years in different parts of Iran as well as in many other countries. In the current study, bacterial agent of olive knot detected in a young orchard was phenotypically and genotypically characterized. Then, pathogen host range and susceptibility to olive knot disease was evaluated in common olive cultivars.

2 MATERIAL AND METHODS

2.1 Sampling procedures and the bacterial pathogens isolation

In spring of 2015, galls were observed on trunk and branches of 4-year-old olive trees in an orchard in Golestan province, located in the north of Iran. Sampling was carried out by cutting knots from different trees. The knots were placed in plastic bags, transported to the laboratory and processed immediately. The knots were surface-disinfected with a paper moistened with ethanol 70 % (Marchi et al., 2005). Small fragments (1-2 mm) were cut aseptically with a sterile scalpel then placed in one ml of sterile distilled water (SDW). After 20 min, a loopful of the resulting suspension was streaked on plates containing King's B medium (KB), and then incubated at 26 °C for 3-5 days. Single colonies were collected and checked for purity. A total of nineteen isolates, PS01-PS19, were obtained from olive knots. A reference strain from

Instituto Valenciano de Investigaciones Agrarias (IVIA 1657-8) was used in all phenotypical and biochemical analyses.

2.2 Phenotypical characterizations of isolates

Physiological and biochemical characteristics of the isolates were determined by standard bacteriological methods including: gram-stain reaction, fluorescent pigment production on KB medium, colony morphology on nutrient agar (NA), levan, oxidase, pectinolytic activity, arginine dehydrolase, and tobacco hypersensitivity reaction (LOPAT) according to Lelliott et al. (1966). Tween 80 hydrolysis, indole production with Kovacs reagent, catalase reaction, nitrate reduction, starch hydrolysis, growth at 37 °C, growth in general media containing 3, 5 and 7 % (wv^{-1}) NaCl, gelatin liquefaction, esculin and casein hydrolysis, H_2S production from L-cysteine and reducing compounds from sucrose based on Schaad et al. (2001). In addition, utilization of sugars and amino acids as a sole carbon and nitrogen source by studied isolates was evaluated.

2.3 Pathogenicity tests

A pathogenicity test was performed for all studied isolates. Bacterial suspension was prepared from pure culture (10^8 CFU ml^{-1}) grown for 48 h on KB medium. Wounds of around one cm were cut in the bark of one-year-old olive stems and inoculated directly with a scalpel dipped in bacterial suspension or bacterial colony. Each isolate was inoculated at five wounding sites. Wounds were protected with parafilm for three days. The inoculated trees were kept in a greenhouse at 25 °C and inspected for knot formation for four months. Negative control trees were inoculated with phosphate buffered saline (PBS).

2.4 DNA extraction

Bacterial isolates were grown for 48 h at 26 °C on KB medium. DNA was extracted from bacterial suspensions (10^9 CFU ml^{-1}) using the protocol described by Llop et al. (1999). The DNA was dissolved in SDW before quantification by spectrophotometer and kept at -20 °C until use. In the direct isolation method, bacterial suspension was adjusted to 10^7-10^8 CFU ml^{-1} in SDW. After adding 100 µl of 0.05 M NaOH to 10 µl bacterial suspension, sample incubated at 95°C for 15 min, and 2 µl of the boiled suspension was used as template for the PCR tests (Rademaker & de Bruijn, 1997).

2.5 PCR amplification

Molecular identification of bacterial isolates was carried out using a universal primer pair for amplification of 16S rRNA fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') in a standard PCR assay (Weisburg et al., 1991). PCR reactions were performed in a 20 µl PCR mixture containing 1X PCR buffer (Fermentas, Germany), 3 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphates, 10 pM of each primer, 1 U Taq DNA polymerase and 50 ng μl^{-1} of template DNA. PCR amplification was carried out under the following conditions: initial denaturation cycle at 94 °C (5 min), 35 cycles at 94 °C (1 min), 62 °C (1 min) and 72 °C (1.5 min), and then one cycle at 72 °C for 7 minutes in a Bio-Rad thermal cycler.

Amplification of *rpoB* gene with oligonucleotide primer pair LAPS (5'-TGGCCGAGAACCAGTTCCGCGT-3') and LAPS27 (5'-CGGCTTCGTCCAGCTTGTTCAG-3') was used in a standard PCR assay (Tayeb et al., 2005). PCR reactions were performed in a 20 µl PCR mix contained 1X PCR buffer (Fermentas, Germany), 3 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphates, 10 pM of each primer, 1 U Taq DNA polymerase and 50 ng μl^{-1} of template DNA. PCR amplification was carried out under following condition: initial denaturation cycle at 94 °C (5 min), 40 cycles at 94 °C (10 s), 50 °C (20 s) and 72 °C (50 s), and then one cycle at 72 °C for 5 minutes in a Bio-Rad thermal cycler. Six µl of amplified products were separated by electrophoresis on a 1 % agarose gel, stained with ethidium bromide and photographed by a gel documentation system. After staining, the results were observed using gel-document (Syngene, USA). Purification of amplified DNA fragments was done with a high pure PCR product purification kit (Roche, Germany) and Sanger sequenced (Macrogen, South Korea).

2.6 Phylogenitic analysis

The 16S rRNA and *rpoB* sequences were compared with available sequences in GenBank using the BLAST search algorithm at NCBI. Alignments were built in ClustalW (Thompson et al., 1994), and subsequently adjusted manually in BioEdit Ver.7.0.9 (Hall, 1999). Phylogenetic relations were inferred from applying the Kimura-2-parameter model (Kimura, 1980) with the neighbor joining (NJ) algorithm (Saitou & Nei, 1987) implemented in MEGA7. The branch support was assessed by computing 1000 bootstrap estimates (Tamura et al., 2007).

2.7 Cultivar susceptibility

Two-year-old plants of six olive cultivars (*Olea europaea* 'Arbequina', 'Arbosana', 'Beldi', 'Koroneiki', 'Manzanilla' and 'Mission') were used for the evaluation of cultivar susceptibility to Psv. The plants were wounded at five sites on the main stem and inoculated with five pathogenic strains (PS1, PS3, PS6, PS10 and PS17), separately. Three plants per cultivar were used (75 sites were inoculated per each cultivar). The

bacterial suspensions were made in phosphate PBS and concentration was adjusted to 10^8 CFU ml^{-1}. Bacterial suspension of 100 µl was injected tangentially into the bark using a sterile needle, and then the hole was covered with parafilm for three days. The negative control plants were inoculated with PBS only. The plants were kept in a greenhouse at 23-26 °C and 75-80 % RH. The disease symptom development was monitored for four months after inoculation. The disease response for each cultivar was evaluated as the proportion of inoculated wound sites developing knots after three months after post inoculations (Penyalver et al., 2006).

2.8 Hosts range test

One and two-year old olive (*Olea europaea* L.), oleander (*Nerium oleander* L.), winter jasmine (*Jasminum nudiflorum* Lindl.), Japanese privet (*Ligustrum japonicum* Thunb.) and common ash (*Fraxinus excelsior* L.) plants were used for determining host range. Inoculation was performed for cultivar susceptibility, as described above. The plants incubated with PBS were used as negative controls (Iacobellis, 2001).

3 RESULTS AND DISCUSSION

Olive knot disease is one of the important diseases caused by *P. savastanoi* pv. *savastanoi*, which can cause significant yield losses. Psv survives epiphytically and penetrates through the wounds, particularly through leaf scars and mechanically caused wounds (e.g. pruning), where bacterial infections and colonization result in a knot formation (Quesada et al., 2010). The typical knot of this disease is caused by phytohormones produced by the bacteria, which cause proliferation of cells surrounding the infection area. Olive knot is present mainly in Mediterranean countries, where climatic conditions often favor the spread of the disease (Moretti et al., 2017). During the research which was conducted in the spring of 2015, we found knot symptoms on trunk and branches of 4-year-old olive trees in the north of Iran. To identify and characterize the bacterial disease agent, we phenotypically and genotypically characterized the pathogenic isolates. In addition, host range and cultivar susceptibility to the disease of common olive varieties were determined.

3.1 Symptomatology and phenotypic identification of the isolates

The galls on trunk and branches were spherical, pale green to brown in color and had a smooth surface (Figure 1). A total of 19 bacterial isolates were recovered from olive galls. Remarkable similarities were observed among isolates in morphological, biochemical and physiological characteristics.

Figure 1: Gall formation on branches as a symptom of olive knot disease

In phenotypic studies, the similarity among the isolates was at least 80 %. All isolates were gram-negative and aerobic, able to produce florescent pigment on KB medium, levan positive and showed hypersensitive reaction on tobacco leaves. The tests for oxidase, potato soft rot and arginine dihydrolase activity were negative. All studied isolates were negative in additional phenotypic tests such as starch hydrolysis, H_2S production from L-cysteine, indole production, cysteine hydrolysis, growth on media containing 3, 5 and 7 % (wv^{-1}) NaCl, reducing compounds from sucrose and hydrolysis of casein, gelatin, esculin and tween 80. The isolates were positive in catalase, nitrate reduction, urease, tyrosinase and growth at 37 °C. The studied isolates utilized some sugars and amino acids as a sole carbon and nitrogen source as well. The phenotypic, biochemical and nutritional characteristics of the isolates are listed in Table 1. According to the results, all isolates belonged to one species, and no particular grouping based on biochemical and physiological characters was found. Phenotypic features of studied isolates were similar to Psv strains isolated from other countries as described before (Penyalver et al., 2000; Campos et al., 2009; Krid et al., 2009). Taghavi and Hassani (2012) detected *P. savastanoi* from winter jasmine, a member of the Oleaceae family, with gall symptoms on shoots from Fars province in Iran. Phenotypic characteristics of the disease agent were similar to characteristics of our isolates, except that the isolates from this study did not produce levan polymer unlike the isolates from winter jasmine where levan production was variable. Result from another study in Italy showed that bacterial agents of olive knot were levan-positive; therefore, they suggested that the production of levan polymer can be variable among Psv stains (Marchi et al., 2005). In sugars utilization capacity, bacterial isolates from this study used sucrose and sorbitol as sole carbon sources, but in previous reports from Iran the *P. savastanoi* isolated from oleander and winter jasmine did not use these sugars (Ghasemi et al., 2006; Taghavi & Hasani, 2012).

Table 1: Phenotypic and biochemical characteristics of all studied strains

Characteristic	Response	Characteristic	Response
Gram staining	-	H_2S production	-
Anaerobic growth	-	L-cysteine	-
Fluorescent pigment on KB	+	Indole production	-
Tobacco hypersensitive reaction	+	Cysteine hydrolysis	-
Catalase activity	+	Nitrate reduction	+
Levan production	-	Urease	+
Oxidase reaction	-	Tyrosinase	-
Potato soft rot	-	Protease	+
Arginine dihydrolase	-	Reducing compounds from sucrose	-
Tobacco hypersensitive reaction	+	Utilization of:	
Hydrolysis of:		Adonitol, Cellobiose, Erythritol,	
Starch	-	DL- Homoserine, Sorbitol,	+
Casein	-	Sucrose, L-Rhamnose, D- Trehalose	
Esculin	-		
Gelatin	-	Arabinose, Citrate, Inositol,	
Tween 80	-	Mannitol, Melibiose, D-Tartrate, L- Tartrate, Xylose	-
3, 5, 7 % NaCl tolerance	-		

3.2 Phylogenetic analyses

The 16S rRNA is the most common gene used in phylogenetic analyses, because of its ubiquity, essential function and evolutionary properties. In addition, multiple copies of this gene with different nucleotide sequence are often present in a bacterium. Phylogenetic analysis based on 16S rRNA is widely used for identification of bacterial genera (Case et al., 2007; Krid et al., 2009; Rajwar & Sahgal, 2016). However, studies have shown that bacterial phylogeny reconstruction using 16S rRNA gene alone does not accurately describe the diversity of microbial community. As a result, alternative housekeeping genes such as the RNA polymerase beta subunit gene (*rpoB*), ATP synthase beta chain (*atpD*), DNA gyrase beta subunit (*gyrB*), 70-KDa heat shock protein (*dnaK*) and recombinase A (*recA*) have been used together with 16S to determine prokaryotes phylogeny (Case et al., 2007; Rajwar & Sahgal, 2016).

Genotypic identification of pathogenic isolates was performed based on two housekeeping genes,

16S rRNA and *rpoB*. We successfully amplified an expected 1500-bp band of 16S rRNA gene from all studied isolates (Figure 2). All 16S rRNA sequences had 100 % similarity with those of Psv strains from GeneBank Database. The partial 16S rRNA sequences of 1377-bp and 1259-bp obtained from isolates PS06 and PS17 were deposited in GenBank under accession numbers MG930024 and MG930040, respectively.

Another gene used in this study was *rpoB*, one of the core housekeeping genes. We successfully amplified an expected 1247-bp band of *rpoB* gene in all studied isolates (Figure 3). Based on *rpo*B sequences, we identified the *P. savastanoi* isolates. All *rpo*B sequences shared 100 % similarity with the Psv strains from GenBank. The partial sequences of 1046-bp obtained from PS06 and PS17 were deposited in GenBank under accession numbers MF695102 and MF695103, respectively.

The phylogenetic tree was reconstructed using two studied Psv isolates (PS06 and PS17), Psv and other *Pseudomonas* sequences deposited in GenBank. The sequences of *P. viridiflava* (Burkholder 1930) Dowson 1939 (JQ267553) were used as an out-group (Figure 4). Phylogenetic tree indicated that our isolates cluster together with *P. savastanoi* (AJ717422) and *P. savastanoi* pv. *savastanoi* (CP008742). These isolates were located in a separate branch from other species and pathovars of pseudomonads (Figure 4). This result confirmed that *rpoB* gene sequences can be applied in identification different species and pathovars of *Pseudomonas*. Tayeb et al. (2005) successfully identified 186 strains belonging to 75 species of *Pseudomonas* sensu stricto and related species based on *rpo*B gene sequences. Now, *rpoB* gene is used routinely for identification of *Pseudomonas* species. Analysis of 16S rRNA and *rpoB* genes partial sequences of 66 fluorescent pseudomonads strains revealed that phylogenetic resolution of the *rpoB* tree was higher than that of the 16S rRNA tree (Mehri et al., 2013). Furthermore, *rpoB* gene sequence analysis has been implemented in identification schemes of several other bacterial species (Renesto et al., 2000; Benie et al., 2016).

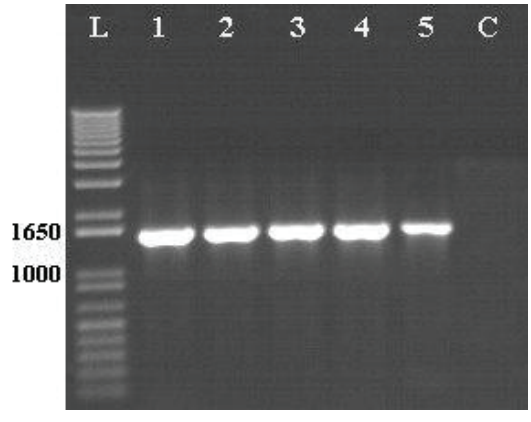

Figure 2: The visualization of PCR amplification product from 16S rRNA gene of *P. savastanoi* pv. *savastanoi* strains isolated from olive trees. Size of the expected product was 1500 bp. L) Ladder, 1) PS01, 2) PS04, 3) PS06, 4) PS17, 5) PS18, and C) water as negative control

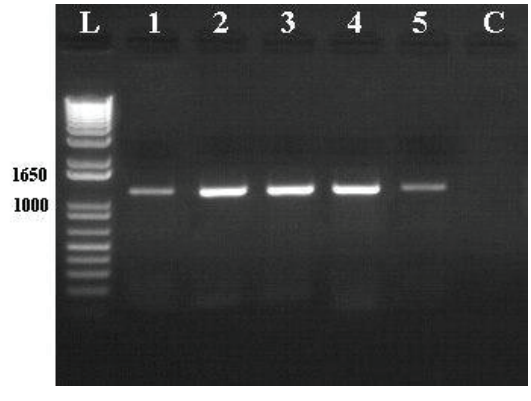

Figure 3: The visualization of PCR amplification product from *rpo*B gene of *P. savastanoi* pv. *savastanoi* strains isolated from olive trees. Size of the expected product was 1247 bp. L) Ladder, 1) PS03, 2) PS06, 3) PS12, 4) PS14, 5) PS17, and C) water as negative control

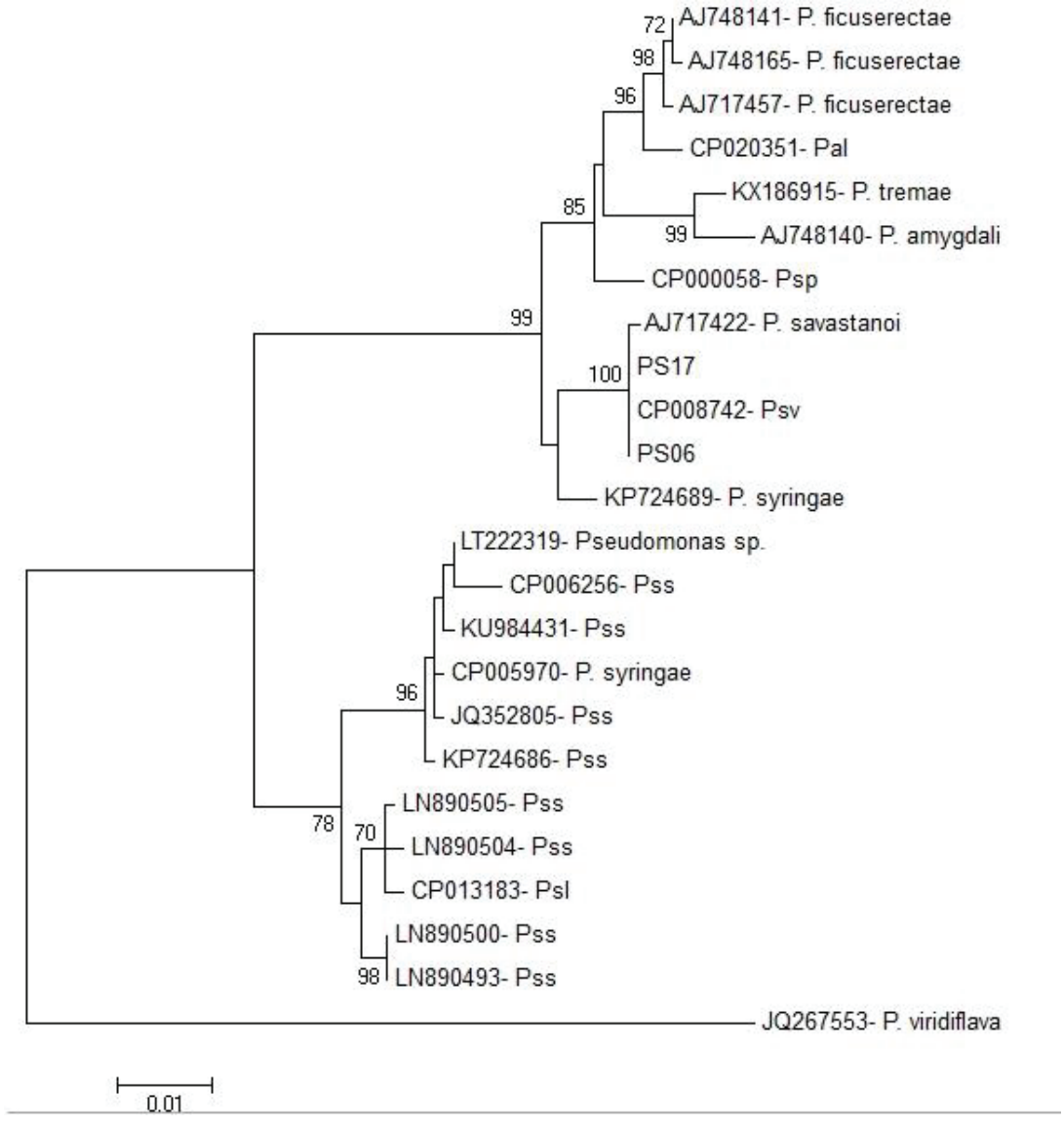

Figure 4: Phylogenetic relationship of bacterial strains isolated from olive trees based on the nucleotide sequences of the *rpoB* gene. PS06 and PS17 are isolates from our study, Psv (*P. savastanoi* pv. *savastanoi*), Pal (*P. amygdali* pv. *lachrymans*), Psp (*P. savastanoi* pv. *phaseolicola*), Pss (*P. syringae* pv. *syringae*) and Psl (*P. syringae* pv. *lapsa*). The tree was reconstructed by using the NJ method, using the genetic distances computed by using the Kimura's two-parameter model. The scale bar represents the unit length of the number of nucleotide substitutions per sites

3.3 Pathogenicity on different hosts

Small galls on olive branches appeared after two weeks and fully developed within three months. The bacterial pathogen was re-extracted from new galls and phenotypically characterized. No galls were formed on oleander, winter jasmine, Japanese privet and common ash shoots as well as on negative control incubated with PBS (Table 2). The absence of gall formation on other plants classifies these strains in pathovar *savastanoi*. Several studies have indicated the host specificity in different of *P. savastanoi* pathovars (Ghasemi et al., 2006; Tegli et al., 2011; Taghavi & Hasani, 2012).

Table 2: Gall formation caused by pathogenic *P. savastanoi* pv. *savastanoi* strains on shoots from different hosts

Host	Gall formation
Ash	-
Oleander	-
Olive	+
Privet	-
Winter jasmine	-
Negative control (SDW)	-

3.4 Cultivars susceptibility

Use of tolerance or low susceptible plant cultivars is an important strategy in controlling plant diseases. There is limited information on the susceptibility of the olive varieties to olive knot disease. In the present study, we infected six olive cultivars with five pathogenic strains to evaluate the cultivar's susceptibility to the disease. Olive knot symptoms were observed on the main stem of all olive cultivars after three months. 'Arbosana', 'Mission' and 'Beldi' cultivars showed the highest susceptibility to pathogenic strains, respectively. The lowest susceptibility was observed in 'Arbequina', but there were no significant differences in the size and mass of the knot, and the time of symptom appearance in studied cultivars (Figure 5). None of control plants developed knots.

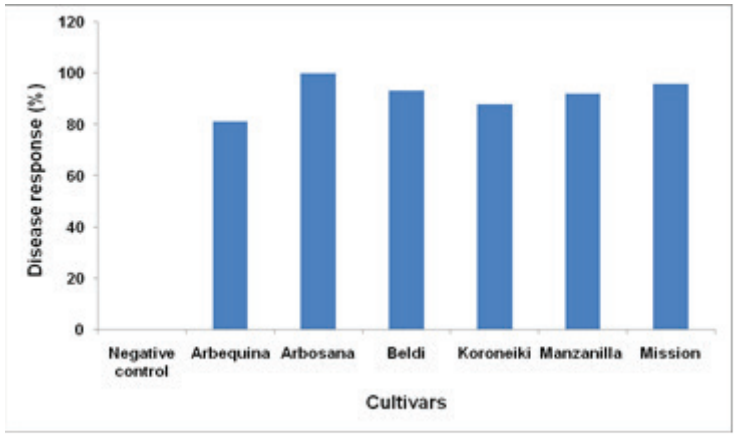

Figure 5: Proportion of wound sites developing knots on two-years-old plants of six olive cultivars. Data are shown average values of five pathogenic *P. savastanoi* pv. *savastanoi* strains (PS1, PS3, PS6, PS10 and PS17) for each cultivar. PBS was used as a negative control

Previous studies from Portugal demonstrated a slight difference in the response of olive cultivars, where the virulence ranged between 36-66 % (Marcelo et al., 1999). Hassani et al. (2003) evaluated the severity of the symptoms by determining the size and mass of the knots after three months. They found that 'Frantoio' was the most and 'Leccino' was the least susceptible cultivar among studied cultivars. Penyalver et al. (2006) determined the proportion and mass of primary knots and the presence of secondary knots on twenty-nine olive cultivars. The cultivars were inoculated with two pathogenic strains at two inoculum doses. Their results indicated that in a low dosage inoculating, large differences in disease response were observed among cultivars infected with both pathogen strains. The proportion of sites with developed knots ranged from 0 to 100 % depending on the cultivar. They also found significant differences in the presence of secondary knots among cultivars and proportion of non-inoculated sites that developed knots (from 0 to 65.5 %), depending on the cultivar. Development of primary knots and presence of secondary knots in each experiment occurred under low inoculum dose. Hence, the severity of the disease was reported to be strongly dependent on the dose of the pathogen used at the wound sites and similar to our results, none of the cultivars was resistant to the disease.

4 CONCLUSIONS

Based on phenotypic and molecular characteristics, the bacterial agent causing olive knot in an orchard located in the north of Iran was *Pseudomonas savastanoi* pv. *savastanoi*. Biochemical and physiological characteristics among the isolates were similar (more than 80 %). Further, phylogenetic analysis based on *rpo*B gene confirmed the classification of the strains to pathovar "*savastanoi*". In biological tests, no cultivar showed resistance to the disease; however, some variation in disease susceptibility was observed. Because the bacterial olive knot disease agent belongs to Iran's list of foreign quarantine pests and diseases, appropriate quarantine and phytosanitary measures were taken to eradicate the disease in the infected orchard.

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Studies on diversity indices and insect pest damage of walnuts in Kashmir, India

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ABSTRACT

Walnut industry, one of the economically important industries of Kashmir is under multitude of stresses like changing weather patterns, international competition, insect pest damage and diseases. Pest damage by various insect species is by for the main cause of walnut damage, thus limiting its production. In this backdrop, the aim of the present study was to check the insect pest diversity and its nature and extent of damage to walnuts. Sampling was done fortnightly in three districts of Central Kashmir *viz.,* Srinagar, Budgam, Ganderbal from June 2014 to November 2015. Quantitative estimation of individual species was made by using various diversity indices and each site varied in species diversity, richness and evenness. A total of nine sites were selected from three different districts and the insects collected belonged to 3 orders, 7 families and 10 species. Order Hemiptera was highly damaging in comparison to Coleoptera and Lepidoptera while in Hemiptera, maximum damage was done by *Chromaphis juglandicola* (Kaltenbach, 1843) and the least by *Apodiphus pilipes* (Horvath,1889). The study provides a baseline data for assessing the biodiversity pattern and damaging potential of walnut pests so as to develop holistic integrated pest management programme.

Key words: *Juglans regia* L.; diversity indices; incidence; infestation; species richness; walnut pests

IZVLEČEK

RAZISKAVE DIVERZITETNIH INDEKSOV IN ŠKOD ZARADI ŠKODLJIVIH ŽUŽELK NA NAVADNEM OREHU V KAŠMIRJU, INDIJA

Pridelovanje orehov, ki je ena izmed ekonomsko najvažnejših kmetijskih dejavnosti v Kašmirju, je soočeno s številnimi izzivi kot so podnebne spremembe, mednarodna konkurenca in škode zaradi škodljivcev in bolezni. Škode zaradi različnih žuželk predstavljajo glavni vzrok zmanjšane pridelave. Namen te raziskave je bil preveriti pestrost škodljivcev in obseg škod, ki jih ti povzročajo pri gojenju oreha. Vzorčenje je bilo izvedeno v štirinajstdnevnih presledkih v treh območjih osrednjega Kašmirja, Srinagar, Budgam, Ganderbal, v obdobju od junija 2014 do novembra 2015. Količinsko določanje posameznih vrst škodljivcev je bilo določeno z različnimi diverzitetnimi indeksi. Vsako izmed območij se je razlikovalo v pestrosti, bogatosti in izenačenosti vrst. Iz vseh treh raziskanih območij je bilo izbranih osem lokacij, na katerih so ujete žuželke pripadale 3 redovom, 7 družinam in 10 vrstam. Škodljivci iz redu Hemiptera so povzročili največ škode, v primerjavi s tistimi iz redov Coleoptera in Lepidoptera, med vrstami iz redu Hemiptera, je največ škode povzročila vrsta *Chromaphagis juglandicola* (Kaltenbach, 1843) in najmanj *Apodiphus pilipes* (Horvath,1889). Raziskava daje osnovne podatke za ocenjevanje vzorcev biodiverzitete in potencialnih škod zaradi škodljivcev na navadnem orehu pri razvoju celostnega integriranega programa upravljanja s škodljivci.

Ključne besede: *Juglans regia* L., diverzitetni indeksi; pojav; okužba; vrstna pestrost; škodljivci navadnega oreha

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1 INTRODUCTION

Regarded as heaven on earth, the state of Jammu & Kashmir is called the biomass state of India due to its immense biodiversity, rich gene pool and priceless resources. Common Walnut (*Juglans regia* L.), one of the prime industries among various other commercial sectors of the valley, is cultivated in districts like Poonch, Pulwama, Anantnag, Ganderbal, Kulgam, Budgam, Kupwara, Baramulla and Srinagar. Among these districts, Shopian was on the forefront in walnut production in the past. The trend has changed and nowadays Kupwara is leading in walnut production (Directorate of Horticulture, 2015). So far as global scenario of walnut production is concerned, China is at the top followed by USA, Iran, Turkey, Ukraine, Romania, France and India while in recent years other countries like Argentina and Chile have also increased production of walnuts manifolds (Martinez et al., 2004). Irrespective of its significance, walnut production is threatened by insect pests and diseases, damaging fruit kernel, leaves, branches and trunk of walnut trees as reported by Mir & Wani (2005). Most of the insect pests reported on walnuts are leaf defoliators, feeding on the leaves, twigs and branches leading to nut drop (Abbas et al., 2015). Among different insect orders, Coleoptera forms an important group of pests feeding on foliage and affecting photosynthetic surface of leaves (Mohandas et al., 2004). Larvae of certain pests form long tunnels inside stem and root and feed on the internal tissue advancing upwards (Khan et al., 2013). The attacked tree eventually accedes to injury by getting hollow inside and finally causes death of a plant. Certain dipteran flies also feed inside the walnut husk, causing blackening of shells and thereby reducing its market value (Boyce, 1934). Walnut aphids directly influence walnut production by accumulating honeydew on the husk, in turn attracting sooty mould. It has phyto-toxic effect leading to general blackening of leaf surface (Boyce, 1934; Bhagat, 1986; Ahmed & Ahmed, 2013). As the diverse insect pests attack and reduce walnut production worldwide, therefore, there is an urgent need to study the pest diversity and damage to walnuts especially in Kashmir where walnuts are organically produced. Conversely, the data will in turn enable us to develop planned integrated pest management strategy for walnut pests.

2 MATERIAL AND METHODS

2.1 Study area and surveys

The field surveys were conducted in three districts of Central Kashmir *viz*., Srinagar (34° 04' 54.36" N, 74° 48' 33.00" E), Budgam (34° 01' 2.05" N, 74° 43' 6.71" E) and Ganderbal (34° 13' 39.11" N, 74° 46' 19.78" E). During surveys, the distribution of insect pests and predators along with their mode of damage was recorded. From each district, three sites were selected based on the accessibility and availability of walnut trees *viz*., S1, S2 & S3 in district Srinagar, G1, G2 & G3 in district Ganderbal and B1, B2 & B3 in district Budgam.

2.2 Sampling methods

For aphid study, sampling was done fortnightly by selecting five trees randomly in each walnut orchard ecosystem. Twenty sub terminal leaves were randomly selected from lower and middle canopy in each direction (East, West, North, South) making a total of 100 leaflets/orchard (Tomanović, 1996; UCIPM, 2011) Similarly, pests were calculated by direct count method In order to check the abundance at each site. For the study of beetle infestation and population counting, collection was done by 'one man - one hour method' by sweep nets in the early morning hours (Khairmode & Sathe, 2014). Likewise, random selection was also done for collecting larvae of butterflies and moths while examining five trees from each orchard and selecting 100 leaves per tree with a total of 500 leaves per orchard. The nut borers were collected by random selection of two branches from opposite directions. The infested and dropped fruits were also analyzed for pest infestation (Mir & Wani, 2005; Khan et al., 2013). Active fliers like bugs were collected by net sweeping method. Each sweep was repeated after a gap of 10 minutes with an overall 10 sweeps at one time (Kumar & Naidu, 2010). Each walnut orchard which was evaluated for pest diversity was free from any kind of pesticide application. The collected insect pest specimens comprised of adults and nymphs (both mature and immature) and were preserved in 75 % alcohol for further identification in Entomological Research Laboratory, Department of Zoology, University of Kashmir.

2.3 Identification

Identification and labelling was done as per the available literature and running taxonomic keys while the specimens which couldn't be identified or doubted were sent to Zoological Survey of India (ZSI), Kolkata, India for further confirmation.

2.4 Estimation of diversity indices

Quantitative estimation of individual species was made using the data derived from field surveys. Margalef's richness index, Shannon-Wiener diversity index, Simpson's diversity index and Pielou's index were applied for studying the diversity and abundance of walnut insect pests. The formulae for various statistical/ diversity indices are as under:

Margalef's richness index (1958)

$$d = (S - 1)/\log_e N$$

Where d = Margalef's richness index
S = Number of species
N = total number of individuals

Shannon-Wiener diversity index (1949)

$$(\mathrm{H}) = -\sum_{\mathrm{i}=1}^{\mathrm{n}} \mathrm{pi}\ \log\ \mathrm{pi}$$

$H_{max} = \log_2 S$
$E = H/H_{max}$ (Evenness)
D = 1-E (Dominance)
Where, pi is the proportion within the sample of the number of individuals of "ith" species and is denoted as $\frac{ni}{N}$.

ni = Number of "ith" individual
N = Total number of individuals
S = Number of species or species richness
H_{max} = Maximum of possible diversity
$E = Evenness = H/H_{max}$

Simpson's diversity index (1949)
Simpson's dominance index of diversity (D):

$$\mathrm{Diversity\ (D)} = (\mathrm{n/N})^2$$

Where, n = number of individuals or amount of each species
N = total number of individuals for the site

Species evenness (J) (1966)

$$J = \frac{H}{\log_e S}$$

Where, H = Shannon-Wiener biodiversity index
S = number of species in the community.

2.5 Statistical analysis

All statistical analysis was performed using SPSS Statistical software (Version 20) and MS excel 2007.

3 RESULTS AND DISCUSSION

The results indicate rich pest diversity on walnut trees at different study sites with an overall of 10 insect species, belonging to 3 orders and 7 families. Out of 10 insect species reported, 8 were major or minor pests while 2 were predatory beetles feeding on aphids. Detailed report on pests, affected plant part and damaging stage in the life cycle of an insect pest is tabulated in Table 1.

3.1 Walnut blue butterfly (*Chaetoprocta odata* (Hewitson 1865))

Commonly called as walnut blue butterfly, *C. odata* is serious monophagous pest of walnuts, defoliating leaves and damaging sprouting buds. Larvae fed on the leaves, pest infestation started from March to May. Adults emerged in June and July, mostly feeding on nectar of flowers of nearby vegetation. Larvae were cylindrical in shape, light green in colour and with each instar development, the colouration changed from light green to dark green finally to dark brown in last instar. Each larval instar's strong mandibles nibbled irregular holes on leaves and also crawled to the emerging buds for feeding. Out of the different larval instars which were feeding voraciously on leaves, second instar was observed to be most damaging. It usually lays eggs on walnut twigs at the end of summer while these overwintering eggs coincide with the sprouting buds and hatch out in next season in March (Figure 1 a-b). Khan et al., (2013) stated *C. odata* as most serious damaging lepidopteron pest infesting walnut trees. They revealed that the pest causes defoliation and damage to sprouting buds affecting both young and old walnut trees. Further, similar results were found by Abbas et al., (2015) in *C. odata*. They described the species as monophagous, having peak season from March to April with only one generation per year.

Table 1: Diversity, period of activity & feeding of insect pests on walnut plantation in Central Kashmir

Name of insects collected	Scientific name	Order	Family	Collected plant part	Activity period	Feeding on	Feeding stage
Walnut aphid	*Chromaphis juglandicola*	Hemiptera	Aphididae	Leaves	Apr-Oct	Sap	Nymph and Adult
Duskey veined aphid	*Panaphis juglandis*	Hemiptera	Aphididae	Leaves	Apr-Oct	Sap	Nymph and Adult
Capsid bug	*Megacoelum stramineum*	Hemiptera	Miridae	Leaves	Apr-Oct	Sap	Nymph and Adult
Stink bug	*Apodiphus pilipes*	Hemiptera	Pentatomidae	Leaves	May-Sep	Sap	Nymph and Adult
Lace bug	*Paracopium cingalensis*	Hemiptera	Tingidae	leaves	Apr-Oct	Sap	Nymph and Adult
Walnut blue butterfly	*Chaetoprocta odata*	Lepidoptera	Lycaenidae	Leaves	Mar-Jul	Foliage & young buds	Larvae
Asian walnut moth	*Ershoviella musculana*	Lepidoptera	Nolidae	Nuts & young shoots	May-Aug	Nuts & young buds	Larvae
Grey weevil	*Myllocerus* spp.	Coleoptera	Curculionidae	Leaves	Apr-Sep	Foliage	Larvae and Adult
Ladybird beetle	*Calvia punctata*	Coleoptera	Coccinellidae	Leaves	Apr-Sep	Aphids	Larvae and Adult
Ladybird beetle	*Oenopia conglobata*	Coleoptera	Coccinellidae	Leaves	May-Aug	Aphids	Larvae and Adult

3.2 Walnut Asian moth (*Ershoviella musculana,* Erschoff, 1874)

One of the most important walnut pests, *E. musculana* feeds voraciously inside nuts and young shoots, leading to early nut drop and also consumes pericarp, resulting in deformed nut. In May, adults emerge and eggs are laid on the surface of young nuts. After hatching, it was observed that caterpillars pierce the nuts through petiole and start feeding inside. Single nut was pragmatic to have 2-3 larvae feeding inside during the study period. The larval instars were seen feeding on nuts from May to August. Newly hatched larva was creamy- white in colour, about 2-3 mm in size with dark brown head with long light hairs on pronotum while the size of each larval instar increases with increase in feeding capacity. The last instar was about 15-20 mm in size with few light brownish- cream hairs on dark brown scutella with light brown coloured body. In August, they start leaving nuts for pupation to continue life cycle next year. The pupation took place inside the loose bark or crevices of walnut trees. The infested nuts had deposition of excrement on the walnut husk which turns to brown & it conversely reduces the yield and market value. Adults were not found damaging walnuts as they mainly fed on flower nectar and hovered on nearby vegetation (Figure 1 c-d). The present study was in agreement with the work of Anonymous (2005) who stated *E. musculana* as an important walnut pest of fruit and young shoots, resulting in the formation of deformed nuts which leads to annual loss of about 70-80 %. Khan (2011) reported the pest for the first time from SKAUST, Shalimar, Srinagar, and J&K. The nature, damage and biology of the studied pest confirm our results.

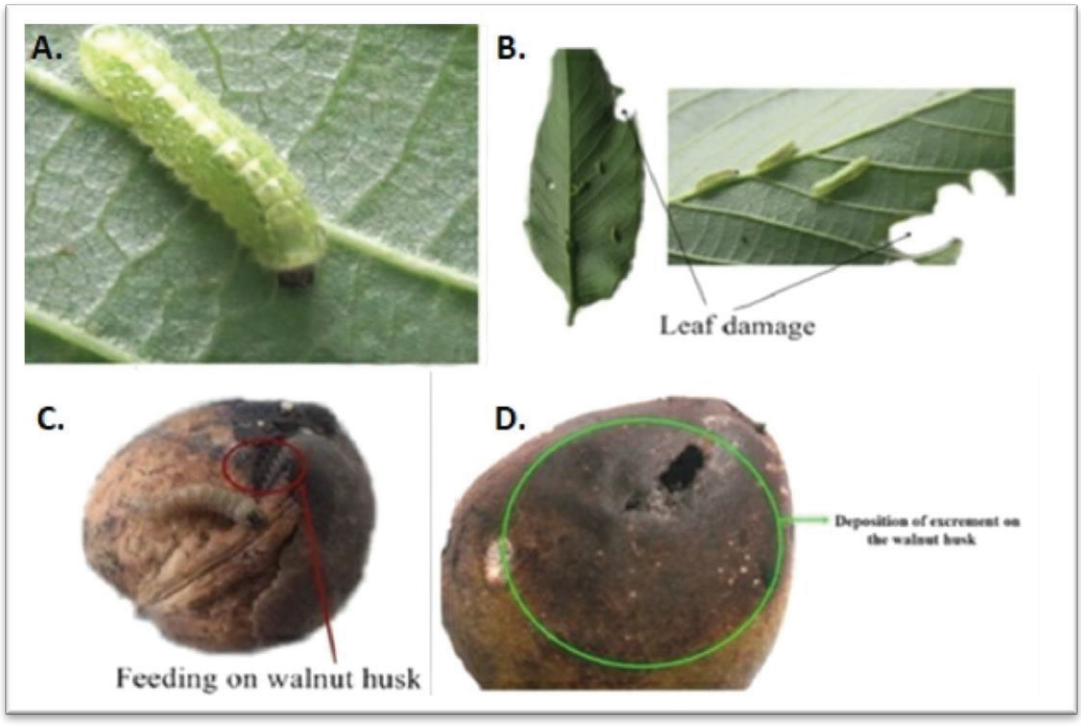

Figure 1: Larvae of *C. odata* and *E.musculana*
A: Damage caused by *C. odata*
B: Larva of *C. odata* feeding on walnut leaf
C: Larva of *E.musculana* feeding on walnut husk
D: Damage caused by *E.musculana* larva

3.3 Aphids; (*Chromaphis juglandicola* Kaltenbach, 1843*)* & *Panaphis juglandis* (Goeze, 1778)

Two aphid species, *Chromaphis juglandicola* and *Panaphis juglandis,*were determined on the studied walnut trees. Infestation due to *C. juglandicola* was very high. Both species are sap suckers and are serious pests, feeding on phloem while the infestation started from April to October with peak infestation in June. The distribution pattern on leaves was also different, *C. juglandicola* was found scattered on the underside of leaves while *P. juglandis* was present on the upper side of leaves, feeding primarily near the mid rib of leaves. Further, *P. juglandis* was observed as minor pest in comparison to *C. juglandicola*. High population of walnut aphids lead to leaf drop, reduced tree vigour, nut quality and size besides shriveling of kernels. Both aphids develop parthenogenetically as well, leading to increase in population densities and nutrient uptake. On the other hand, excretion of honey dew attracts black sooty mould fungus which reduces light penetration to leaves, make them black and cause sunburn to fruits and thereby, reducing market value of fruits.

During the study period, both aphid species maintained distance and never fed on same leaf, strongly reinforces the Gause's competition exclusive principle, which states that no two species having same ecological niche can coexist together. *C. juglandicola* and *P. juglandis* were never seen feeding on same leaf during the study period. *C. juglandicola* is important factor that limit the populations of *P. juglandis,* which can be attributed to the excretion of acidic honeydew. Both aphids overwinter as egg stage, hatch out in early spring and produce young ones without mating which lead to the production of many generations per year and resulting in the development of many colonies in summer season. In the case of dusky-veined aphid (*P. juglandis*) winged males and wingless females appear in September and cause general blackening of leaves while walnut aphid (*C. juglandicola*) cause early leaf drop and reduced nut size. (Figure 2 e-h). During the present investigation it was observed there was high infestation of walnut aphid population per leaflet which showed close congruity with result 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 line with the studies carried out by Ginzel (2010) who reported both these aphid species as a contributing factor for reduced tree vigour, nut size and yield. Our results were strongly reinforced by the finding of the Mosz (2002) who had found aphid damage on walnut trees in the spring and summer and consume cell contents of the leaves.

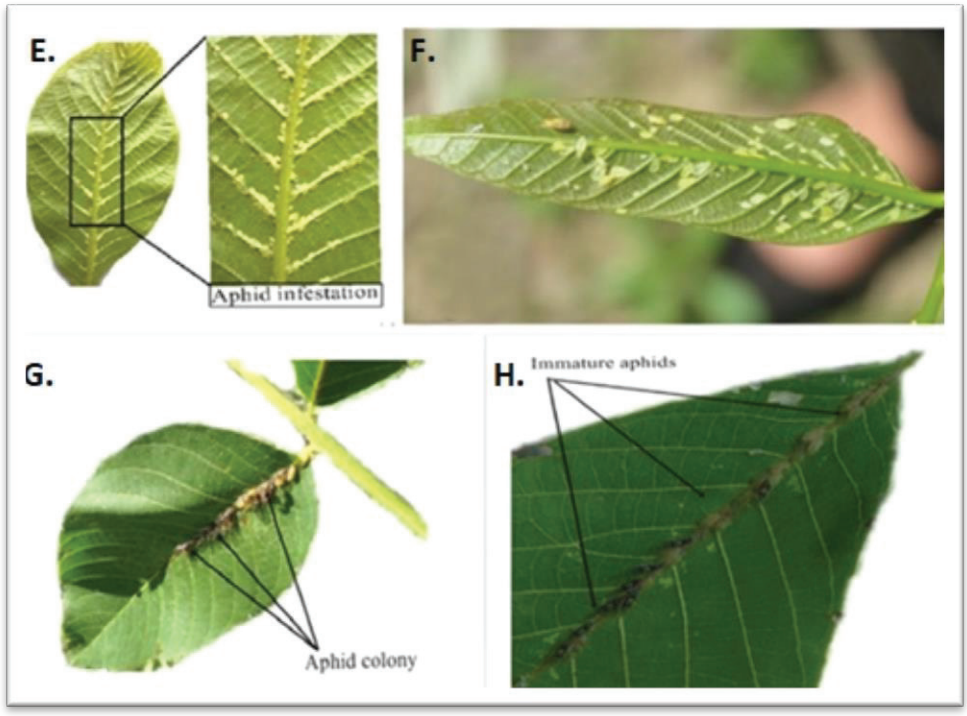

Figure 2: Adults and nymphs of *C. juglandicola* and *P. juglandis*
E: *C. juglandicola* scattered on lower surface of leaf
F: Adults and immatures of *C. juglandicola* feeding on lower surface of young leaf
G: *P. juglandis* feeding near mid rib of walnut leaf
H: Immature aphid colony feeding on mid rib of walnut leaf (upper surface)

3.4 Stink bug (*Apodiphus pilipes* Horvath, 1889)

Being a plant feeder, it sucks the sap of leaves from May to September. It has well developed rostrum which is inserted into the plant tissue for feeding (Figure 3i) while infestation is mainly on young developing buds and leaves. High infestation may cause stunted growth and sticky appearance. During the present study, it was observed that both adults and nymphs attacked walnut foliage notwithstanding, the population of *A. pilipes* was much weaker in comparison to other pests even though abundance was observed at few sites only. Less frequent appearance of the *A. pilipes* on walnuts can be attributed to apple trees as its prime host while acting as a visiting pest on walnuts owing to high competition in their own niche (Bhat, 2007).

3.5 Lace bug (*Paracopium cingalensis* Walker, 1873)

During the present study, it was found for the first time feeding on the sap of walnut tree leaves being active from April to October. Adults and nymphs were found to feed on the abaxial surface of leaves resulting in the formation of bumps. Being gregarious in nature they usually feed with protusible mouth parts on leaves making cholorotic patches resulting in development of galls on walnut leaves which leads to leaf drop, leaf discolouration and leaf blackening due to brownish black excrement deposited on them (Figure 3j). Our results are in line with the findings of McGavin (1993), Gull et al., (2018) and Deckert & Scheiding (2006) whose findings revealed that tingids are exclusively herbivorous and feed on specific hosts causing damage to ornamental, friut and other crops, however such pests are more responsible for causing transmission of disease causing pests to plants.

3.6 Capsid bug (*Megacoelum stramineum* Walker,1873)

*Megacoelum stramineum***,** commonly called as capsid bug, is a new report on walnuts as no data sets are available to confirm its feeding potential on walnuts although as per available literature, it has a wide host range. Adults usually feed on shoots, undersurface of walnut leaves from April to October (Figure 3k). The visible symptoms include small and round sunken spots on leaves while infestation appears from spring to early autumn with peak abundance in summer. The present results were reinforced by work of Udikeri et al., (2014) who carried out its damaging potential on crops.

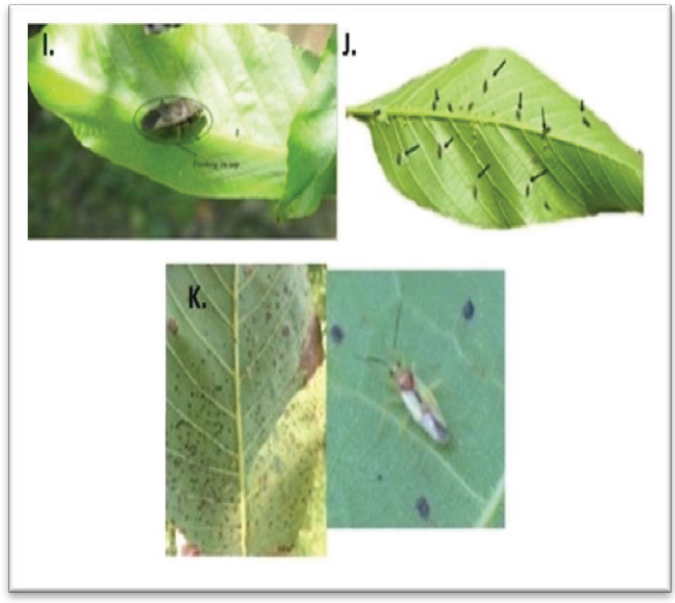

Figure 3: *A. pilipes*, *P. cingalensis* and *M. stramineum* feeding in walnut leaf sap
I: *A. pilipes* feeding on the sap of walnut leaf
J: *P. cingalensis* feeding on the sap of walnut leaves (Lower surface)
K: *M. stramineum* excreata along with black soot

3.7 Grey weevil (*Myllocerus* spp.)

It is commonly called as Grey weevil and is the most serious pest causing defoliation of trees by feeding on the leaf margins and is active from April to September. It nosh inwardly along the leaf veins, preferring new foliage and young shoots. During the present work, adults were serious defoliators while grubs were concealed feeders. Adults usually fed on leaves, making holes of 2-3 cm and then gradually eating up the entire leaf leaving behind mid rib only. Further, it curles the leaftips and are generally called as leaf rollers which leads to general blackening of the tips of leaves as was revealed by Mir & Wani, (2005). (Figure 4 l-n).

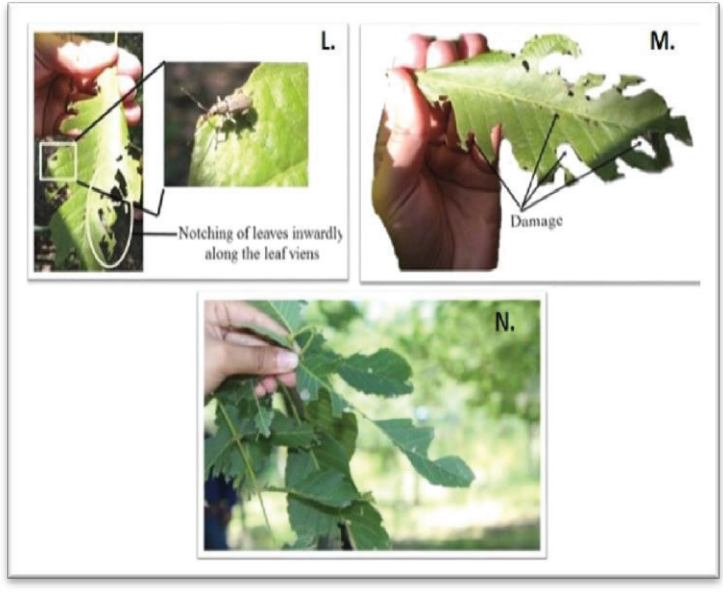

Figure 4: Feeding and damaging pattern shown by *Myllocerus* spp.
L: *Myllocerus* spp. feeding on foliage of walnut leaves.
M: Notching of leaves caused *Myllocerus* spp.
N: Damage caused by *Myllocerus* spp. leaving behind only mid rib

3.8 Diversity indices

Diversity indices were studied in three districts of Central Kashmir *viz.*, Srinagar (S), Budgam (B) and Ganderbal (G) with three sites chosen at each district such as S1, S2 and S3 for Srinagar, B1, B2 and B3 for district Budgam and G1, G2 and G3 for district Ganderbal. The insect pest community was analyzed for species diversity by applying Shannon- Weiner index which combines the effect of richness and evenness. The mean abundance of pests with significant differences is presented in Table 2. During the analyses, Shannon- Weiner index decreased with decrease in total number of species. On comparing the data of various sites of Central Kashmir, it was found that the diversity index in Srinagar showed the highest value at S3 (1.41), followed by S2 (1.25) & S1 (1.10). In Budgam, peak value was recorded at B2 (1.57) followed by B1 (1.40) and B3 (1.31). In Ganderbal district, higher values were found at site G1 (1.31) followed by G2 (1.12) and G3 (1.00). The Simpson's diversity index did not show any noticeable variations among the study sites. However, the diversity index recorded the highest value in Srinagar at S1 (0.48) followed by S2 (0.41) and the lowest one at S3 (0.31). The diversity index fluctuated at Budgam from B3 (0.35) to B1 (0.32) and lower at B2 (0.24). However, In Ganderbal maximum value was found at G3 (0.44) followed by G2 (0.43) and G1 (0.36). The Margalef's diversity index was applied to measure the distribution pattern and richness of species at a particular site (Figures 5-7). According to the evaluated results, the insect heterogeneity was found different at different sites of Central Kashmir. The upper most range was found at S2 (7.69), followed by site S1 (5.43) and site S3 (5.36). At Budgam, peak value was at site B3 (5.53) with lower values at B2 (5.32) and least at B1 (4.96). However, minimum value of diversity was found at G3 (2.43) with maximum at G2 (6.51) and G1 (5.29) at Ganderbal (Figure 8x).

The Pielou's evenness index showed higher value at site S3 (0.79) followed by site S2 (0.64) and site S1 (0.62) in Srinagar. At Budgam evenness was found the highest at site B2 (0.87) followed by site B1 (0.78) and the lowest at site B3 (0.73). On comparing the evenness at three sites of Ganderbal, species were more evenly present at G1 (0.73) followed by site G3 (0.72) and G2 (0.69) (Figures 5-7).

Figure 5: Diversity indices of various pest species of district Srinagar
O: Distribution of pest in walnut orchard at site S1 of district Srinagar
P: Distribution of pest in walnut orchard at site S2 of district Srinagar
Q: Distribution of pest in walnut orchard at site S3 of district Srinagar

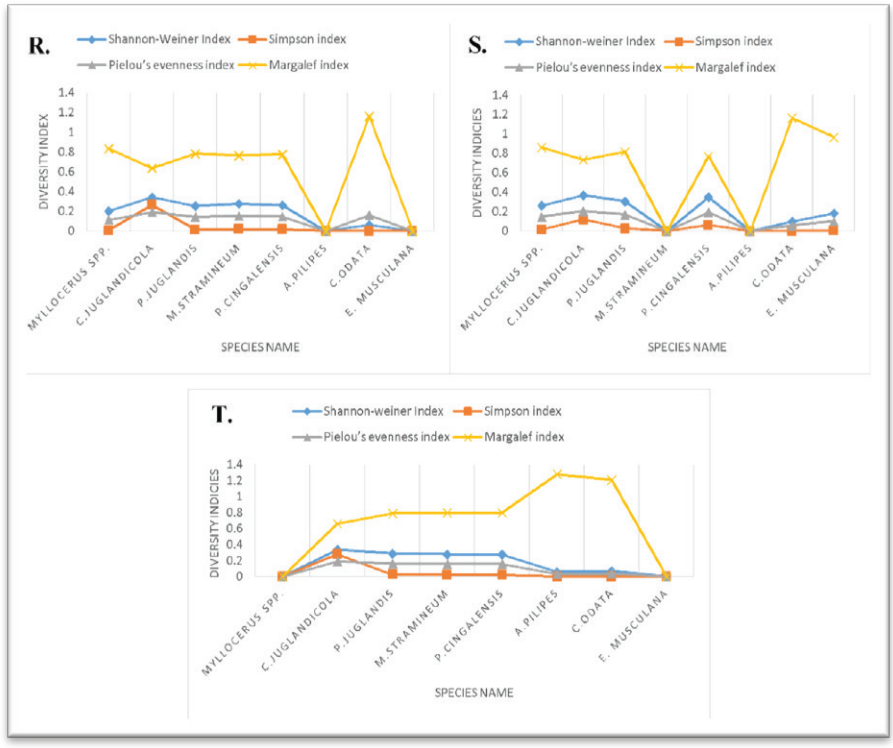

Figure 6: Diversity indices of various pest species of district Budgam
R: Distribution of pest in walnut orchard at site B1 of district Budgam
S: Distribution of pest in walnut orchard at site B2 of district Budgam
T: Distribution of pest in walnut orchard at site B3 of district Budgam

Figure 7: Diversity indices of various pest species of district Ganderbal
U: Distribution of pest in walnut orchard at site G1 of district Ganderbal
V: Distribution of pest in walnut orchard at site G2 of district Ganderbal
W: Distribution of pest in walnut orchard at site G3 of district Ganderbal

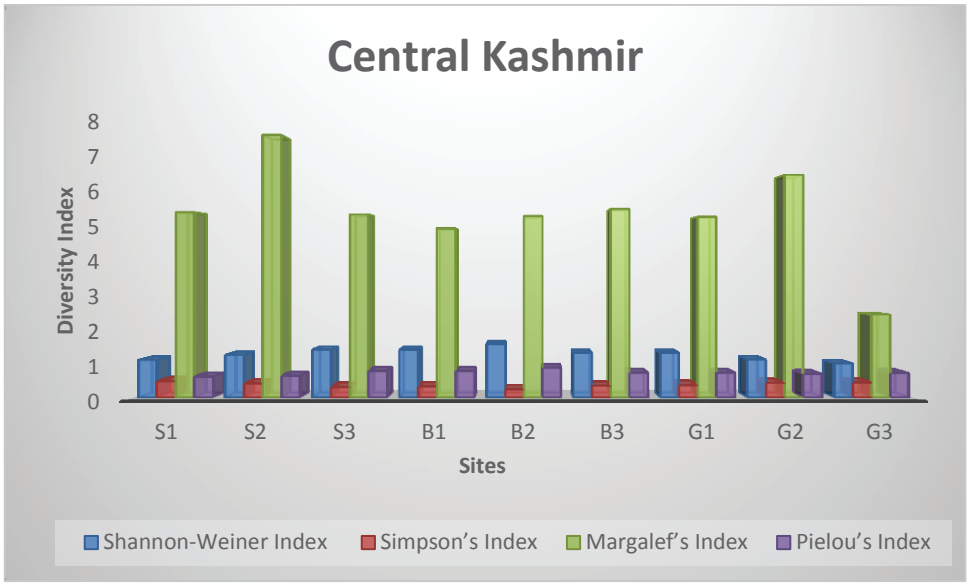

Figure 8X: Various diversity indices of insect orders infesting walnut orchards at different sites of Central Kashmir

The overall average diversity of pests infesting walnut orchards of Central Kashmir depicted that Shannon-Weiner diversity index (H) was utmost at Budgam (1.43) and least at Srinagar (1.25). However, Simpson's diversity index (D) was higher at Ganderbal (0.41) and lower at Budgam (0.30). Margalef's diversity index (MI) had uppermost value at Srinagar (6.16) and lowermost at Ganderbal (4.74) while as Pielou's index depicted the evenness values having higher values at Budgam (0.80) and lower at Srinagar (0.68) (Figure 8y).

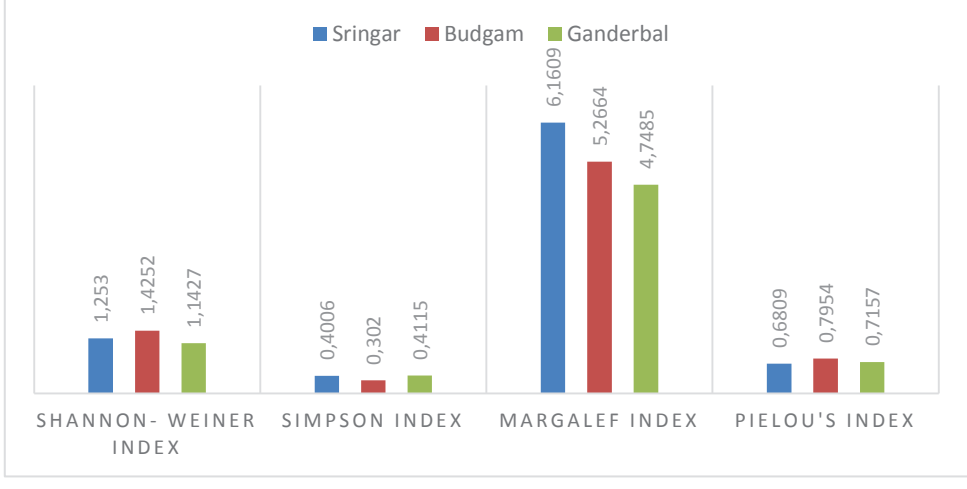

Figure 8Y: Average values of diversity indices in different districts of Central Kashmir

Table 2: Mean of pest abundance in Srinagar, Budgam & Ganderbal districts, Jun 2014- Nov, 2015.

Species	Srinagar	Budgam	Ganderbal
C. juglandicola	225.23±141.63[b]	169.00±128.09[ab]	135.07±91.36[a]
P. juglandis	46.32±38.86[a]	49.06±35.56[a]	39.91±31.50[a]
Myllocerus spp.	26.19±11.46[a]	38.83±20.16[b]	21.35±12.08[a]
P. cingalensis	66.36±34.95[a]	56.63±29.34[a]	65.78±30.00[a]
C. odata	10.64±5.71[a]	10.50±7.36[a]	8.57±6.49[a]
E. musculana	22.57±3.39[a]	25.14±2.54[a]	22.50±5.72[a]

Significant $p < 0.05$, Non-significant $p > 0.05$; Mean values with different superscripts are significantly different ($p < 0.05$, Tukeys HSD)

The aim of the present study was to focus on the different walnut insect pests which reduce the yield of walnuts and directly influences the people who are involved in the commercial trading system at national level. Any manipulation in the plant communities by the human activities results in the more susceptible insect pest attack (Altieri 1991). A total of 8 insect pests were collected belonging to three insect orders, i.e. Coleoptera, Hemiptera and Lepidoptera were recorded on the walnut trees of Central Kashmir. Mir & Wani (2005), Khan et al. (2011, 2013) mentioned most of the species on walnut, which were recorded during present investigation. However, newly recorded two pest species, i.e. *Paracopium cingalensis* and *Megacoelum stramineum* were for the first time recorded on walnuts of Central Kashmir. UCIPM in 2011 reported about 17 species of arthropod infesting walnuts. Ginzel (2010) mentioned about at least 10 species infesting walnut and studied their activities as pest. Diversity of insects enables us to understand the relationship between the habitat and ecosystem (Denys & Tschantke 2002). The results revealed that each site varied in the species diversity, species richness and evenness prevailing on walnut ecosystem. The result depicted that all the sites of three districts of Central Kashmir had similar diversity levels as reported in Shannon-Wiener diversity index, Pielous's index, Margalef's index and Simpson index. However, each site at three districts of Central Kashmir had different values when examined with the four measures of diversity. This variation observed in the pests infesting walnut reflects that there is variation in the distribution and abundance of pests infesting walnut. Perfecto et al., (1997) have also observed that natural and unobserved habitats have significant impact on insect diversity within heterogeneous environment.

Shannon – Weiner diversity index was used to calculate the diversity of each site of Central Kashmir which included three districts viz., Srinagar, Budgam and Ganderbal. It was found to be 1.25, 1.43 and 1.14 indicating sites of Central Kashmir were more or less similarly diversified. This was in line with the findings of Chakraborty (2014). Simpson's index (λ) gives the probability that two individuals, when selected at random from population, belong to particular species (Ambrose 2004). Higher the value less is the dominance of each species prevailing in a particular community. Our results showed 0.40, 0.30, and 0.41 for Srinagar, Budgam and Ganderbal respectively which shows all sites of Central Kashmir have more or less equal diversity index. Higher values may indicate a healthy environment for insects. The values calculated for districts Srinagar (6.16), Bugdam (5.27) and Ganderbal (4.74) portrayed that Srinagar had high value for species richness and it may be attributed to the presence of higher number of species infesting untreated walnut trees and forms good ecosystem for higher diversity. According to Hart & Horwitz (1991) the habitat heterogeneity simply has more number of arthropod species where different types of plant species are found. Various factors such as resource availability for both adults and larva plays significant role in increasing the richness of particular area as observed by Pinheiro & Ortiz (1992). One possible explanation for direct correlation between the food plant and richness of species associated to it could be potentially due to higher number of niches associated to plant which exists within there (Hutchinson, 1959). Species richness provides an advantageous measure of diversity when total number of species in the community is obtained (Magurran, 1988). In the present study evenness ranged from 0 to 1 which signifies that scale ranging from near

0, indicates low evenness or high single specie dominance, to 1 which indicates abundance of all species or maximum evenness (Routledge, 1980). Pielous's index was high at Ganderbal (0.72) which indicated that the species are more evenly distributed there.

Many researchers have studied the diversity of insects and their association and interactions with the plant community. Panzer & Schwartz (1998) investigated that 49 % of variance in insect species is due to plant species richness among the studied areas. The present study revealed the decrease in diversity count from season to season and site to site can be attributed to low availability of food resources, which was previously reported by Thomazini & Thomazini (2002). Phylogenetic diversity (May, 1990) and endemism (Jetz et al., 2004) also influence variation of species diversity. Other factors which influence the diversity is competition, succession and the most important predation which causes the change in species evenness without change in richness (Tramer Elliot, 1969; Magurran, 1988). The indices values of the present study were in close proximity with the findings of Reddy & Moos (2015) on calculating insect diversity, species richness and evenness of the walking mango tree with H = 1.417, D = 0.306, MI = 8.17 and Mh =5.435. Our results were strongly reinforced by the findings of Abbas et al. (2015) who found the biodiversity and dynamics of macro-invertebrate population in wheat weeds in agro-ecosystem and recorded diversity (H = 3.36) and evenness = 0.402 when Species richness (S) = 72, however when S = 58, diversity (H = 3.23) and evenness (E = 0.79) on wheat. Thus, it can be concluded that when species richness decreases the value of H decreases and E increases and was in conformity with our result.

In the present study order Hemiptera was the predominant order and comprised about 89 % of the pests infesting walnut. Similar results were found by Rajadurai & Thiagarajan (2003) who reported 18 heteropterans and 10 homopterans infesting mulberry and found it dominant order causing high infestation. During the pest investigation each season showed fluctuations in the total species richness and abundance, pest activity was high during spring and summer. On set of autumn there was reduced pest load as there was sharp decline in the abundance of pests and in winter no pest diversity was observed. Similar result was found by Kutschbach- Brohl et al., (2010). Seasonal fluctuations can be explained as all the insect species have different phenologies and resulted difference in activity periods is depending on temperature (Booij, 1995). The present study also correlates with the findings of Daiqin & Jackson (1996) and Finch et al., (2008). The change in diversity index values is attributed to rainfall and other environmental factors. Cartea et al. (2009) found the similar results when studied the lepidopteron pest population infesting *Brassica* in Spain for six years and concluded that environment is directly correlated with pest population. Presence of food is one of the important factors for maintenance of diversity in a particular ecosystem. This is strongly reinforced by the findings of Perrins et al. (1991) who concluded that the presence of any species is restricted by the distribution of its habitat and within that habitat there must be enough food availability and other resources so that species exists. The predominant natural enemies were ladybird beetles however, population was not much pronounced. Manjunath et al. (1989) also reported that parasites and predators are inadequate to check pest build up thus, management strategies are very essential to check further losses. Species richness and abundance are both essential aspects for the structure of community. Several studies have concluded the community structure can be changed with the change in the relative abundance of species even when species richness remains the same (Magurran, 1988; Stirling & Wilsey, 2001).

4 CONCLUSION

Walnut industry is one of the prime industries of Kashmir with over 90 % of its demand in the country met by Kashmir only. Nonetheless unlike apples, this sector is yet to flourish at international market due to various factors and non-seriousness of government as well as other stake holders notwithstanding, the importance is quintessential for its existential threat. In this backdrop, this piece of research work has been conducted to check its current status and insect pest diversity with their overall effect on quality and quantity of walnuts. A total of nine sites were selected from three different districts and the insects pests collected belonged to 3 orders 7 families and 10 species. The abundance of pests infesting walnut orchards showed the same seasonal pattern with increase in spring, reaching maximum in summer and then decreasing in autumn. A wide range of abundance (4944 individuals at site B1 of the district Budgam to 1207 individuals at site G3 of the district Ganderbal) and number of species (7 at site S1 to 4 at site G3 of the district Ganderbal) clearly demonstrated that there are slight differences amongst these sites in the prevailing factors that affect pest community. Most of the sites had consistent number of the pests collected during the study period. The study also depicted that order Hemiptera was highly damaging in comparison to Coleoptera and Lepidoptera

while in Hemiptera, the strongest damage was done by *C. juglandicola* and the weakest one by *A. pilipes* (Figure 8z). Observations of these sites did not reveal any major changes in habitat between the study period of one and half year. Diversity indices, i.e. Shannon-Weiner index, Simpson index, Pielou's index and Margalef's index varied in different sites and time of the year, which is attributed to the change in quality and quantity of food as well as temperature fluctuations. The work represented here would be very helpful in controlling the walnut pests in different seasons to yield better quality and quantities of walnut in Kashmir.

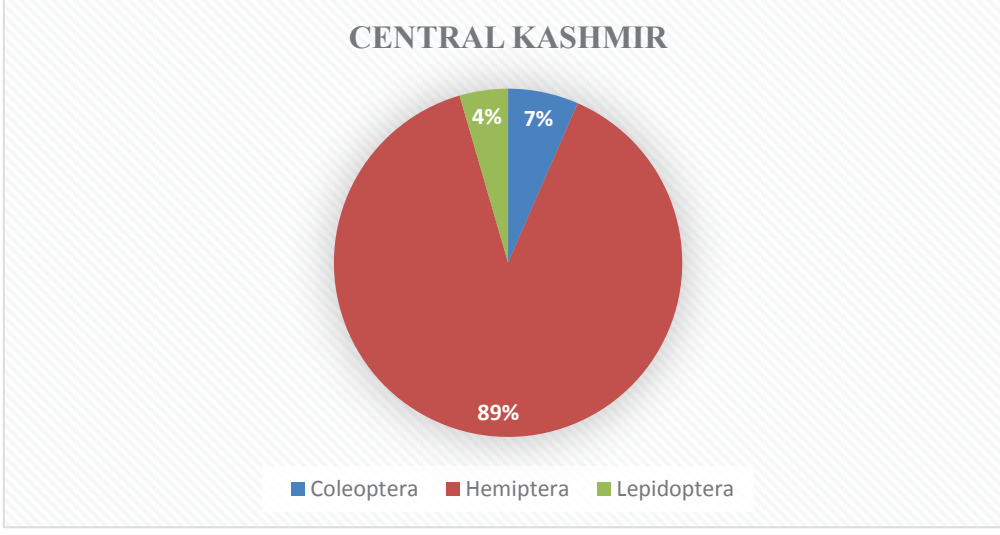

Figure 8Z: Overall composition of insect orders infesting walnut orchards in Central Kashmir (%).

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Evaluation of arthropods diversity on apple crop ('Red Delicious') in Sidi Naâmane area (Tizi-Ouzou), Algeria

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ABSTRACT

Arthropods fauna contributes significantly to biodiversity and ecosystem functioning. In this context an inventory of arthropods communities upon ecological apple plot, 'Red Delicious' is realized in Sidi Naâmane area (Tizi-Ouzou, Algeria). This study was conducted from November 2014 to December 2015, by combining different sampling techniques: sweep net, barber pot and colored traps.
The results showed a total of 113 species distributed into 64 families, 10 orders and 3 classes, which are Arachnida, Enthognata and Insecta. The colored traps sampling method allowed collecting 63 species (with 30 % of pests), among which *Coruna* sp. is the most noted, with relative abundance of 6.77 %. The barber traps sampling method allowed collecting 56 species (with 42.88 % of pests), among which *Harpalus paratus* Casey, 1924 is the most frequent, with 6.51 %. The sweep net sampling method allowed collecting 80 species (with 33 % of predators), among which *Coccinella algerica* Kovář, 1977 is the most noted, with relative abundance of 5.32 %. The Shannon diversity index values ranged from 5.33 bits for the Barber pot traps method to 5.58 bits for colored traps and 5.90 bits for the sweep net technique.

Key words: inventory; arthropods; apple; 'Red Delicious'; Sidi Naâmane; Algeria

IZVLEČEK

OVREDNOTENJE RAZNOLIKOSTI ČLENONOŽCEV NA JABLANI ('Red Delicious') V OBMOČJU SIDI NAÂMANE (TIZI-OUZOU), ALŽIRIJA

Favna artropodov prispeva v veliki meri k raznolikosti in delovanju ekosistemov. V tem smislu je bil izveden popis členonožcev v sadovnjaku rdečega delišesa ('Red Delicious') v območju Sidi Naâmane (Tizi-Ouzou, Alžirija). Raziskava je bila opravljena od novembra 2014 do decembra 2015, s kombinacijo različnih vzorčevalnih tehnik kot so lovljenje z mrežo, z lovilnimi lončki in z obarvanimi pastmi.
Celokupno so na vzorčeni površini našli 113 vrst, ki so spadale v 64 družin, 10 redov in v 3 razrede, ki so bili Arachnida, Enthognata in Insecta. Vzorčevalna metoda z obarvanimi pastmi je dala ulov 63 vrst (s 30 % škodljivcev), med katerimi so bile vrste iz rodu *Coruna* sp. najpogostejše, z relativno abundanco 6,77 %. Z vzorčevalno metodo lovilnih lončkov se je ujelo 56 vrst (z 42,88 % škodljivcev), med njimi je bila vrsta *Harpalus paratus* Casesy, 1924 najpogostejša, z 6,51 %. Z metodo lovljenja z mrežo je bilo ujetih 80 vrst (s 33 % plenilcev), med njimi je bila alžirska polonica *Coccinella algerica* Kovář, 1977 najpogostejša, z relativno abundanco 5,32 %. Shannonov diverzitetni indeks je bil v območju od 5,33 za lovilne lončke, do 5,58 za obarvane pasti in 5,90 za lovlenje z mrežo.

Ključne besede: popis; artropodi; jablana; 'Red Delicious'; Sidi Naâmane; Alžirija

1 INTRODUCTION

The apple crop extends to all temperate climates areas of the globe; it is ranked as first fruit trees cultivated worldwide (Chouinard et al., 2000). Fruit trees like any plant species form a favorable environment for the spread of pests and infectious diseases.

Arthropods occupy a special place in the ecosystem, they are good biological indicators, and occupy very diverse ecological niches (Clere and Bretagnolle, 2001).

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Arthropods can be harmful to crops but also helpful such as parasites and predators involved in regulating populations of pests.

Sustainable arboriculture aims to produce quality fruits with a minimization of negative impacts on the environment and human health, caused by the misuse of pesticides against pests (Dubuis, 2010). Therefore, it is necessary today to develop new methods for protecting apple and consider their negative impacts by implementing new agricultural practices that integrate sound management of pests and respect the balance of environment and human health.

For this, it is fundamental to understand the relationships between insect pests and their host plants and thus know their spatial and temporal dispersion in a region (Debouzie and Thioulouse, 1986).

The objective of the work is to study the arthropod fauna associated with apple crop, and identifying possible predators and parasites that can intervene in the regulation of pest populations in order to envisage a rational control program and more respectful of the environment.

2 MATERIALS AND METHODS

This study was conducted in a 'Red Delicious' orchard not subject to treatment by pesticides. The parcel is located in the Sidi Naâmane area (36°45'29'' Nord, 3°59'02'' East) (Tizi-Ouzou, Algeria) in a Mediterranean climate characterized by a sub-humid bioclimatic stage with temperate winter.

The study was conducted from November 2014 to December 2015, covering vegetation, flowering and fruiting periods of Malus domestica Borkh. plants.

2.1 Geographical location of the study area

The ecological apple orchard is situated in Sidi Naâmane area (Tizi-Ouzou) at 100 km East from Algiers and 30 km south of the Mediterranean coasts (Fig.1).

In an orchard of 1600 trees, a plot of 100 trees is isolated for the study. Sampling of arthropod populations was performed by using three methods namely colored air traps (on the foliage), Barber pot and sweep net.

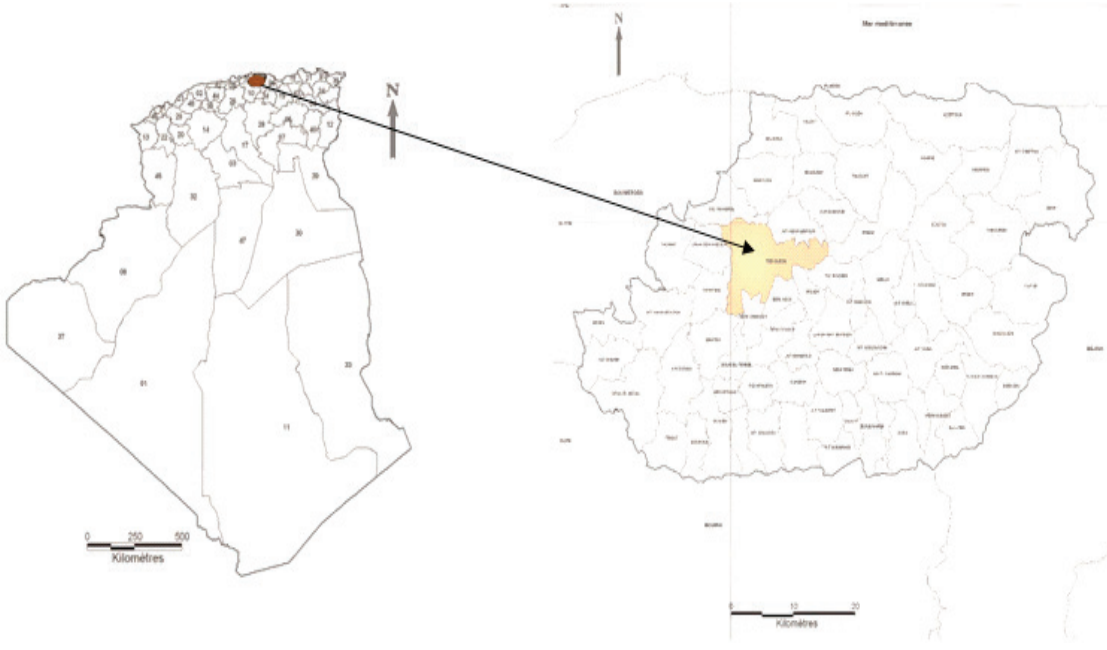

Figure 1: Location of the study area in Algeria (Google maps, 2019)

2.2 Sampling methods

Nine Barber pots were disposed in quadra, filled to 2/3 of their content with soapy water; for collecting walkers arthropod; they are visited once a week. The content was collected and put in jars with labels on which were indicated the date of collection and the trap concerned.

For colored traps, nine yellow basins are suspended by an iron wire to apple trees and filled with soapy water to two-third of their height; for collecting arthropods lodged in the foliage. The water trap is renewed after each removal.

Sweep net moving is applied in herbaceous layer between the rows of the study plot once a week during the study period, by dislodging arthropods hidden in vegetation.

All samples collected in the field are brought back to the laboratory for being sorted and identified under a binocular microscope. The determination of arthropods species, based on morphological characters and their chaetotaxy, was performed by using different identification keys Sergent (1909); (Perrier, 1927, 1932, 1961); (Seguy, 1923, 1924); (Piham, 1986); (Chinery, 1988); (Delvare & Aberlenic, 1989).

2.3 Data processing

For treating the results obtained, different indices were used.

The total wealth is calculated for each sampling method. It is the total number of species that includes the population considered in an ecosystem (Ramade, 2003).

The relative abundance (centesimal frequency) Fc (%) was also evaluated; it gives the percentage of individuals of a species Ni relative to the total number of individuals N (Dajoz, 1971).

$$Fc = Ni \times 100 / N$$

According to Barbault (1981), species diversity is measured by various indexes; the most used is the Shannon-Weaver. It is calculated by the following formula:

$$H' = - qi \log2 qi$$

H'**:** diversity index expressed in bits units
qi: the probability of encountering the species i
The equitability index is the ratio of observed diversity H' to the maximum diversity' max**:** E = H'/H' max (Blondel, 1979). Knowing that H' max is calculated using the following formula:

$$H' max = Log 2 S$$

S: total wealth
H'max: is expressed in bits

3 RESULTS

During this study which focused on the inventory of arthropods fauna associated to apple trees in an ecological orchard not subjected to pesticide treatments, 113 species were captured, distributed in 64 families belonging to 10 orders and to 3 classes.

3.1 Total wealth and relative abundance

The collected arthropod in a 'Red Delicious' apple plot using different trapping methods allowed us to identify 113 species belonging to three classes: Arachnida, Enthognata and Insecta. The Insecta class is the best represented with eight orders. The total wealth of the species caught by the three trapping methods was 80 species for the sweep net; 63 species for colored traps and 56 species for Barber pots (Table 1).

Table 1: Total wealth of species caught by different sampling methods

Traps	Sweep net	Colored traps	Barber pots
Total wealth	80 Species	63 Species	56 Species

Centesimal frequency (CF) of arthropod orders captured in an apple plot using different sampling methods is shown in Table 2. Centesimal frequency of species identified according to the order, family and gender are presented in Table 3.

Table 2: Centesimal frequency of arthropod species captured using different sampling methods

Orders	Sweep net CF %	Barber pot CF %	Colored traps CF %
Spiders	5.32	6.51	2.19
Collembola	0	7.75	0
Neuroptera	0.25	0	1.75
Hymenoptera	36.38	17.08	37.13
Diptera	20.03	6.69	25.3
Heteroptera	7.35	0	6.77
Homoptera	2.66	3.52	12.23
Coleoptera	24.84	50.35	14.63
Dermaptera	0.38	2.29	0
Orthoptera	2.79	5.81	0
Total	100	100	100

The most dominant order recorded for sweep net and colored traps is Hymenoptera with relative abundance of 36.38 % and 37.13 % respectively for Barber potgs, the most dominant order is Coleoptera with relative abundance equal to 50.35 %.

Table 3: Centesimal frequency of arthropod species captured using different sampling methods

Classes	Orders	Families	Species	Centesimal frequency (%)		
Arachnida	Spiders	Opilionidae	*Phalangida* sp. Sundevall, 1833	0.38	0	0.65
		Dysderidae	*Dysdera crocata* Koch, 1838	0	1.41	0
		Gnaphosidae	*Gnaphosidae* sp. Pocock, 1898	0	0.70	0
		Thomisidae	*Thomisus* sp. Walckenaer, 1805	0.38	0	0.44
		Philodromidae	*Tibellus* sp. Simon, 1875	1,52	0	0
		Pisauridae	*Pisaura mirabilis* Clerck, 1757	1.14	0.53	0
		Salticidae	*Salticidae* sp. Blackwall, 1841	1.52	0	1.09
		Lycosidae	*Lycosidae* sp. Sundevall, 1833	0.38	3.87	0
Enthognata	Collembola	Entomobryidae	*Entomobrya nivalis* Rondani, 1861	0	0.18	0
			Orchesella cincta Linnée, 1758	0	2.46	0
		Sminthuridae	*Sminthurus viridis* Lubbock, 1862	0	5.10	0
Insecta	Neuroptera	Chrysopidae	*Chrysoperla carnea* Stephens, 1836	0.25	0	1.75
	Hymenoptera	Apidae	*Eucera panonica* Linnée, 1758	1.65	0	1.31
			Bombus terrestris Linnée, 1758	0.38	0.53	0.87
			Panurgus sp. Panzer, 1806	0.89	0.70	3.93
			Apis mellifera Linnée, 1758	2.92	1.58	3.27
			Eucera longicornis Linnée, 1758	0.76	0	0.44
		Scoliidae	*Colpa quinquecinta* Latreille, 1802	2.79	0	1.09
			Scolia sp. Fabricius, 1775	0.38	0	0
		Andrenidae	*Andrena* sp. Fabricius, 1775	1.14	0.53	3.71
		Colletidae	*Hylaeus meridionalis* Forster, 1871	0.51	0	0
		Pompilidae	*Priocnemis* sp. Latreille, 1802	1.65	0	2.62
		Ichneumonidae	*Coruna* sp. Walker, 1833	1.14	0	6.77
			Diplazon sp. Fabricius, 1781	0.89	0	0
		Tenthredinidae	*Tenthredo marginella* Linnée, 1758	0.76	0	0

	Formicidae	*Pheidol pallidula* Nylander, 1849	0	0	1.09
		Cataglyphis viaticus Forster, 1850	0	1.06	0
		Cataglyphis bicolor Forster, 1850	0	2.99	0
		Aphaenogaster sp. Mayr, 1853	0	2.11	0
		Messor barbarus Linnée, 1767	1.14	4.92	0
		Componotus lateralis Olivier, 1792	0.25	2.11	0
		Plagiolepis sp. Mayr, 1861	0	0.53	0
	Pteromalidae	*Pteromalus puparum* Linnée, 1758	1.77	0	3.49
	Eupelmidae	*Eupelmus* sp. Walker, 1833	1.39	0	0.44
	Brachonidae	*Cotesia* sp. Linnée, 1758	0.50	0	0
		Brachonidae sp. Latreille, 1829	0.25	0	0
	Vespidae	*Lasioglossum calceatum* Scopoli, 1763	3.42	0	2.84
		vespula germanica Fabricius, 1793	0.38	0	0
		Polistes gallicus Linnée, 1761	4.69	0	2.62
	Sphecidae	*Sceliphron destillatorium* Klug, 1801	0.63	0	0.44
	Megachilidae	*Osmia cornuta* Latreille, 1805	0.38	0	0
		Megachile centuncularis Linnée, 1758	1.65	0	0
		Megachile fertoni Pérez, 1896	0.89	0	0
	Halictidae	*Halictus* sp. Latreille, 1804	1.14	0	1.53
	Trichogrammatidae	*Trichogramma daumalae* Westwood, 1833	2.03	0	0.65
Diptera	Culicidae	*Anopheles* sp. Meigen, 1818	0.25	0	2.40
		Culiseta sp. Felt, 1904	0.51	0	0.65
		Culex sp. Linnée, 1758	0	0	1.31
		Culex pipiens Linnée, 1758	1.52	0	3.71
	Ceratopogonidae	*Culicoides albicans* Winnertz, 1852	0.63	0	0.87
	Calliphoridae	*Calliphora vicina* Robineau-Desvoidy, 1830	1.77	0.70	1.53
		Calliphora vomitoria Linnée, 1758	0.63	0.35	0.65
		Calliphoridae sp. Hough, 1899	0	0	0.44
		Lucilia ceasar Linnée, 1758	1.90	1.06	2.18
	Tephritidae	*Ceratitis capitata* Weidemann, 1824	0	0	3.93
	Stratiomidae	*Chloromyia formosa* Duncan, 1837	2.66	0.53	2.18
	Tabanidae	*Chorisops* sp. Meigen, 1820	1.14	0	0
	Syrphidae	*Melanostoma mellinum* Linnée, 1758	3.04	0	0
		Eristalis tenax Linnée, 1758	0.89	0.35	1.09
		Syrphus ribesii Linnée, 1758	1.01	0	0.44
	Tipulidae	*Tipula oleracea* Linnée, 1758	1.52	0	0.65
		Tipula lateralis Linnée, 1758	1.8	0	0
	Empididae	*Empis grisea* Fallen, 1816	0.76	0.53	0
		Empis sp. Linnée, 1758	0	0	0.65
	Muscidae	*Graphomya maculata* Scopoli, 1763	0.51	0	0.87
	Fannidae	*Fannia* sp. Robineau-Desvoidy, 1830	0	0	0.44
	Chironomidae	*Chironomus plumosus* Linnée, 1758	0	3.17	1.31
Heteroptera	Scutelleridae	*Eurygaster maura* Linnée, 1758	0.38	0	1.75
		Eurygaster testudinaria Geoffroy, 1758	0	0	0.87

		Pentatomidae	*Rhaphigaster nebulosa* Poda, 1761	0.63	0	0
			Dolycoris baccaum Linnée, 1758	0.51	0	0.44
		Lygaeidae	*Nysius* sp. Dallas, 1852	4.18	0	0.65
			kleidocerys resedae Panzer, 1797	0.76	0	0.87
		Reduvidae	*Rhynocoris erythropus* Linnée, 1767	0	0	1.09
			Reduvius sp. Fabricius, 1775	0.89	0	0
		Cydnidae	*Cydnus atterimus* Forster, 1771	0	0	1.09
	Homoptera	Aphididae	*Aphis fabae* Scopoli, 1763	1.14	1.23	5.46
			Aphis pomi De Geer, 1773	1.52	0.70	3.71
			Dysaphis plantaginea Passerini, 1860	0	1.58	3.05
	Coleoptera	Apionidae	*Apion* sp. Schoenherr, 1823	0.89	1.40	2.84
		Buprestidae	*Anthaxia dimidiata* Thunberg, 1789	1.77	0.35	1.31
		Mordellidae	*Variimorda villosa* Schrank, 1781	2.28	0	1.09
		Coccinelidae	*Thea vigintiduopunctata* Linnée, 1758	0.51	0.35	0.44
			Coccinella quatuordecimpunctata Linnée, 1758	0.38	0	0
			Coccinella algerica Kovář, 1977	5.32	0.70	2.84
			Hippodamia variegata Goeze, 1777	3.42	0.53	2.18
		Bruchidae	*Bruchidius* sp. Fabricius, 1792	0.76	0	0
		Cetoniidae	*Oxytheria funesta* Poda, 1761	4.94	1.23	1.09
		Scarabaeidae	*Scarabaeus* sp. Linnée, 1758	0	2.64	0
			Geotropus sp. Linnée, 1758	0	1.40	0
			Rhizotrogus maculicollis Villa & Villa, 1833	0	5.98	0
			Rhizotrogus aestivus Olivier, 1789	0	2.11	0
		Carabidae	*Carabus auratus* Linnée, 1760	0.51	2.82	0
			Macrothorax morbilusus Fabricius, 1792	0	4.75	0
			Cicindella campestris Linnée, 1758	0	1.94	0
			Harpalus paratus Casey, 1924	0.25	6.51	0
			Bembidion sp. Latreille, 1802	0.76	4.58	0
		Curculionidae	*Lixus* sp. Fabricius, 1801	1.14	2.64	0.44
			Phyllobius sp. Germar, 1824	1.65	1.23	1.09
		Staphilinidae	*Ocypus olens* Muller, 1764	0	3.87	0
		Elateridae	*Drilus flavescens* Olivier, 1790	0	3.35	0
			Agriotes lineatus Linnée, 1767	0	0.53	1.31
		Meloidae	*Meloe proscarabaeus* Linnée, 1758	0	0.88	0
		Histeridae	*Hister* sp. Linnée, 1758	0.25	0.53	0
	Dermaptera	Forficulidae	*Forficula auricularia* Linnée, 1758	0.38	2.29	0
	Orthoptera	Oedipodidae	*Oedipoda germanica* Latreille, 1804	0.63	1.23	0
		Grillidae	*Acheta domestica* Linnée, 1758	0	0.88	0
			Grillus campestris Linnée, 1758	0.38	2.11	0
		Acrididae	*Anacridium aegyptium* Linnée, 1764	0.76	0.53	0
		Blattelidae	*Ectobius* sp. Stephens, 1835	0	1.06	0
		Tetrigidae	*Acrida ungarica* Herbst, 1786	1.01	0	0
Total	10		64 113 Species	100	100	100

The colored traps allowed us to collect 63 species, represented mainly by *Coruna* sp. with 6.77 %, may be parasitic of *Aphis fabae* with relative abundance of 5.46 %. The species *Thomisius* sp., *Eupelmus* sp., *Sceliphron destillatorium, Calliphoridae* sp., *Syrphus ribesii, Fannia* sp., *Dolycoris baccaum, Thea vigintiduo punctata,* and *Lixus* sp. presented a low centesimal frequency of 0.44 %.

Barber pot allowed us to collect 56 species, represented mainly by *Harpalus paratus* with relative abundance of 6.51 % which are natural predators of various pests, followed by *Rhysotrogus maculicolis* with 5.99 %. The lowest relative abundance was recorded for the species *Entomobrya nivalis* with 0.18 %.

The sweep net allowed us to collect 80 species, represented mainly by the species *Coccinella algerica* with relative abundance of 5.32 %, followed by *Nyisus* sp. with relative abundance of 4.18 % which are active natural predators of pests such as aphids. The species which presented a low centesimal frequency was *Chrysoperla carnea, Componotus lateralis, Hister* sp., *Harpalus paratus, Anopheles* sp, *Brachonidae* sp. recording a value of 0. 25 %.

3.2 Species centesimal frequency according to their trophic relationships

The relative abundance obtained for species according to their trophic relationships is illustrated for sweep net (Fig. 2), for colored traps (Fig. 3) and for barber pots (Fig. 4).

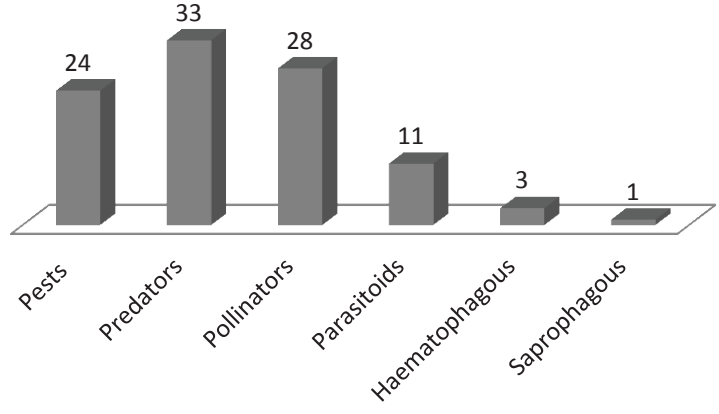

Figure 2: Relative frequency of species caught using sweep net following their diet

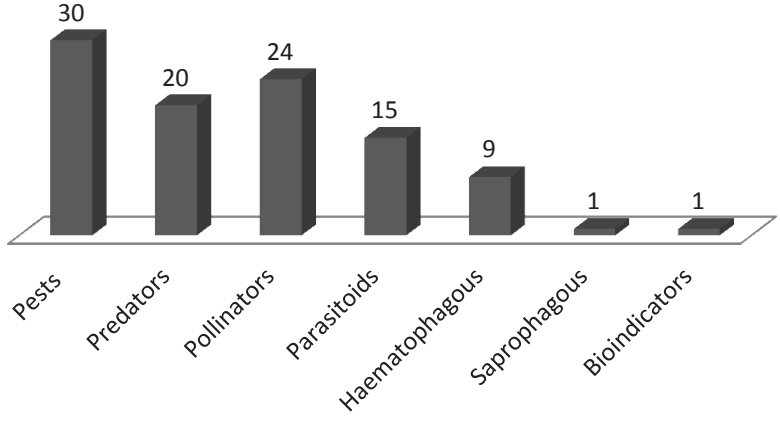

Figure 3: Relative frequency of species caught using colored traps following their diet.

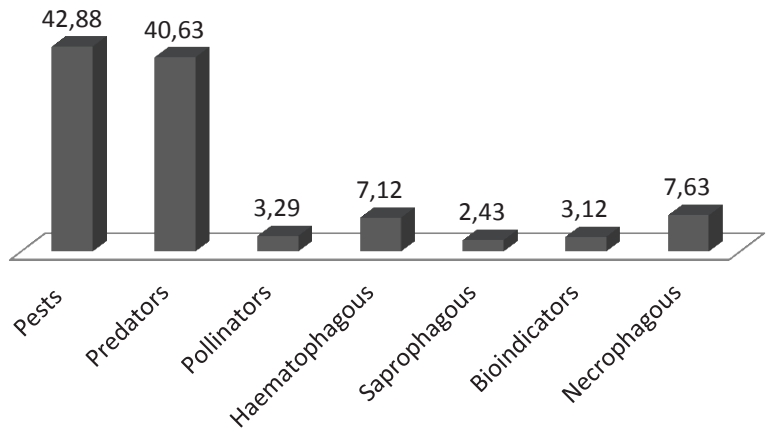

Figure 4: Relative frequency of species caught using barber pots following their diet

The best represented group using sweep net is predators with relative abundance of 33 %, whereas the least abundant group is saprophagous with only 1 %.

The best represented group using colored traps is pests with 30 %, whereas the least abundant group are saprophagous and bioindicators with only 1 %.

When using barber pots, the best represented group is pests with relative abundance of 42.88 %, while the group of saprophagous is the least represented recording only 2.43 %.

3.3 Shannon Weaver diversity index and evenness index (E)

Shannon-Weaver diversity index (H '), maximum diversity (H'max.) and equitability (E) applied to species trapped by the different sampling techniques are presented in Figure 5.

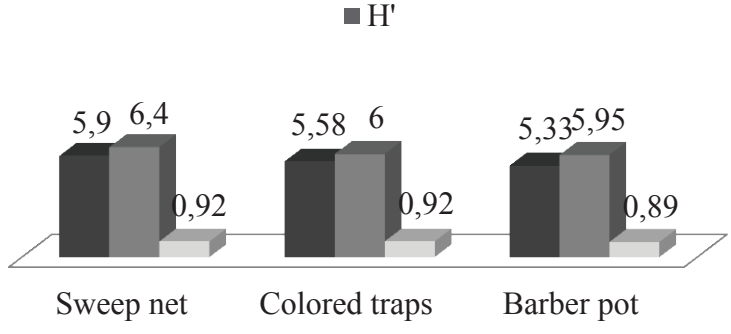

Figure 5: Shannon-Weaver diversity values H' and evenness of species trapped by the various traps

Shannon-Weaver diversity values for the various species caught by trapping methods are equal to H' = 5.90 bits; H max = 6.40 bits for sweep net; H' = 5.58 bits; H max = 6 bits for colored traps and H' = 5.33 bits; H max = 5.95 bits for Barber pots. The species evenness values are E = 0.92 for the sweep net and colored traps; and E = 0.89 for barber pots. A fairly high evenness is recorded for three sampling methods (sweep net, colored traps and barber pots) this value approaches a value of 1 which reflects a balance between the middle of species.

4 DISCUSSIONS AND CONCLUSION

The background knowledge of arthropods restricted to the apple crop in the region of Tizi Ouzou is a first step towards developing effective approaches for insect-pest control and for auxiliary species conservation. Our inventory upon ecological orchard not subjected to pesticide treatments, revealed 113 species distributed in 64 families belonging to 10 orders and to 3 classes.

Guettala-Frah (2009) has identified 348 insect species distributed in 97 families and 13 orders on apple orchard of the Aures region (Algeria) during three years (2001 to 2003).

(Allili, 2008) mentioned 23 species belonging to 19 families divided into eight levels of three classes in a pear orchard Birtouta (Algiers). (Frah et al., 2015) during his study on arthropodofauna in Sefiane (Batna) estimated the total wealth to S = 71 using barber pot, S = 63 for colored traps, and S = 54 for sweep net.

(Ounis et al., 2014) during an estimation of soil fauna biodiversity in an apricot orchard, report that the order of Coleoptera dominate with a percentage equal to 46.67 %.

According to the diet, (Guettala-Frah, 2009), in his study on the economic impact and bioecology of the main apple pests in the Aurés region, recorded 69.72 % of pests, followed predators with a percentage equal to 15.98 %, and 4.76 % for parasitoid. Finally, saprophages, necrophages and coprophages account for low levels of less than 3 %. (Mahdjane, 2013) obtained a frequency of 57.4 % of pests, followed by predators with a value of 20.63 % and polyphagous with 18.87 %, in her inventory of apple insects in Tadmait area, Tizi-Ouzou.

According to (Blondel, 1979), a community is even more diversified as the diversity index is higher. (Guermah and Medjdoub-Bensaad, 2016) report a value of H = 4.31 bits with a maximum range of H max = 6.64 bits applied to arthropods sampled by using the sweep net in the Tizi Ouzou region. Using the trapping trap technique for the study of arthropod biodiversity in 3 steppes in Djelfa area, (Guerzou et al., 2014) report variations in diversity values between 1.9 and 3.7 bits in Taicha, 3.02 and 3.5 bits in El Khayzar and 3.6 and 4.0 bits in Guayaza. (Frah et al., 2015) during his study on arthropodofauna upon an olive orchard in Sefiane (Batna) report a value of H = 4.7 bits, Hmax = 6.1 using barber pot; H = 4.6 bits, Hmax = 6 for colored traps and H = 5.2 bits, Hmax = 5.8 for sweep net. (Guermah and Medjdoub-Bensaad, 2016) found an evenness of 0.65; (Ounis and al., 2014) find a fairness varying from 0.12 to 0.47. (Frah et al., 2015) during his study on arthropodofauna upon an olive orchard in Sefiane (Batna) found an evenness of 0.77 using barber pot and colored traps, and 0.90 for sweep net.

The proliferation of arthropods and their diversity is favored by the absence of phytosanitary treatment in the study plot. Therefore, we notice the presence of a very varied auxiliary fauna composed of predators and parasitoids with significant values to maintain the pest populations at an economically acceptable level.
The identification of these arthropods and their trophic relationship constitutes an important scientific base, likely to contribute to the establishment of an appropriate integrated control strategy within these agro-ecosystems, from the perspective of an alternative approach to the use of pesticides and the preservation of biodiversity and the environment.

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Study of genetic diversity in different wheat species with various genomes based on morphological characteristics and zinc use efficiency under two zinc-deficient growing conditions

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ABSTRACT

Screening of cash crops to tolerate and grow under low levels of micronutrients is important issue in the plant breeding programs. Thus, the study screened the tolerance of 50 wheat genotypes to zinc (Zn) deficiency in the calcareous soil. The Zn treatment was carried out with application of 5 mg kg^{-1} (+Zn) and without (-Zn) to the collected soils with initial Zn extractable of 0.5 mg Zn kg^{-1} soil. The results revealed that the supplementary application significantly increased shoot dry matter, shoot Zn concentration and shoot Zn content compared to the without Zn application (control), but Zn utilization decreased under Zn application. There was considerable genetic variation in Zn efficiency (55 - 118 %), shoot Zn concentration (11.8 - 27.0 and 14.3 - 39.6 mg kg^{-1} DM under deficient and sufficient Zn, respectively), shoot Zn content (0.56 - 2.02 and 0.90 - 2.83 µg $plant^{-1}$, under deficient and sufficient Zn, respectively) and Zn utilization efficiency (39 - 87.2 and 31.2 - 71.5 mg DM μg^{-1} Zn under deficient and sufficient Zn, respectively) within wheat genotypes. Cluster analysis based on Zn efficiency, and shoot dry matter at deficient and adequate Zn conditions classified the genotypes into four clusters. Over the two conditions, the most Zn-efficient and Zn-unefficient genotypes were 'Ankara-98' and 'Altintoprak-98' and 'Pg"S' and 'Zarin', respectively. Most durum genotypes had a greater Zn efficiency than modern bread wheat genotypes, therefore these genotypes could be effectively used to breed the new cultivars with high Zn efficiency for calcareous soils.

Key words: durum wheat; bread wheat; zinc concentration; zinc deficiency; zinc efficiency; biofortification

Abbreviations: Zn - Zinc, DAS - days after sowing, DM - dry matter, PVC - plastic pots, FC - field capacity, DARI - Dryland Agricultural Research Institute, AAS - atomic absorption spectrophotometer, ANOVA - analysis of variance, DMRT - Duncan's multiple range test, SE - standard error, SOD - superoxide dismutase, CA - carbonic anhydrase.

IZVLEČEK

PREUČEVANJE GENETSKE RAZNOLIKOSTI DVEH VRST PŠENICE Z RAZLIČNIMA GENOMOMA NA OSNOVI MORFOLOŠKIH LASTNOSTI IN UČINKOVITOSTI IZRABE CINKA V DVEH RAZMERAH NJEGOVE POMANKLJIVE OSKRBE

Preverjanje poljščin na rastno strpnost majhnim koncentracijam mikrohranil je pomemben izziv v rastlinskih žlahtniteljskih programih. V raziskavi je bila preverjena toleranca 50 genotipov pšenice na pomanjkanje cinka (Zn) na apnenčastih tleh. Obravnavanja s cinkom so obsegala uporabo (5 mg Zn kg^{-1} , +Zn) in neuporabo cinka (-Zn) v tleh z začetno vsebnostjo ekstraktibilnega Zn 0,5 mg Zn kg^{-1} tal. Izsledki so pokazali, da je dodajanje cinka značilno povečalo vsebnost suhe snovi poganjkov in vsebnost cinka v njih v primerjavi s kontrolo, a hkrati zmanjšalo učinkovitost njegove izrabe. Med genotipi je bila ugotovljena znatna genetska variabilnost v učinkovitosti izrabe cinka (55 - 118 %), v koncentraciji Zn v poganjkih (11,8 - 27,0 in 14,3 - 39,6 mg kg^{-1} DM v razmerah pomankljive in zadostne oskrbe s cinkom), v vsebnosti Zn (0,56 - 2,02 in 0,90 - 2,83 µg na rastlino, v razmerah pomankljive in zadostne oskrbe s cinkom) in v učinkovitosti izrabe cinka v ramerah pomankljive (39 - 87,2) in zadostne oskrbe s cinkom, (31,2 – 71,5 mg DM/µg Zn). Klasterska analiza, osnovana na učinkovitosti izrabe Zn in vsebnosti suhe snovi poganjkov v razmerah zadostne in pomankljive oskrbe s cinkom je genotipe razdelila v štiri skupine. V obeh rastnih razmerah sta Zn najučinkoviteje izrabljala genotipa 'Ankara-98' in 'Altintoprak-98' in najmanj učinkovito genotipa 'PgS' in 'Zarin'. Večina genotipov trde pšenice je imelo večjo učinkovitost izrabe cinka kot genotipi krušne pšenice, zato bi te lahko učinkovito uporabili pri žlahtnenju novih sort pšenice, ki bi dobro uspevale na apnenčastih tleh z veliko učinkovitostjo izrabe cinka.

Ključne besede: trda pšenica; krušna pšenica; vsebnost Zn v tleh; pomankanje Zn; učinkovitost izrabe Zn; biofortifikacija

Okrajšave: Zn – cink, DAS – dnevi po setvi, DM – suha snov, PVC – plastični lonci, FC – poljska kapaciteta, DARI – Dryland Agricultural Research Institute, AAS – atomski absorpcijski spektrofotometer, ANOVA – analiza variance, DMRT – Duncanov test, SE – standardna napaka, SOD – superoksid dismutaza, CA – karboanhidraza

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1 INTRODUCTION

Zinc deficiency is one of the common restricting factors in crops production, especially cereals, in world (Alloway, 2008). This scarcity is severed in calcareous soils of rainfed areas due to low availability caused by high levels of calcium carbonates, low total Zn contents, high pH and high phosphate in the soil (Alloway, 2009). Thirty percent of world's cultivated soils are estimated to be inadequate in zinc, chiefly in the Mediterranean region and Asia (Suzuki et al., 2006; Alloway, 2009). The investigations has been estimated that approximately up to 40 % of the soils under wheat production areas of Iran are encountered with a level of Zn-deficiency which has drastically influenced the crop performance (Broadley et al., 2007; Esfandiari et al., 2016; Esfandiari and Abdoli, 2016). Thus, in these areas loss of yield is the main concern of farmers. To deal with the problem, applications of different Zn-source of chemical fertilizers are proposed to enhance the plant growth and development, and finally increase crop yield (Sadeghzadeh et al., 2009; Bharti et al., 2013; Abdoli et al., 2014; Guo et al., 2016; Esfandiari et al., 2016).

Sensitivity to Zn deficiency in plants is species specific phenomena and among cereals, wheat is more sensitive than rye, triticale and barley (Cakmak et al., 1997, Cakmak et al., 1999; Blum, 2014). Also durum wheat has a more sensitivity to this deficit (Genc and McDonald, 2008). Studies have shown large variations in performance of bread and durum genotypes in Zn-deficient soils (Rengel and Graham, 1995; Cakmak et al., 1996, Cakmak et al., 1999; Kalayci et al., 1999; Torun et al., 2000; Moshiri et al., 2010; Velu et al., 2012; Abdoli et al., 2016; Yilmaz et al., 2017; Esfandiari et al., 2018). Therefore, the selection and breeding of tolerant genotypes to low Zn content in the soil are logical ways to overcome the Zn deficiency in wheat and other crops (Genc and McDonald, 2008; Chatvaz et al., 2010). There is very promising progress in breeding of Zn biofortified cereal genotypes, particularly through the HarvestPlus program (Gomez-Coronado et al., 2016). Generally, the combination of plant breeding and agronomic biofortification is the most affordable and reasonable approach to attenuate Zn deficiency-related problems in humans, however also in crop production (Cakmak, 2008; Gomez-Coronado et al., 2016).

The aims of this study were (i) to screen fifty genotypes of durum and bread wheat for their potential to use of Zn element at early growth stages, (ii) to identify the most Zn-efficient and Zn-inefficient wheat genotypes to be utilized in further genetic studies, and (iii) assess the impact of Zn application on shoot dry matter, Zn concentration and content, and Zn utilization efficiency in wheat.

2 MATERIALS AND METHODS

2.1 Plant materials

Wheat genotypes including eight winter bread wheat (*Triticum aestivum* L.) and forty-two winter durum wheat (*Triticum durum* L.) were obtained from Dryland Agricultural Research Institute (DARI), Maragheh of Iran. The details of wheat genotypes are shown in Table 1.

2.2 Soil preparation and crop management

The used soils were collected from severely Zn-deficient soils of Moghanlou, Bijar state in the Kourdistan city of Iran (47° 56' E, 36° 08' N; 1478 m elevation from sea level), where previous study proved the decline of wheat yield due to Zn deficiency (Esfandiari, unpublished; Abdoli, 2017). The soil details of the location are shown in the Table 2. Critical Zn concentration deficiency was considered when the concentration declined below to 0.5 - 0.6 mg kg^{-1} (Sims and Johnson, 1991). Plastic pots (PVC, 20 × 35 cm) were filled with 3.5 kg soil of the combined samples and for Zn treatment pots the concentration raised up to 5 mg Zn kg^{-1} soil form the $ZnSO_4.7H_2O$ source based on the soil Zn concentrations of the sample (+Zn) and without Zn fertilization (-Zn). Before sowing, the soils in pots were mixed homogenously with a basal treatment of 200 mg N $(Ca(NO_3)_2.4H_2O)$ kg^{-1} and 100 mg P (KH_2PO_4) kg^{-1} fertilizers. Fourteen seeds from every genotype were sown into each pot, and the pots were thinned to seven seedlings per pot after emergence and daily watered by using deionized water. The field capacity (FC) was determined by the gravimetric method following the method suggested by Souza et al. (2000), and the irrigation treatment was carried out based on the distinction between the mass of the dry soil and wet soil after saturation. Plants were harvested after 45 days of treatment; Zn concentration and content in shoot, as well as shoot dry mass, were measured.

2.3 Determination of Fe and Zn concentration and contents

After the mentioned time, the seedling samples were oven dried at 75 ºC for 48 hours and weighted, then samples were ashed at 550 ºC for 8 hours and dissolved in 1 % (v/v) hydrochloric acid (Chapman and Pratt, 1961). Concentrations of Zn and Fe within the digested solutions were determined by Atomic Absorption

Spectrophotometer (model: AAS-6300 Shimadzu) and the expressed based on plant dry mass (mg kg^{-1} DM). Content of Zn in the shoot (μg $plant^{-1}$) were measured by multiplying amount of seedling dry matter by amount of Zn concentration in the shoot (Genc et al., 2000).

Table 1: Name, description and 1000 grain mass (g) of durum and bread wheat genotypes

No.	Genotype	Wheat type	1000 grain mass (g)	Description/Origin
1	Altintoprak-98	Durum	39	Turkish variety
2	Ankara-98	Durum	43	Turkish variety
3	Cheheldaneh	Durum	-	Local variety for cold
4	Mirzabey-2000	Durum	39	Turkish variety
5	Imren	Durum	36	Turkish variety
6	Berkmen-469	Durum	31	Turkish variety
7	Tunca-79	Durum	30	Turkish variety
8	G-1252	Durum	-	Turkish variety
9	Kunduru-414-44	Durum	33	Turkish variety
10	Durbel	Durum	35	Turkish variety
11	Gokgol-79	Durum	33	Turkish variety
12	Ammar-9	Durum	33	CIMMYT
13	Pinor-2001	Durum	36	Turkish variety
14	Gerdish	Durum	-	Local variety for cold
15	Sarayolla	Durum	36	Turkish variety
16	Chesit-1252	Durum	39	Turkish variety
17	Geromtel-1	Durum	36	CIMMYT
18	Fatasel-185	Durum	37	Turkish variety
19	Altin-40-98	Durum	36	Turkish variety
20	Turabi	Durum	37	Turkish variety
21	Cakmak-79	Durum	37	Turkish variety
22	Tyten-2002	Durum	38	Turkish variety
23	Zardak	Durum	-	Local variety
24	Kiziltan-91	Durum	41	Turkish variety
25	Meram-2002	Durum	39	Turkish variety
26	Haurani	Durum	-	ICARAD material
27	Za-14-105	Durum	40	-
28	Ter-1//Mrf1/Stj2	Durum	35	-
29	Kumbet-2000	Durum	39	Turkish variety
30	Haran-95	Durum	41	Turkish variety
31	61-130	Durum	-	ICARAD material
32	Kunduru-1149	Durum	38	Turkish variety
33	Bcr/Gro1//Mgnl1	Durum	31	-
34	Selcuklu-97	Durum	35	Turkish variety
35	Yelken-2000	Durum	40	Turkish variety
36	GAP	Durum	41	Turkish variety
37	Saji	Durum	-	Iranian released variety for moderate cold condition
38	SonQarak-98	Durum	37	Turkish variety
39	Eminbey	Durum	41	Turkish variety
40	Viya-2005	Durum	43	Turkish variety
41	Kunduru	Durum	-	Turkish variety
42	Pg"S	Durum	-	ICARAD material
43	Azar-2	Bread	42	Iranian released variety
44	Homa	Bread	42	Iranian released variety
45	Pishgam	Bread	43	Iranian released variety
46	Ohadi	Bread	43	Iranian released variety
47	Sardari	Bread	40	Local variety
48	Gascogen	Bread	-	Iranian released variety
49	Rasad	Bread	-	Iranian released variety
50	Zarin	Bread	39	Iranian released variety

Table 2: Physical-chemical properties of the soil used in the experiment

Physical properties	Amount	Chemical properties	Amount
Calcium carbonate, $CaCO_3$ (%)	20	Extractable Fe (mg kg^{-1})	3.1
Organic matter (%)	0.5	Extractable Zn (mg kg^{-1})	0.5
pH (H_2O)	7.2	Extractable Cu (mg kg^{-1})	0.7
Electrical Conductivity, EC_e (dS m^{-1})	2.3	Extractable P (mg kg^{-1})	6.1
Silt (%)	45	Available N (%)	0.092
Clay (%)	39	Available P (mg kg^{-1})	6.1
Sand (%)	16	Available K (mg kg^{-1})	360
Texture	Clay-loam		

2.4 Estimated of Zn efficiency and Zn utilization efficiency

Zinc efficiency ratio expressed as relative shoot growth and was calculated as the percentage of shoot dry matter produced under Zn-deficiency relative to shoot dry matter produced under Zn fertilization. Zn utilization efficiency was calculated by dividing amount of produced shoot dry matter by content of Zn in the shoot [mg DM μg^{-1} Zn] (Genc and McDonald, 2004; Genc et al., 2006).

2.5 Statistical analysis

The experiment was performed as a factorial based on completely randomized block design (RCBD) with three replications at out-glasshouse in 2013-14 at University of Maragheh, Maragheh, Iran. Analysis of variance (ANOVA) was performed using SAS software ver. 9.1 (SAS Institute, 2011) and also Duncan's Multiple Range Test (DMRT) was used to compare the means ($P \leq 0.05$) (Duncan, 1955). The data were analyzed using SPSS software ver. 16 (SPSS, 2007) for cluster analysis of genotypes based on Square Euclidean distance and Ward method. The figures were drawn using Excel software ver. 10 and the means ± standard error (SE) was used to compare the data.

3 RESULTS

3.1 Shoot dry matter and zinc efficiency

Shoot dry matter was influenced by genotype and Zn application (Table 3), and significant genetic differences were observed at both deficient and sufficient Zn supplies. Shoot dry matter varied from 33 ± 3 mg $plant^{-1}$ in 'Zarin' to 105 ± 5 mg $plant^{-1}$ in 'Ankara-98' at Zn deficient condition, and 41 ± 3 mg $plant^{-1}$ in 'Durbel' to 108 ± 12 mg $plant^{-1}$ in 'Gascogen' at Zn sufficient condition (Figure 1A). Zn application increased averages of shoot dry matter of genotypes from 54 mg $plant^{-1}$ to 68 mg $plant^{-1}$, which means 26 % rise in shoot dry matter, especially in durum wheats (Figure 1A). Shoot dry matter suppress due to Zn deficiency was different among the genotypes. At day 45, decreases in shoot growth and dry matter were more distinct in durum wheat genotypes (particularly in 'Pg"S', 'Kunduru-414-44' and 'Viya-2005'). There was a positive relationship between shoot dry matter at deficient and sufficient Zn condition ($r = 0.591$, $P < 0.001$, $n = 50$, Figure 2).

Zn efficiency of genotypes was ranged from 55 to 118 % in 'Pg"S' and 'Altintoprak-98', respectively (Figure 1B). Mean Zn efficiency in bread wheats (83 %) was higher than durum wheats (73 %), but some durum wheats such as 'G-1252', 'Tunca-79', 'Durbel', 'Ammar-9', 'Ankara-98' and 'Berkmen-469' had greater Zn efficiency than the bread wheats.

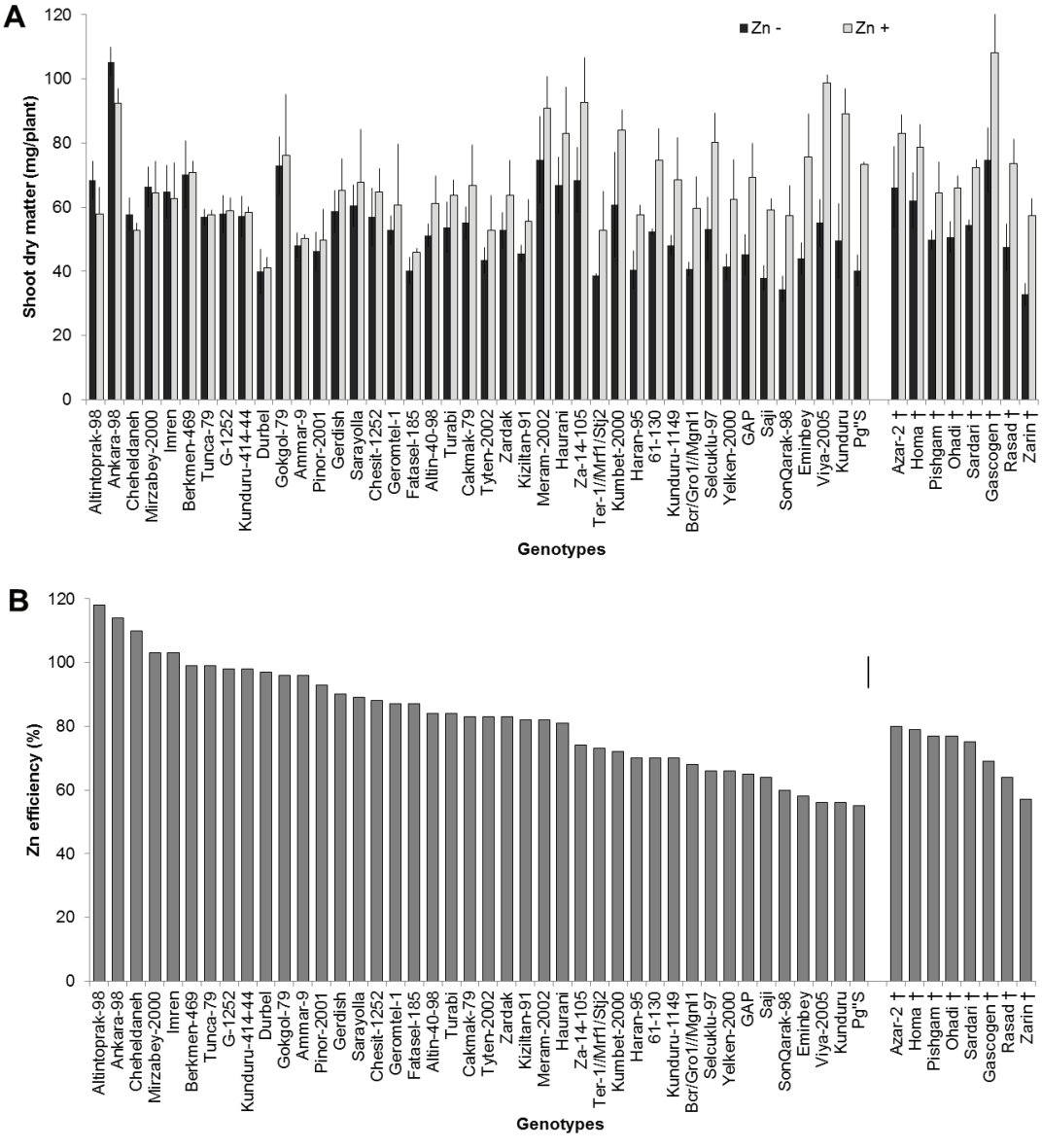

Figure 1: Effects of Zn fertilization (5 mg Zn kg^{-1} soil) on A: shoot dry matter (mg $plant^{-1}$) and B: Zn efficiency (%) in durum and bread wheat genotypes at 45 DAS. Vertical lines indicate standard error (SE) and vertical bar on the corners represent DMRT ($P < 0.05$) for the comparison between the genotypes. Zinc efficiency was calculated as [(shoot dry matter at -Zn/shoot dry matter at +Zn) × 100]. † Bread wheat.

Table 3: Analysis of variance (mean square) for the measured traits of in durum and bread wheat genotypes

Source of variance	df	Mean squares				
		Shoot dry matter	Shoot Zn concentration	Shoot Fe concentration	Shoot Zn content	Zn utilization efficiency
Replication	2	116 ns	1292 **	61041 **	3.87 **	5691 **
Zn fertilization (Zn)	1	13920 **	3184 **	13200 **	36.0 **	17144 **
Genotypes (G)	49	885 **	90.7 **	2984 **	0.938 **	395 **
Zn × G	49	228 ns	31.0 ns	1845 **	0.213 ns	122 ns
Error	198	206	33.9	505	0.245	123
CV (%)	-	23.4	25.6	12.9	34.8	22.7

ns, * and **: Non-significant and significant at the 5 % and 1 % levels of probability, respectively.
df: degrees of freedom, CV: coefficient of variance.

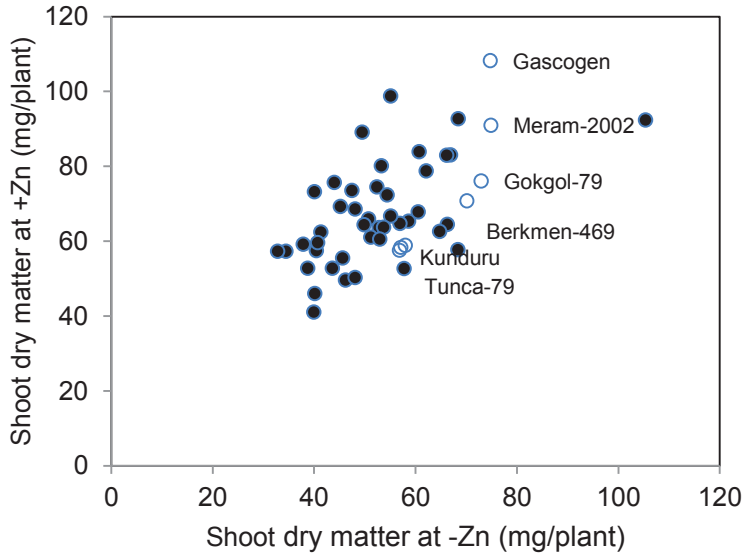

Figure 2: The relationship between shoot dry matter at deficient (-Zn) and sufficient Zn (+Zn) condition in durum and bread wheat genotypes at 45 DAS (r = 0.591, P < 0.001, n = 50). The 'Gascogen' and 'Meram-2002' genotypes which are Zn efficient and also responsive to Zn fertilizer, and also 'Gokgol-79', 'Berkmen-469', 'Kunduru' and 'Tunca-79' which are Zn efficient but not responsive to Zn fertilizer (empty circles). Closed circles represent reminder of genotypes studied.

3.2 Zn concentration and content in the shoot

Zn fertilization significantly affected ($P < 0.001$) shoot Zn concentration and content, with significant differences ($P < 0.001$) among genotypes (Table 3). Large genotypic diversity in shoot Zn concentration were observed under both no Zn application condition (11.8 mg Zn kg^{-1} DM in 'Ammar-9' to 27.0 mg Zn kg^{-1} DM in 'Saji') and with Zn application (14.3 mg Zn kg^{-1} DM in 'Pishgam' to 39.6 mg Zn kg^{-1} DM in 'Sarayollah') (Table 4). Although, shoot Zn concentration was higher in plants supplied with Zn (Table 4). Zn fertilization resulted in 28 % increase in Zn concentration. According to Figure 4 there was no significant correlation between shoot Zn concentration and dry matter production. Zinc content ranged from 0.56 µg $plant^{-1}$ in 'Ter-1//Mrf1/Stj2' to 2.02 µg $plant^{-1}$ in 'Ankara-98', and 0.90 µg $plant^{-1}$ in 'Pishgam' to 2.83 µg $plant^{-1}$ in 'Ankara-98' at deficient and sufficient Zn conditions, respectively (Table 5). Moreover, shoot Zn content was significantly correlated with shoot dry matter (r = 0.70, $P < 0.001$) and shoot Zn concentrations (r = 0.51, $P < 0.001$) (Figure 4).

Table 4: Effects of Zn fertilization (5 mg Zn kg^{-1} soil) on shoot Zn and Fe concentration (mg kg^{-1} DM) in durum and bread wheat genotypes at 45 DAS

No.	Genotype	Shoot Zn concentration (mg kg^{-1} DM)			Shoot Fe concentration (mg kg^{-1} DM)		
		-Zn	+Zn	Mean	-Zn	+Zn	Mean
1	Altintoprak-98	19.2 ± 2.8	27.9 ± 2.6	23.5 a-i	143 ± 19	184 ± 38	163 f-m
2	Ankara-98	19.5 ± 4.0	30.8 ± 2.8	25.1 a-h	177 ± 39	154 ± 20	166 e-l
3	Cheheldaneh	20.6 ± 5.4	25.1 ± 1.7	22.9 a-i	180 ± 25	182 ± 7	181 d-i
4	Mirzabey-2000	24.8 ± 5.7	31.2 ± 3.0	28.0 ab	179 ± 29	182 ± 25	181 d-i
5	Imren	23.7 ± 4.3	29.2 ± 0.0	26.4 a-f	181 ± 25	203 ± 5	192 c-f
6	Berkmen-469	21.2 ± 3.8	30.4 ± 3.6	25.8 a-g	185 ± 19	207 ± 25	196 b-e
7	Tunca-79	24.3 ± 4.4	31.4 ± 7.3	27.9 a-c	180 ± 26	132 ± 12	156 h-m
8	G-1252	22.4 ± 5.7	31.4 ± 7.3	26.9 a-d	158 ± 25	165 ± 5	162 f-m
9	Kunduru-414-44	25.5 ± 6.8	34.4 ± 8.1	30.0 a	219 ± 25	212 ± 19	215 a-c
10	Durbel	14.5 ± 3.2	23.9 ± 0.5	19.2 d-j	215 ± 16	154 ± 9	184 d-h
11	Gokgol-79	19.0 ± 2.8	28.1 ± 2.6	23.6 a-i	149 ± 29	158 ± 22	154 h-m
12	Ammar-9	11.8 ± 1.4	20.4 ± 0.7	16.1 ij	205 ± 8	144 ± 2	174 d-k
13	Pinor-2001	19.0 ± 2.8	19.5 ± 4.1	19.2 d-j	159 ± 14	176 ± 14	168 e-l
14	Gerdish	23.4 ± 4.3	29.8 ± 2.3	26.6 a-f	244 ± 16	224 ± 14	234 a
15	Sarayolla	19.8 ± 3.4	39.6 ± 17	29.7 a	173 ± 34	157 ± 4	165 e-l
16	Chesit-1252	15.9 ± 0.5	30.4 ± 4.1	23.1 a-i	165 ± 15	172 ± 20	169 e-k
17	Geromtel-1	15.3 ± 2.2	24.9 ± 6.7	20.1 b-j	178 ± 16	167 ± 8	172 d-k
18	Fatasel-185	16.0 ± 2.9	25.0 ± 2.0	20.5 b-j	242 ± 15	188 ± 6	215 a-c
19	Altin-40-98	23.8 ± 3.4	32.0 ± 1.3	27.9 a-c	181 ± 16	176 ± 3	178 d-j
20	Turabi	19.1 ± 2.9	20.3 ± 0.0	19.7 b-j	141 ± 19	159 ± 2	150 i-m
21	Cakmak-79	18.0 ± 3.3	29.5 ± 2.4	23.8 a-i	229 ± 19	200 ± 20	215 a-c
22	Tyten-2002	16.6 ± 2.2	24.5 ± 1.7	20.6 b-j	183 ± 23	153 ± 7	168 e-k
23	Zardak	22.5 ± 5.5	26.1 ± 1.6	24.3 a-i	223 ± 28	157 ± 1	190 c-g
24	Kiziltan-91	22.2 ± 3.1	22.4 ± 0.2	22.3 a-j	190 ± 16	191 ± 10	191 c-f
25	Meram-2002	20.1 ± 2.4	27.8 ± 2.4	24.0 a-i	151 ± 25	176 ± 21	163 f-m
26	Haurani	25.1 ± 9.4	26.1 ± 3.0	25.6 a-g	178 ± 24	171 ± 16	174 d-k
27	Za-14-105	21.7 ± 3.1	25.4 ± 2.1	23.5 a-i	177 ± 17	183 ± 23	180 d-i
28	Ter-1//Mrf1/Stj2	14.5 ± 1.5	24.0 ± 3.1	19.2 d-j	190 ± 20	164 ± 2	177 d-j
29	Kumbet-2000	14.3 ± 0.7	31.7 ± 3.8	23.0 a-i	243 ± 18	149 ± 11	196 b-e
30	Haran-95	16.9 ± 2.6	21.6 ± 1.7	19.2 d-j	157 ± 22	159 ± 6	158 g-m
31	61-130	16.3 ± 0.2	24.0 ± 4.6	20.2 b-j	162 ± 13	110 ± 9	136 lm
32	Kunduru-1149	16.9 ± 1.4	31.5 ± 2.7	24.2 a-i	160 ± 13	165 ± 19	163 f-m
33	Bcr/Gro1//Mgnl1	16.9 ± 3.3	18.2 ± 0.4	17.5 g-j	161 ± 25	146 ± 10	153 h-m
34	Selcuklu-97	21.0 ± 4.9	32.5 ± 2.8	26.8 a-d	172 ± 15	170 ± 20	171 e-k
35	Yelken-2000	24.5 ± 3.3	30.2 ± 3.7	27.4 a-d	229 ± 27	215 ± 9	222 ab
36	GAP	19.6 ± 3.0	24.1 ± 0.9	21.8 a-j	167 ± 34	167 ± 9	167 e-l
37	Saji	27.0 ± 5.2	32.2 ± 8.1	29.6 a	179 ± 22	163 ± 15	171 e-k
38	SonQarak-98	24.0 ± 6.2	28.9 ± 6.2	26.5 a-f	132 ± 22	162 ± 2	147 j-m
39	Eminbey	16.6 ± 3.1	20.8 ± 0.4	18.7 e-j	138 ± 23	160 ± 2	149 i-m
40	Viya-2005	26.2 ± 2.7	26.8 ± 1.9	26.5 a-f	177 ± 17	168 ± 19	172 d-k
41	Kunduru	18.1 ± 1.7	28.8 ± 3.5	23.5 a-i	270 ± 28	136 ± 11	203 b-d
42	Pg"S	17.8 ± 0.9	24.1 ± 3.7	20.9 b-j	210 ± 16	157 ± 13	183 d-h
43	Azar-2 †	18.2 ± 3.3	23.5 ± 2.9	20.8 b-j	175 ± 27	133 ± 18	154 h-m
44	Homa †	19.2 ± 3.4	19.8 ± 1.9	19.5 c-j	163 ± 24	126 ± 14	144 k-m
45	Pishgam †	13.8 ± 2.1	14.3 ± 1.4	14.0 j	152 ± 27	141 ± 9	147 j-m
46	Ohadi †	17.6 ± 2.6	18.9 ± 0.2	18.3 f-j	145 ± 21	176 ± 6	161 f-m
47	Sardari †	16.3 ± 2.5	18.7 ± 0.0	17.5 g-j	170 ± 29	137 ± 9	153 h-m
48	Gascogen †	20.7 ± 3.1	22.9 ± 2.4	21.8 a-j	173 ± 25	208 ± 23	190 c-g
49	Rasad †	16.4 ± 2.1	17.8 ± 0.6	17.1 h-j	149 ± 27	116 ± 8	133 m
50	Zarin †	17.1 ± 3.1	18.5 ± 2.6	17.9 g-j	138 ± 31	179 ± 12	158 g-m
	Mean	19.5 b	26.0 a		180 a	167 b	

Means followed by the same letters in each column and each factor are not significantly different at 5 % level, according to Duncan's Multiple Range Test. Mean ± SE (n = 3). † Bread wheat.

Table 5: Effects of Zn fertilization (5 mg Zn kg^{-1} soil) on shoot Zn content (µg $plant^{-1}$) and Zn utilization efficiency (mg DM $µg^{-1}$ Zn) in durum and bread wheat genotypes at 45 DAS

No.	Genotype	Shoot Zn content (µg $plant^{-1}$)			Zn utilization efficiency (mg DM $µg^{-1}$ Zn)		
		-Zn	+Zn	Mean	-Zn	+Zn	Mean
1	Altintoprak-98	1.34 ± 0.30	1.57 ± 0.09	1.45 b-l	54.4 ± 7.7	36.5 ± 3.3	45.5 c-i
2	Ankara-98	2.02 ± 0.32	2.83 ± 0.18	2.42 a	55.3 ± 10	33.0 ± 2.7	44.2 c-i
3	Cheheldaneh	1.16 ± 0.25	1.32 ± 0.09	1.24 c-l	54.6 ± 12	40.2 ± 2.8	47.4 c-i
4	Mirzabey-2000	1.60 ± 0.33	2.01 ± 0.32	1.80 a-d	46.7 ± 14	32.6 ± 2.9	39.7 g-i
5	Imren	1.59 ± 0.46	1.83 ± 0.33	1.71 b-f	45.4 ± 9.1	34.3 ± 0.0	39.9 g-i
6	Berkmen-469	1.47 ± 0.30	2.14 ± 0.23	1.80 a-d	50.6 ± 9.9	33.8 ± 3.6	42.2 e-i
7	Tunca-79	1.40 ± 0.31	1.82 ± 0.46	1.61 b-j	44.3 ± 8.9	34.9 ± 6.7	39.6 g-i
8	G-1252	1.36 ± 0.46	1.89 ± 0.52	1.62 b-i	49.9 ± 11	35.0 ± 6.9	42.5 e-i
9	Kunduru-414-44	1.51 ± 0.48	1.98 ± 0.39	1.74 b-f	45.0 ± 12	32.0 ± 6.1	38.5 hi
10	Durbel	0.60 ± 0.22	0.98 ± 0.08	0.79 l	75.5 ± 16	41.9 ± 0.8	58.7 b-d
11	Gokgol-79	1.40 ± 0.32	2.05 ± 0.40	1.73 b-f	54.7 ± 7.5	36.2 ± 3.1	45.5 c-i
12	Ammar-9	0.57 ± 0.09	1.03 ± 0.06	0.80 l	87.2 ± 11	49.1 ± 1.7	68.2 ab
13	Pinor-2001	0.91 ± 0.25	1.05 ± 0.39	0.98 h-l	55.1 ± 8.2	56.1 ± 11	55.6 b-g
14	Gerdish	1.38 ± 0.30	1.99 ± 0.42	1.69 b-h	46.4 ± 10	34.0 ± 2.7	40.2 f-i
15	Sarayolla	1.16 ± 0.10	2.27 ± 0.52	1.71 b-f	54.3 ± 11	34.1 ± 10	44.2 c-i
16	Chesit-1252	0.91 ± 0.17	1.99 ± 0.39	1.45 b-l	63.2 ± 2.1	33.9 ± 4.1	48.6 c-i
17	Geromtel-1	0.83 ± 0.18	1.76 ± 0.99	1.29 c-l	68.4 ± 9.9	45.3 ± 9.6	56.9 b-d
18	Fatasel-185	0.65 ± 0.16	1.15 ± 0.12	0.90 j-l	66.3 ± 11	40.5 ± 3.1	53.4 b-i
19	Altin-40-98	1.20 ± 0.09	1.94 ± 0.24	1.57 b-k	43.8 ± 6.4	31.4 ± 1.3	37.6 i
20	Turabi	0.98 ± 0.07	1.29 ± 0.10	1.14 e-l	54.8 ± 7.9	49.3 ± 0.1	52.1 c-i
21	Cakmak-79	1.02 ± 0.28	1.91 ± 0.23	1.47 b-l	59.7 ± 11	34.3 ± 2.7	47.0 c-i
22	Tyten-2002	0.73 ± 0.14	1.26 ± 0.22	1.00 g-l	62.3 ± 8.3	41.3 ± 2.9	51.8 c-i
23	Zardak	1.16 ± 0.23	1.66 ± 0.30	1.41 b-l	49.7 ± 11	38.6 ± 2.4	44.2 c-i
24	Kiziltan-91	1.01 ± 0.12	1.24 ± 0.16	1.13 e-l	46.9 ± 6.7	44.6 ± 0.3	45.8 c-i
25	Meram-2002	1.48 ± 0.30	2.56 ± 0.47	2.02 ab	51.4 ± 6.9	36.5 ± 3.2	44.0 c-i
26	Haurani	1.84 ± 0.89	2.25 ± 0.66	2.04 ab	50.9 ± 15	39.3 ± 4.1	45.1 c-i
27	Za-14-105	1.42 ± 0.09	2.40 ± 0.54	1.91 a-c	48.5 ± 8.1	39.9 ± 3.1	44.2 c-i
28	Ter-1//Mrf1/Stj2	0.56 ± 0.07	1.34 ± 0.48	0.95 i-l	70.8 ± 7.5	43.1 ± 5.4	57.0 b-d
29	Kumbet-2000	0.84 ± 0.18	2.66 ± 0.34	1.75 b-f	70.4 ± 3.7	32.4 ± 3.9	51.4 c-i
30	Haran-95	0.66 ± 0.07	1.25 ± 0.14	0.96 i-l	61.8 ± 8.7	47.0 ± 3.6	54.4 b-g
31	61-130	0.85 ± 0.01	1.70 ± 0.08	1.28 c-l	61.3 ± 0.6	44.5 ± 7.4	52.9 b-i
32	Kunduru-1149	0.82 ± 0.12	2.10 ± 0.28	1.46 b-l	59.9 ± 4.8	32.1 ± 2.5	46.0 c-i
33	Bcr/Gro1//Mgnl1	0.68 ± 0.11	1.09 ± 0.21	0.89 kl	64.5 ± 14	55.0 ± 1.3	59.8 bc
34	Selcuklu-97	1.18 ± 0.47	2.58 ± 0.25	1.88 a-d	54.0 ± 14	31.2 ± 2.5	42.6 e-i
35	Yelken-2000	1.04 ± 0.21	1.80 ± 0.18	1.42 b-l	42.5 ± 6.5	34.0 ± 3.8	38.3 hi
36	GAP	0.85 ± 0.05	1.68 ± 0.30	1.27 c-l	54.0 ± 9.8	41.7 ± 1.6	47.9 c-i
37	Saji	1.06 ± 0.28	1.85 ± 0.35	1.46 b-l	40.6 ± 9.4	34.7 ± 7.2	37.7 i
38	SonQarak-98	0.78 ± 0.11	1.66 ± 0.39	1.22 c-l	48.0 ± 13	37.4 ± 6.8	42.7 d-i
39	Eminbey	0.76 ± 0.20	1.59 ± 0.31	1.17 d-l	65.8 ± 15	48.0 ± 1.0	56.9 b-e
40	Viya-2005	1.47 ± 0.34	2.65 ± 0.24	2.06 ab	39.0 ± 3.9	37.7 ± 2.5	38.4 hi
41	Kunduru	0.86 ± 0.12	2.52 ± 0.12	1.69 b-h	56.2 ± 5.3	35.7 ± 3.9	46.0 c-i
42	Pg"S	0.71 ± 0.07	1.77 ± 0.29	1.24 c-l	56.6 ± 2.8	43.3 ± 6.1	50.0 c-i
43	Azar-2 †	1.26 ± 0.45	1.93 ± 0.21	1.59 b-k	59.1 ± 11	43.8 ± 5.0	51.5 c-i
44	Homa †	1.25 ± 0.37	1.55 ± 0.16	1.40 b-l	56.3 ± 12	51.5 ± 4.7	53.9 b-g
45	Pishgam †	0.68 ± 0.07	0.90 ± 0.10	0.79 l	76.6 ± 13	71.5 ± 7.1	74.1 a
46	Ohadi †	0.88 ± 0.11	1.25 ± 0.07	1.07 f-l	59.2 ± 8.1	52.9 ± 0.7	56.1 b-f
47	Sardari †	0.90 ± 0.16	1.36 ± 0.05	1.13 e-l	64.9 ± 12	53.4 ± 0.0	59.2 bc
48	Gascogen †	1.49 ± 0.02	2.52 ± 0.50	2.00 ab	50.3 ± 7.1	44.5 ± 4.2	47.5 c-i
49	Rasad †	0.78 ± 0.18	1.31 ± 0.13	1.05 f-l	63.2 ± 8.2	56.2 ± 1.8	59.8 bc
50	Zarin †	0.57 ± 0.15	1.06 ± 0.18	0.80 l	62.1 ± 11	56.0 ± 7.1	59.1 bc
	Mean	1.07 b	1.77 a		56.4 a	41.3 b	

Means followed by the same letters in each column and each factor are not significantly different at 5 % level, according to Duncan's Multiple Range Test. Mean ± SE (n = 3). † Bread wheat.

3.3 Fe concentration in the shoot

Shoot Fe concentration was influenced by genotype and Zn fertilization, and significant genetic differences were evident at both deficient and adequate Zn supply ($P < 0.001$) (Tables 3, 4). The amount of Fe in the shoots varied among genotypes and ranged from about 133 to 234 mg Fe kg^{-1} DM. Results showed that the shoot Fe concentration ranged from 132 ± 22 mg Fe kg^{-1} DM in 'SonQarak-98' to 270 ± 28 mg Fe kg^{-1} DM in 'Kunduru' at deficient Zn supply, and 110 ± 9 mg Fe kg^{-1} DM in '61-130' to 224 ± 14 mg Fe kg^{-1} DM in 'Gerdish' at adequate Zn supply (Table 4).

3.4 Zn utilization efficiency

Zn fertilization significantly affected ($P < 0.001$) Zn utilization efficiency, with significant variations ($P < 0.001$) among genotypes (Table 3). Zn utilization efficiency (shoot dry matter produced per unit of Zn) also varied among the genotypes and was affected by Zn fertilization. Unlike to shoot Zn concentration and content, Zn utilization efficiency decreased in all wheat genotypes by Zn fertilization ('Ammar-9' and 'Viya-2005', the highest and lowest decrease, respectively). Under Zn deficiency, Zn utilization efficiency varied from 39.0 ± 3.9 to 87.2 ± 11 in 'Viya-2005' and 'Ammar-9', respectively. At Zn application, it varied from 31.2 ± 2.5 to 71.5 ± 7.1 in 'Selcuklu' and 'Pishgam', respectively (Table 5).

3.5 Genetic variation revealed by Zn efficiency and shoot dry matter

The result of cluster analysis for studied genotypes is presented in Figure 3. In the present study, cluster analysis separated 50 wheat genotypes into four main groups (Figure 3). Twenty-five wheat genotypes were placed in the first group (G-I), which these genotypes included 'Altintoprak-98', 'Cheheldaneh', 'Mirzabey-2000', 'Imren', 'Berkmen-469', 'Tunca-79', 'G-1252', 'Kunduru-414-44', 'Durbel', 'Gokgol-79', 'Ammar-9', 'Pinor-2001', 'Gerdish', 'Sarayolla', 'Chesit-1252', 'Geromtel-1', 'Fatasel-185', 'Altin-40-98', 'Turabi', 'Cakmak-79', 'Tyten-2002', 'Zardak', 'Kiziltan-91', 'Pishgam' and 'Ohadi'. These wheat genotypes had high Zn efficiency, and shoot dry matter values, thus they were considered the most desirable genotypes for both growth conditions. The second group (G-II) consists of twelve durum wheat genotypes and three bread wheat genotypes ('Ter-1//Mrf1/Stj2', 'Haran-95', '61-130', 'Kunduru-1149', 'Bcr/Gro1//Mgnl1', 'Selcuklu-97', 'Yelken-2000', 'GAP', 'Saji', 'SonQarak-98', 'Eminbey', 'Pg"S', 'Sardari', 'Rasad' and 'Zarin'). In this group, all genotypes had low Zn efficiency, thus they were susceptible to Zn deficiency and only suitable for non-Zn deficiency (adequate Zn) conditions. Six durum wheat genotypes as well as three bread wheat genotypes ('Meram-2002', 'Haurani', 'Za-14-105', 'Kumbet-2000', 'Viya-2005', 'Kunduru', 'Azar-2', 'Homa' and 'Gascogen') were clustered in the third group (G-III). Finally, the fourth group (G-IV) consists of one ('Ankara-98') genotype and this genotype have high shoot dry matter in both deficient and adequate Zn conditions (Figure 3).

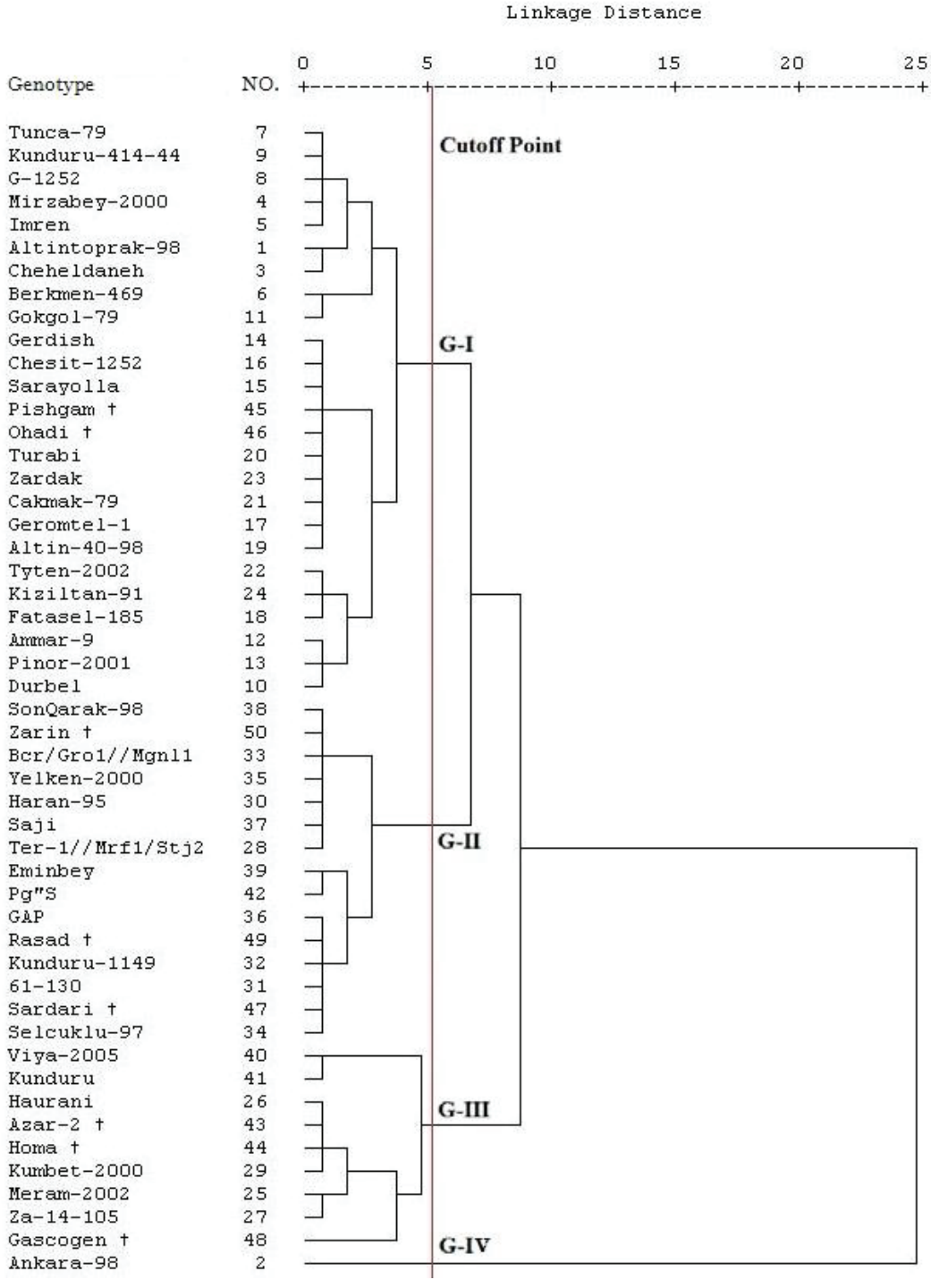

Figure 3: Dendrogram of 50 durum and bread wheat genotypes resulted from UPGMA cluster analysis based on mean Zn efficiency (%), and shoot dry matter (mg plant^{-1}) at deficient and adequate Zn supply. † Bread wheat.

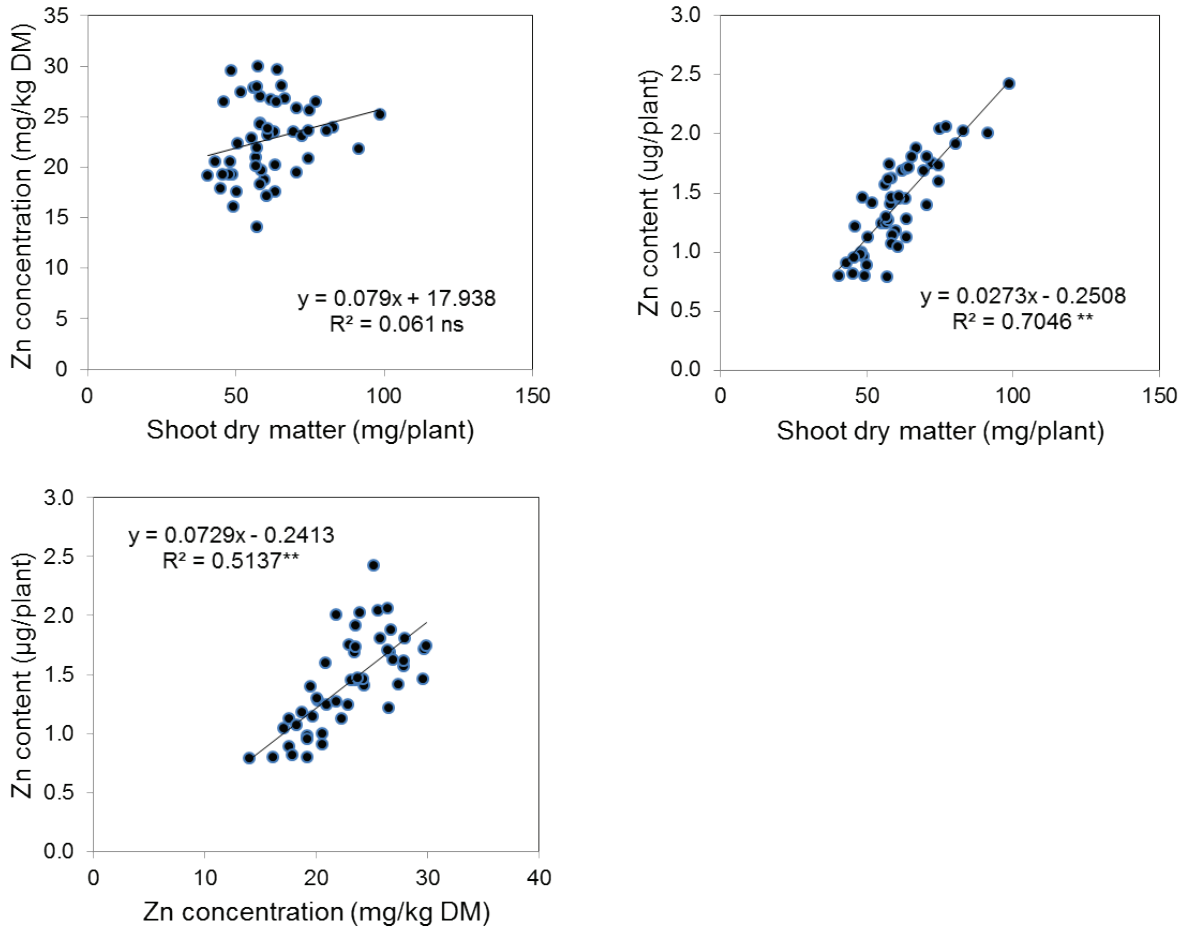

Figure 4: Relationship between shoot dry matter with shoot Zn concentration and content, also shoot Zn content with shoot Zn concentration in eight bread wheat and forty two durum wheat genotypes grown for 45 DAS. ns, * and **: Non-significant and significant at the 5 % and 1 % levels of probability, respectively

4 DISCUSSION

Wheat genotypes exhibited a variation in their performance, which has been exploited in this study, and there was great difference in Zn efficiency between durum and bread wheat genotypes (Figures 1A, B). At the current experiment, we did not measure the Zn content and concentration at seeds, however, since, the seeds were harvested from the homogenous plants not treated with chemical fertilizers, so, the differences observed in Zn efficiency seemingly is due to genetic make-up dissimilarities. McDonald et al. (2008) reported the same differences on the Zn content and concentration at the controlled growing conditions with diverse durum genotypes. Genc and McDonald (2008) in their research on the variation of Zn content and concentration in seeds noted that, due to the weak correlation between Zn efficiency and Zn content or concentration of seed, the related difference observed was main part due to the genetical differences as well. Most of durum wheats (26 genotypes) had higher Zn efficiency than Zn efficient bread wheats and there were no durum wheats with lower Zn efficiency than Zn-inefficient bread wheat except ‘Eminbey’, ‘Viya-2005’, ‘Kunduru’ and ‘Pg"S’ (Figure 1B). Cakmak et al. (1999) presented that durum wheat had the least Zn-efficiency among cereals, and this was partly attributed to the lack of D genome. However, Cakmak et al. (1999) reported in *Aegilops tauschii* Coss. (DD) demonstrated genetic variation in Zn efficiency within this species as well. In the present study, the existence of Zn-inefficient bread wheat genotype (‘Zarin’) despite the presence of the D genome, and equivalent or greater Zn efficiency in some durum wheats compared to bread wheat show that the D genome might not necessarily be the source of Zn efficiency.

The higher Zn efficiency of durum and bread wheat genotypes can also use to produce new cultivars of wheat through plant breeding program. However, this targeted breeding approach requires screening of a large number of genotypes or cultivars of both species for identification of Zn efficiency sources. In such screening studies, it is important to remember that donors should be selected based on their performance under contrasting Zn availability. It is obvious that high yielding genotypes below Zn deficiency and responsive to Zn fertilizer ('Gascogen' and 'Meram-2002' bread and durum wheat genotypes, respectively) are extremely desirable for cropping on Zn-deficient soils (Figure 1A), whereas those with high Zn efficiency simply due to low yield potential under Zn sufficiency are not ('Kunduru-414-44' and 'Tunca-79'). Moreover, genotypes with high yield under Zn deficiency, and also responsive to Zn fertilizers can be identified simultaneously by two level testing where the second level aims to identify Zn-efficient and responsive genotypes (Figures 1A, B). Therefore, identification and cultivation of Zn-efficient genotypes that could use Zn efficiently is a realistic alternative to Zn fertilizer application in some edaphic environments (Hacisalihoglu et al., 2004; Gomez-Coronado et al., 2016).

Our results revealed significant variation among durum and bread wheat genotypes for dry matter and other measured traits (Figure 1; Tables 5, 4). One of the helpful test in breeding programs is seedling test, it could be possible to screened and predict yield response in short time. According to some previous work, there were significant correlations between seedling responses and yield in bread wheat (Kalayci et al., 1999). Genc et al. (2000) reported that Zn efficiency at the seedling stage were higher than maturity or vice versa in some genotypes. On the other hand, it seems that some efficient genotypes are identified and enter the crossing program or the next generation (Graham, 1984). In previous studies Rengel (1999), Gao et al. (2005), Genc et al. (2006) and Genc and McDonald (2008) evaluated differences in Zn efficiency in Zn-efficient and Zn-inefficient wheat by a number of Zn efficiency mechanisms such as Zn uptake by the roots, translocation to the shoots and physiological efficiency (utilization). In this research we did not study on Zn uptakes and transportation in roots and shoot. Thus, the evaluation of relative importance of these individual components was impossible. However, Zn uptake was the main factor in determination of Zn efficiency in barley and bread wheat, respectively (Gao et al., 2005; Sadeghzadeh et al., 2009). But, Hacisalihoglu et al. (2001) showed that there is no correlation between Zn efficiency and Zn compartmentation or xylem translocation in wheat. Furthermore, it was reported that superoxide dismutase (SOD) and carbonic anhydrase (CA) were two importance enzymes to improve Zn efficiency (Hacisalihoglu et al., 2001), therefore it seems that Zn-efficient genotype with more efficient biochemical utilization of cytoplasmic Zn could be response to Zn deficiency, and this may be an important contributor in wheat phenotypic characteristics.

Soil Zn application at 5 mg kg^{-1} significantly decreased Fe concentration in the shoots of wheat genotypes (Table 4). Decrease in Fe concentration in plant was observed and this may be attributed to its increased uptake with the application of Zn showing synergistic effect with Zn. Our findings are contradictory to Rathore et al. (1974), who showed that increasing either element (Zn, Fe and/or Mn) decreased the toxic effect of others and implied a mutual antagonistic effect on Zn uptake. As found in the previous studies (Cakmak et al., 2004; Peleg et al., 2008), there was a close positive relationship between grain Zn and Fe concentrations, and this correlation seems to be specific.

Cluster analysis based on Zn efficiency, and shoot dry matter at deficient and adequate Zn conditions classified the genotypes into four clusters (Figure 3). Cluster analysis has been generally used for description of variation between genotypes and grouping based on Zn efficiency, shoot dry matter and stress tolerance indices (Genc and McDonald, 2004).

5 CONCLUSIONS

The present study showed the existence of genotypic variation for tolerance to Zn deficiency among bread and durum wheat genotypes, which offers potential for the improvement of Zn efficiency in wheat breeding programs. In addition, Zn fertilization improved shoot dry matter and shoot Zn content and concentration of bread wheat genotypes compared to durum wheat genotypes under calcareous soil. Screening Zn tolerant genotypes using cluster analysis discriminated 'Ankara-98' and 'Altintoprak-98' genotypes as the most Zn-efficient and 'Pg"S' genotype among durum wheat and 'Zarin' genotype among bread wheat as the most Zn-inefficient. Moreover, it is necessary to test of more cultivars or genotypes of both wheat species in future to reveal greater Zn efficiency values than those recognized here. Also, seedling responses measured in the present study need to be affirmed at maturity in future studies.

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Interrelationships among traits and morphological diversity of wheat (*Triticum aestivum* L.) accessions in base collection of Plant Genetic Resources Institute, Albania

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ABSTRACT

The object of the study was the evaluation of the morphological variability of 92 wheat germplasm (*Triticum aestivum* L.) part of *ex situ* collection of Plant Genetic Resources Institute, Agricultural University of Tirana. Principal components and cluster analysis were carried out involving 8 quantitative traits, such as tiller capacity, plant height, spike length, number of spikelet per spike, number of seeds per spikelet, number of seeds per spike, seed size and of seeds per spike. Plant height showed positive significant correlation with yield contributing traits as spike length (r = 0.560) and the number of spikelet's per spike (r = 0.305). The number of grains per spike had a significant positive relationship with the mass of grains per spike. Three principal components exhibited about 66.42 % of variability where two PCs components influenced mostly the variability (PC1 with 28.1 % and PC2 with 24.43 %). Accessions were grouped into three major clusters based on complete linkage, suggesting for a variance at the level of 27.50 % within a class and 72.50 % between classes. The results suggested that plant height, spike length, number of spikelet per spike were the most important characters in differentiating the genotypes.

Key words: bread wheat; cluster; PC; morphology; traits; variability

IZVLEČEK

RAZMERJA MED LASTNOSTMI IN MORFOLOŠKO RAZNOLIKOSTJO AKCESIJ KRUŠNE PŠENICE (*Triticum aestivum* L.) IZ OSNOVNE ZBIRKE Plant Genetic Resources Institute, ALBANIJA

Predmet raziskave je bilo ovrednotenje morfološke variabilnosti 92 genotipov navadne pšenice (*Triticum aestivum* L.) iz *ex situ* zbirke Inštituta za genetske resurse (Plant Genetic Resources Institute), Kmetijske univerze v Tirani (Agricultural University of Tirana). Analiza glavnih komponent in klasterska analiza sta bili izvedeni na 8 količinskih lastnostih kot so sposobnost bilčenja, višina rastlin, dolžina klasa, število klaskov v klasu, število semen v klasku, število semen na klas, velikost semen in masa semen na klas. Višina rastlin je pokazala značilno pozitivno korelacijo z lastnostmi, ki so povezane s pridelkom kot je dolžina klasa (r = 0,560) in število klaskov na klas (r = 0,305). Število zrn na klas je imelo značilno pozitivno povezanost z maso zrn na klas. Tri glavne komponente so pokazale 66,42 % variabilnosti od katerih sta dve prispevali večji del (PC1 28,1 % in PC2 24,43 %). Akcesije so se združevale v tri glavne povezane skupine, kar kaže, da je bila variabilnost znotraj razreda 27,50 % in 72,50 % med razredi. Rezultati kažejo, da so najpomembnejše lastnosti, po katerih se razlikujejo genotipi višina rastlin, dolžina klasa in število klaskov na klas.

Ključne besede: krušna pšenica; grozd; PC, morfologija; lastnosti; variabilnost

1 INTRODUCTION

The Albanian Gene Bank has 3317 accessions of different crops in long-term storage (base collection), where wheat crop place an important role. Among 594 of wheat (*Triticum durum* Desf. and *Triticum aestivum* L.) accessions, approximately 270 accessions belong to bread wheat genotypes. The gene bank has the aim not only to preserve the germplasm but also to make available the plant resources into breeding programs, to improve cultivars or to develop new ones.

The evaluation of genetic variability based on morphological characters especially those of economic interest could also be used to select appropriate materials in breeding programs for crop improvement (Dos Santos et al., 2009). As previously reported (Al Khanjari et al., 2008), quantitative traits are often used to assess and describe the wheat characters due to their role in the estimation of genetic diversity and discrimination of closely related types. They were used to identify duplicates, to establish core collections in gene banks, to investigate relationships between

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landraces and their wild relatives, and for the most important tool, to prioritize material for use in breeding programs (Ariyo, 1993; Pecetti and Annicciarico, 1992).

Genetic diversity of wheat genotypes can be evaluated using morphological, which contribute toward grain yield as plant height, spike length, number of spikes per spike and grain (Maqbool et al., 2010). The correlation coefficient analysis is useful in the identification of characters that are positively correlated with yield (Maqbool et al., 2010; Bode et al., 2012). The evaluation of phenotypic variability by multivariate analysis gives the possibility to include a large number of accessions and to identify the most suitable resources for special traits (Goel et al., 2015).

Therefore the object of this study was the characterization of 92 accessions of bread wheat (*Triticum aestivum* L.), part of the base collection of the Albanian Gene Bank (Agricultural University of Tirana), in terms of diversity in morphological traits and association between each pair of these traits.

2 MATERIALS AND METHODS

The study was based on the characterization of the diversity of bread wheat (*Triticum aestivum* L.) germplasm. The plant material was characterized by a survey on land and laboratory, during the year 2016 in the experimental field of the Agricultural University of Tirana. The agronomic characters were measured after harvesting, using 20 plants from each accession.

2.1 Plant materials

In this study, 92 accessions of bread wheat (*Triticum aestivum* L., Table 1.) were used, part of the base collection of PGRI former Albanian Gene Bank (https://eurisco.ipk-gatersleben.de/apex/f?p = 103:25), conserved during 2001-2003.

Table 1: List of the 92 wheat (*Triticum aestivum* L.) accessions object of the study

Code/ AGB	Accession name	Acquire date	Origin	Growth class	Growth habit
0152	BL-76 x MEC 5/1-8 - 4	08.01.03	ALB	winter	upright
0153	LINJA FIKSE VRINE 6/1-1-1-3-4	08.01.03	ALB	winter	upright
0154	YAV x GTA"S"(2)-SO 179 4/3-1-11-1	08.01.03	ALB	winter	upright
0155	LLUCHIANENCO x PAVON 76 3/2-1-2-3	08.01.03	ALB	winter	upright
0156	Ç 2278 x LLUCHIANENCO 11/2-2-1-4-5	08.01.03	ALB	winter	upright
0157	REGINA x L 2076 10/3-3-1-6 258H 1983	08.01.03	ALB	winter	upright
0158	SLLOVENKA x MAJ x L68/3-2 7/6-6-1-1-2	08.01.03	ALB	winter	upright
0159	KAMZA 10 x MEC 7-1-7-1	08.01.03	ALB	winter	upright
0160	MEC x 519 CM 9160 2/1-1-12-3	08.01.03	ALB	winter	upright
0161	KAMZA 10 x MEC 49/1-4	08.01.03	ALB	winter	upright
0221	LP 3-3	26.06.01	ALB	winter	upright
0222	David x Mec	26.06.01	ALB	winter	upright
0223	Dajti	26.06.01	ALB	winter	upright
0224	LVS	26.06.01	ALB	winter	upright
0225	Ni-496	26.06.01	ALB	winter	upright
0226	Ni-594	26.06.01	ALB	winter	upright
0227	Ni-792	26.06.01	ALB	winter	upright
0228	Ni-886	26.06.01	ALB	winter	upright
0229	Ni-896	26.06.01	ALB	winter	upright
0239	-	26.06.01	ALB	winter	upright
0240	-	26.06.01	ALB	winter	prostrate

0241	-	26.06.01	ALB	winter	upright
0242	-	26.06.01	ALB	winter	upright
0243	-	26.06.01	ALB	winter	prostrate
0244	-	26.06.01	ALB	winter	prostrate
0245	-	26.06.01	ALB	winter	prostrate
0246	-	26.06.01	ALB	winter	prostrate
0247	-	26.06.01	ALB	winter	prostrate
0248	-	26.06.01	ALB	winter	prostrate
0249	-	26.06.01	ALB	winter	prostrate
0250	-	26.06.01	ALB	winter	prostrate
0251	-	26.06.01	ALB	winter	upright
0252	-	01.05.02	ALB	winter	upright
0253	-	26.06.01	ALB	winter	upright
0254	-	26.06.01	ALB	winter	upright
0255	-	26.06.01	ALB	winter	upright
0256	-	26.06.01	ALB	winter	upright
0257	-	26.06.01	ALB	winter	upright
0258	-	01.05.02	ALB	winter	upright
0259	-	26.06.01	ALB	winter	upright
0260	-	26.06.01	ALB	winter	upright
0261	-	26.06.01	ALB	winter	upright
0262	-	01.05.02	ALB	winter	upright
0263	-	26.06.01	ALB	winter	upright
0264	-	01.05.02	ALB	winter	upright
0265	-	26.06.01	ALB	winter	upright
0266	-	01.05.02	ALB	winter	upright
0267	-	26.06.01	ALB	winter	upright
0268	-	26.06.01	ALB	winter	upright
0269	-	26.06.01	ALB	winter	prostrate
0270	-	26.06.01	ALB	winter	upright
0271	-	26.06.01	ALB	winter	upright
0272	-	26.06.01	ALB	winter	upright
0273	-	01.05.02	ALB	winter	upright
0274	-	26.06.01	ALB	winter	prostrate
0275	-	26.06.01	ALB	winter	prostrate
0276	-	26.06.01	ALB	winter	prostrate
0277	-	26.06.01	ALB	winter	upright
0278	-	26.06.01	ALB	winter	upright
0279	-	26.06.01	ALB	winter	prostrate
0280	-	26.06.01	ALB	winter	prostrate
0281	-	26.06.01	ALB	winter	upright

0282	-	26.06.01	ALB	winter	prostrate
0283	-	26.06.01	ALB	winter	prostrate
0284	-	26.06.01	ALB	winter	prostrate
0285	-	26.06.01	ALB	winter	prostrate
0286	-	26.06.01	ALB	winter	upright
0287	-	26.06.01	ALB	winter	prostrate
0288	-	26.06.01	ALB	winter	prostrate
0289	-	26.06.01	ALB	winter	upright
0290	-	26.06.01	ALB	winter	prostrate
0291	-	26.06.01	ALB	winter	upright
0292	-	26.06.01	ALB	winter	upright
0293	-	26.06.01	ALB	winter	prostrate
0294	-	01.05.02	ALB	winter	prostrate
0295	-	26.06.01	ALB	winter	upright
0296	-	26.06.01	ALB	winter	upright
0297	-	26.06.01	ALB	winter	upright
0298	-	26.06.01	ALB	winter	upright
0299	-	26.06.01	ALB	winter	upright
0300	-	26.06.01	ALB	winter	upright
0301	-	26.06.01	ALB	winter	upright
0302	-	26.06.01	ALB	winter	upright
0321	-	08.01.03	ALB	winter	upright
0322	-	08.01.03	ALB	winter	upright
0323	-	08.01.03	ALB	winter	upright
0324	-	08.01.03	ALB	winter	upright
0325	-	08.01.03	ALB	winter	upright
0326	-	08.01.03	ALB	winter	upright
0327	-	08.01.03	ALB	winter	upright
0328	-	08.01.03	ALB	winter	upright
0329	-	08.01.03	ALB	winter	upright

2.2 Experimental site

The study was conducted at the Experimental Station of Institute of Plant Genetic Resources Valias, Tiranë. It lies at an altitude of 40 m above sea level and at Latitude 41°24'6.14"N and Longitude 19°44'9.93"E.

2.3 Methods (Experimental Design)

Experiment carried one replication during the autumn season 2016. During the crop year, the accessions were evaluated for different characters of quantitative type as: tiller capacity (TC), plant height/cm (PH), spike length/cm (SL), number of spikelet per spike (SpS), number of seeds per spikelet (GSp), number of seeds per spike (GS), seed size/mm (SeS) and of seeds per spike/g (WGS). Morphological characterization of the accessions was done according to international standards (IPGRI, 1985).

2.4 Statistical analyses

Statistical tests were carried out by the Statistical Package for Social Sciences (version 21) and JPM.

3 RESULTS

To obtain a successful breeding program, it is essential the information that researchers can get on the variability of germplasm within a crop spices. Morphological characterizing of the individual wheat accessions is useful in selection of the adaptable parents in the hybridization process. To assess the genetic diversity among 92 bread wheat germplasm, 8 quantitative traits were used and the estimated variation coefficient was high for agronomic traits as PH, SL, GS and WGS, similar with others authors (Ali et al., 2008; Sabaghina et al., 2014). Regarding PH trait it was observed a variation from 82.9 cm to 180.3 cm, for WGS among 92 accessions the minimum value measured was 0.28 g and the maximum 5.738 g, high variance resulted in GS trait (from ± 12.8 number of seeds per spike to ± 71, Table 2). AGB 0262 recorded the highest value for tiller capacity (± 3.8) whereas the 92 accessions presented an average of 2.56 for the same trait. Sabaghina et al. (2014) reported a higher tiller number (ranging from 1 to 6) measured at 56 bread wheat genotypes. Among the mean value of genotype for plant height trait, accession AGB 0268 recorded the highest mean value (±180.3 cm) and genotype AGB 0258 resulted with the lowest plant height (±82.9 cm). The results are higher from those reported by Sabaghina et al. (2014) plant height variation from 54.9 cm to 109.53 cm, whereas Mahmood et.al. (2006) obtained results ranging from 62 cm to 110 cm, while Aliu et.al. (2010) reported a range from 71 to 79 cm in different bread wheat genotypes.

The variation of plant height trait classified the 92 accessions in different classes (Table 3) where the major number of genotypes resulted from 91-100 cm. Similar results are reported by Peltonen et al. (2007) for the same trait.

Grain yield is influenced by spike properties and the spikelet number plays a very important role in the wheat grain yield (Sabaghina et al., 2014). Spike length in this study varied from 6.40 cm in AGB 0288 to 17.83 cm. Results presented are higher from those reported from other authors (Peltonen et al., 2007; Sabaghina et al., 2014; Xhulaj et al., 2017). Comparing the mean values for SL and number of spikelet's per spike traits, the maximum values were observed in accession AGB 0251 (respectively 17.83 cm and 26.6 cm) followed by AGB 0268. Observations revealed that most of the wheat germplasm (50 accessions) were classified together for spike length trait measured between 9.1 to 11 cm (Table 4).

Table 2: Descriptive statistics of quantitative traits in 92 accessions of bread wheat (*Triticum aestivum* L.)

Statistics	TC	PH	SL	SpS	GSp	GS	SeS	WGS
Observations	92	92	92	92	92	92	92	92
Minimum	2.000	82.900	6.400	10.600	2.100	12.800	3.000	0.280
Maximum	3.800	180.300	17.830	26.600	4.000	71.000	9.000	5.738
Range	1.800	97.400	11.430	16.000	1.900	58.200	6.000	5.458
Mean	2.564	125.280	9.985	19.883	2.994	38.523	6.184	1.788
Variance	0.210	646.808	3.893	10.101	0.119	141.03	0.931	0.578
Standard deviation	0.458	25.432	1.973	3.178	0.345	11.876	0.965	0.760
Variation coefficient %	17.9	20.3	19.8	16.0	11.5	30.8	15.6	42.5
Standard error of ±	0.048	2.666	0.207	0.333	0.036	1.245	0.101	0.080
Mean absolute deviation	0.395	22.330	1.468	2.421	0.223	8.835	0.711	0.543

TC - tiller capacity; PH - plant height/cm; SL - spike length/cm; SpS - number of spikelet per spike; GSp - number of seeds per spikelet; GS - number of seeds per spike; SeS - seed size/mm and WGS - of seeds per spike/g.

In terms of the number of spikelet per spike considerable variance consist between accessions for SpS trait, AGB 0323 recorded the lowest value (10) and AGB 0251 and AGB 0268 the highest value (26.6). Most of the accessions (39) were classified between 19 to 21 numbers of spikelet per spike measured after harvest (Table 6).

Regarding the number of grains per spikelet character, AGB 0322 had the highest mean value (4.0) and AGB 0266 recorded the lowest value (2.1). Among the 92 bread wheat genotypes 23 of the accessions recorded 2.0 to 2.9 number of GSp while 69 of the wheat accessions resulted with 3.0 to 4.0 number of GSp. According to Othmani et al. (2015) this trait is regarded as the main wheat yield component and an increased grain number has been produced by spikes per unit or more grains per spike due to a higher spikelet number.

Data revealed that wheat genotype AGB 0326 presented the highest mean values for two traits, number of seeds per spike (±71) and seed size trait (±9 mm). Regarding GS trait, 92 accessions were grouped in different classes, from 5.43 % presented with 12.0 to 20.0 numbers of grains per spike, till 44.56 % (3.0 to 40.0 GS).

According to Okamoto et al. (2013) the grain number and mass as two main components in wheat grain yield are determined at different times of the growing season. This author suggested that seed mass best-explained genotype by environmental interaction for wheat grain yield. The 92 accessions revealed a high variation regarding this trait, where 26.08 % of them recorded values from 0.2 g to 1.2 g (Table 5), and most of the wheat germplasm (58.69 %) recorded values from 1.3 g to 2.3 g of the same trait. Observation showed that only one accession, AGB 0285 recorded the highest value in mass of seeds per spike (±5.73 g). Seed mass parameter also is important in wheat increasing seed germination percent, seedling emergence, tiller capacity, spike density and yield (Bellatreche et al., 2017).

Seed size trait recorded a high variation from 3.00 mm to 9.00 mm, representing one of the main components of the wheat yield, and increasing grain size continues to be a major breeding target (Sabaghina et al., 2014). Among 92 wheat germplasm in 16.3 % of them seed size varied from 3.0 mm to 5.0 mm, whereas most of the accessions (60.86 %) presented values from 5.0 mm to 7.00 mm for the same trait (Table 5). AGB 0276 had the lowest values for two traits WGS (0.28 g) and seed size (3 mm) followed for this last trait by AGB 0278 and AGB 0221.

Table 3: Classification of 92 wheat (*Triticum aestivum* L.) accessions according to PH trait

Plant height class/cm	frequency	accession
>80	0	
81-90	5	AGB 0221, AGB 0223, AGB 0226, AGB 0227, AGB 0258
91-100	19	AGB 0152, AGB 0153, AGB 0157, AGB 0158, AGB 0159, AGB 0161, AGB 0224, AGB 0225, AGB 0228, AGB 0229, AGB 0255, AGB 0261, AGB 0302, AGB 0321, AGB 0324, AGB 0325, AGB 0326, AGB 0327, AGB 0328
101-110	10	AGB 0154, AGB 0156, AGB 0160, AGB 0222, AGB 0257, AGB 0272, AGB 0294, AGB 0299, AGB 0300, AGB 0322
111-120	8	AGB 0243, AGB 0254, AGB 0274, AGB 0277, AGB 0278, AGB 0295, AGB 0301, AGB 0329
121-130	8	AGB 0155, AGB 0263, AGB 0271, AGB 0287, AGB 0290, AGB 0296, AGB 0297, AGB 0298
131-140	14	AGB 0241, AGB 0242, AGB 0245, AGB 0252, AGB 0259, AGB0260, AGB 0262, AGB 0264 AGB 0269, AGB 0279, AGB 0280, AGB0282, AGB 0288, AGB 0323
141-150	12	AGB 0240, AGB 0246, AGB 0253, AGB 0256, AGB 0265, AGB 0267, AGB 0270, AGB 0273, AGB 0275, AGB 0276, AGB 0291, AGB 0293
151-160	6	AGB 0239, AGB 0247, AGB 0248, AGB 0249, AGB 0250, AGB0292
161-170	7	AGB 0251, AGB 0266, AGB 0281, AGB 0283, AGB 0285, AGB0286, AGB 0289
171-180	2	AGB 0244, AGB 0284
<181	1	AGB 0268

Table 4: Classification of 92 wheat (*Triticum aestivum* L.) accessions according to SL trait

Spike length		
class/cm	frequencyy	accession
> 6	0	
6.1-8	29	AGB 0255, AGB 0258, AGB 0271, AGB 0273, AGB 0275, AGB 0276, AGB 0278, AGB 0279, AGB 0280, AGB 0287, AGB 0288, AGB 0290, AGB 0294, AGB 0321, AGB 0322, AGB 0325, AGB 0327, AGB 0328, AGB 0329
9.1-11	50	AGB 0152, AGB 0156, AGB 0157, AGB 0158, AGB 0159AGB 0160, AGB 0161 AGB 0222, AGB 0223, AGB 0224, AGB 0227, AGB 0241, AGB 0245, AGB 0246, AGB 0247, AGB 0249, AGB 0250, AGB 0252, AGB 0253, AGB 0257, AGB 0259, AGB 0260, AGB 0261, AGB 0262, AGB 0263, AGB 0264, AGB 0267, AGB 0270, AGB 0272, AGB 0274, AGB 0277, AGB 0281, AGB 0282, AGB 0283, AGB 0284, AGB 0285, AGB 0286, AGB 0291, AGB 0293, AGB 0295, AGB 0296, AGB 0297, AGB 0298, AGB 0299, AGB 0300, AGB 0301, AGB 0302, AGB 0323, AGB 0324, AGB 0326
12.1-13	8	AGB 0240, AGB 0242, AGB 0244, AGB 0256, AGB 0265, AGB 0269, AGB 0289, AGB 0292
14.1-16	4	AGB 0239, AGB 0248, AGB 0266, AGB 0268
<17.1	1	AGB 0251

Table 5: Accessions of bread wheat (*Triticum aestivum* L.) frequency (%) distribution

Trait					
Number of seeds per spike		Seed size		Mass of seeds/spike	
Class/nr.	Frequency %	Class/mm	Frequency %	Class/g	Frequency %
12-20	5.43	3.0-5.0	16.30	0.2-1.2	26.08
21-30	14.13	5.0-7.0	60.86	1.3-2.3	58.69
31-40	44.56	7.0-9.0	22.82	2.4-3.4	14.13
41-50	20.65	>9.0	0.00	4.6-5.6	1.08
51-60	10.86				
61-70	3.26				
>70	1.08				

Table 6: Classification of 92 wheat (*Triticum aestivum* L.) accessions according to SpS trait

Number of spikelets per spike		
class/nr.	frequency	accession
10-12	2	AGB 0153, AGB 0323
13-15	3	AGB 0242, AGB 0243, AGB 0288
16-18	28	AGB 0152, AGB 0154, AGB 0155, AGB 0156, AGB 0221, AGB 0226, AGB 0228, AGB 0252, AGB 0253, AGB 0255, AGB 0257, AGB 0258, AGB 0261, AGB 0262, AGB 0272, AGB 0273, AGB 0274, AGB 0278, AGB 0279, AGB 0281, AGB 0282, AGB 0289, AGB 0290, AGB 0294, AGB 0302, AGB 0328, AGB 0329, AGB 0271
19-21	39	AGB 0158, AGB 0249, AGB 0224, AGB 0227, AGB 0240, AGB 0246, AGB 0247, AGB 0159, AGB 0250, AGB 0225, AGB 0227, AGB 0254, AGB 0241, AGB 0286, AGB 0160, AGB 0259, AGB 0263, AGB 0275, AGB 0280, AGB 0283, AGB 0287, AGB 0161, AGB 0260, AGB 0264, AGB 0276, AGB 0293, AGB 0284, AGB 0300, AGB 0295, AGB 0296, AGB 0265, AGB 0297, AGB0298, AGB 0299, AGB 0301, AGB 0324, AGB 0325, AGB 0326, AGB 0327
22-24	9	AGB 0157, AGB 0223, AGB 0245, AGB 0256, AGB 0269, AGB 0270, AGB0291, AGB 0321, AGB 0322
25-27	11	AGB 0222, AGB 0244, AGB 0248, AGB 0251, AGB 0266, AGB 0267, AGB 0268, AGB 0292, AGB 0239, AGB 0277, AGB 0285

3.1 Correlation Coefficient Analysis

Correlation of morphological traits was calculated by studying the data of bread wheat germplasm (Table 7). Correlations measure the interdependence between a pair of characters. Knowledge of correlation is required to obtain the expected response of other traits when selection is applied to the trait of interest in a breeding program (Maqbool et al., 2010). Plant height showed positive significant correlation with yield contributing traits as spike length (r = 0.560) and the number of spikelet's per spike (r = 0.305).

Same results are reported from previous studies (Maqbool et al., 2010; Xhulaj et al., 2017). While significant negative correlation is observed among TC and SpS (r = -0.358). The number of spikelet per spike had a positive correlation with spike length trait (r = 0.589). Whereas number of grains per spike had a significant positive relation with if grains per spike trait (r = 0.719), supported by other works (Khaliq et al., 2004; Xhulaj et al., 2017).

3.2 Principal component analysis

The average data was analyzed using principal component analysis. According to the data (Table 8), three principal components exhibited about 66.42 % of variability where two PC components influenced mostly the variability (PC1 with 28.1 % and PC2 with 24.43 %; Figure 1).

Table 7: Correlation matrix among the morphological traits (Pearson (n))

Variables	TC	PH	SL	SpS	GSp	GS	SeS	WGS
TC	**1**	-0.126	-0.282	-0.358	-0.013	0.241	-0.102	-0.015
PH	-0.126	**1**	0.560	0.305	-0.012	-0.053	-0.104	0.029
SL	-0.282	**0.560**	**1**	0.589	0.095	0.102	0.046	0.196
SpS	-0.358	**0.305**	**0.589**	**1**	0.064	-0.018	-0.024	0.168
GSp	-0.013	-0.012	0.095	0.064	**1**	0.177	0.231	0.136
GS	0.241	-0.053	0.102	-0.018	0.177	**1**	0.275	**0.719**
SeS	-0.102	-0.104	0.046	-0.024	0.231	0.275	**1**	0.283
WGS	-0.015	0.029	0.196	0.168	0.136	0.719	0.283	**1**

Table 8: Eigen values and percentage of total variance for PCA in 92 accessions of wheat

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Eigen value	2.248	1.955	1.111	0.898	0.712	0.534	0.320	0.221
Variability (%)	28.101	24.435	13.887	11.220	8.904	6.678	4.006	2.768
Cumulative %	28.101	52.537	66.424	77.644	86.548	93.226	97.232	100.000

Table 9: Eigenvectors contribution in 92 accessions of wheat

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
TC	-0.277	0.279	-0.544	**0.426**	0.097	0.555	-0.097	-0.202
PH	**0.378**	-0.284	-0.349	0.309	0.493	-0.353	-0.412	0.145
SL	**0.555**	-0.193	-0.127	0.112	0.106	0.178	0.720	-0.255
SpS	**0.484**	-0.234	0.044	-0.119	-0.407	0.562	-0.420	0.194
GSp	0.169	0.228	**0.431**	0.775	-0.320	-0.167	-0.038	-0.036
GS	0.234	**0.578**	-0.285	-0.078	-0.087	-0.094	0.213	0.679
SeS	0.166	**0.362**	**0.518**	-0.089	0.660	0.345	-0.105	0.007
WGS	**0.356**	**0.481**	-0.172	-0.285	-0.159	-0.254	-0.262	-0.610

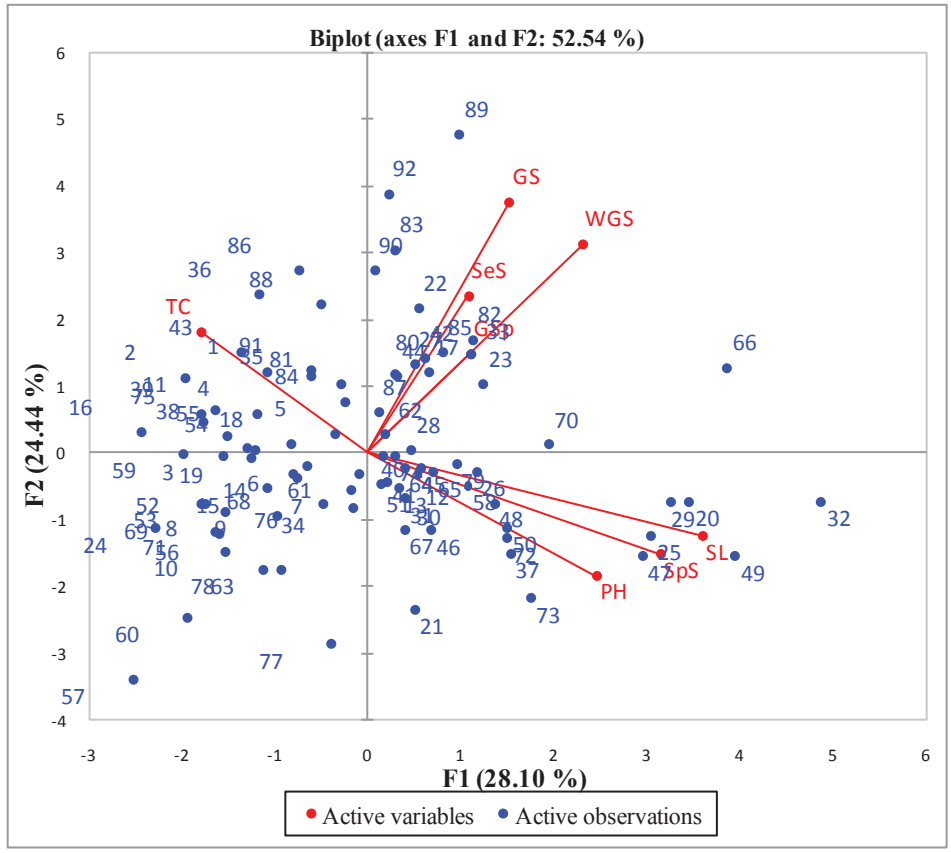

Figure 1: Principal component biplot of 92 wheat (*Triticum aestivum* L.) accessions

The first PC was related with plant height, spike length, number of spikelets per spike and of seeds per spike traits (Table 9) giving about 28.1 % of the variability but poor in tiller capacity. In the second PC traits as number of seeds per spike, seed size and WGS contribute at the level of 24.43 % of variability. The third principal component exhibited positive effects for seed size and number of seeds per spikelet (13.88 %), and maximum variation was observed for tiller capacity, plant height and number of seeds per spikelet at fourth, PC but poor in SL, SpS, GS and WGS. Different authors (Escobar-Hernandez et al., 2005; Othmani et al., 2015) used principal component method for grouping of germplasm. In addition to cluster analysis, biplot has been applied to study relation among studied traits in a set of genotypes (Aghaee et al., 2010; Peterson et al., 2005; Yan and Fregeau – Reid, 2008). Biplot (genotype by trait) explained the percentage variance associated with each principal component obtained by drawing a graph between Eigen values and principal components number.

The biplot (Figure 1) suggest that the best or the incompatible wheat genotypes in most of the traits, since they had the longest distance from the origin for the two principal components were AGB 0251 (32), AGB 0326 (89), AGB 0268 (49), AGB 0239 (20), AGB 0261 (42), AGB 0261 (29), AGB 0244 (25) , AGB 0227 (17), AGB 0266 (47), and AGB 0329 (92). This group is followed by others genotypes with similar high results in different traits as AGB 0302 (83), AGB 0327 (90), AGB 0241 (22), AGB 0255 (36), AGB 0323 (86), AGB 0285 (66) and AGB 0292 (73).

Therefore it seems that for the first PC genotypes (numbered at Figure 1) 32, 29, and 20 have the highest values mostly for spike length and number of spikelets per spike traits, while genotypes as 49, 47, 25, 73, resulted with the highest values basically for plant height trait, and the other related cultivars as 37, 72, 50 which fall in its sector were suitable for PH too. The genotype 66 is different from the other genotypes in its sector in relation for of seeds per spike trait. The genotypes that presented not suitable performance for the measured traits within the first component, with lower distance from the origin of the biplot were AGB 0276 (57), AGB 0226 (16), AGB 0153 (2), AGB 0278 (59) and AGB 0243 (24).

According to the PC analyze the wheat genotypes that presented the highest variability for the traits in the second component especially for number of seeds per

spike, seed size and of seeds per spike were 36, 89, 86, 88, 90, 83 and 92. Within this component the group of genotypes AGB 0240 (21), AGB 0279 (60), AGB 0282 (63), AGB 0296 (77), AGB 0275 (56) and AGB 0297 (78) resulted with the lowest performance for the measured traits (Figure 1).

The vector view of the biplot suggest a strong positive correlation among traits as WGS and GS, GSp, SeS; between SpS and SL, PH; also among SeS and GSp, GS as indicated by the small obtuse angles between their vectors. The correlation between WGS and PH, SpS, SL; among SeS and SL; between GSp and SL, SpS; and finally GS and SL was near zero as indicated by the near perpendicular vectors. The vectors indicated by the near angle of approximately 180 degrees, suggest for the existence of a strong negative correlation between TC and SpS, SL and PH; also between SeS and PH.

Comparing the Eigen values for each factor using the minimum Eigen value criterion, there are 3 main PC with Eigen values > 1.00 (Table 9 and Figure 1) that influence the genetic variability among 92 wheat genotypes. PC1 showed 28.1 % of variability with Eigen value 2.24 in germplasm which then reduced gradually. After the fourth PC little variance was observed and it ended at 2.76 % with Eigen value 0.22.

From the graph (Figure 1) the maximum variation was present in the first PC. So the selection of genotypes with desirable characters from this PC will be useful for further breeding programs.

3.3 Cluster analysis

The 92 wheat accessions were grouped according to quantitative traits into three major clusters based on complete linkage, whereas each cluster was statically different from each other (Figure 2). Cluster 1 consisted of 41 accessions, cluster 2 of 32 wheat accessions and the third cluster with 19 accessions (Table 10).

The variance calculated within the classes ranged from the maximum variance within class 249.63 at the second class to the minimum variance value 129.31 at third class.

The maximum distance to centroid at first class is 32.10, at second class 29.54 and for the third class 19.76. The results suggest for variance decomposition for the optimal classification at the level of 27.50 % within the class and 72.50 % between classes. The two clusters with most similarity observed are cluster two and three (27.06 D2 Euclidean distance among them), and with the highest cluster distance value (60.87 units) of dissimilarity were cluster one and three. This dissimilarity was basically due to traits as plant height, spike length, number of grains per spike and seeds . Similar results related to wheat germplasm grouping were reported before (Maqbool et al., 2010; Xhulaj et al., 2017). Two most closely wheat genotypes within the first cluster are AGB 0296 and AGB 0297 (2.19 Euclidean distance), similar for traits as plant height and number of seeds per spikelet. These two accessions were joined from another sub-cluster formed by AGB 0225 and AGB 0159 (2.395 Euclidean distance) similar for plant height and of seeds per spike. Similarity is observed among AGB 0261 and AGB 0228 basically for number of spikelets per spike, also sub-clustered together for high level of similarity especially for spike length trait and plant height are AGB 0265 and AGB0240. Wheat genotypes with the lowest level of similarity at the second cluster are AGB 0296 and AGB 0297 (4.413 distance) and AGB 0287 with AGB 0155 (4.461 Euclidean distance) fully similar for number of seeds per spike trait. Similarity for tiller capacity sub-cluster genotypes AGB 0271 and AGB 0155. Within the third cluster major similarity is observed among AGB 0281 and AGB 0283 especially for tiller capacity, plant height and number of seeds per spike, joined to this pair AGB 0284 for traits as spike length, number of spikelets per spike and seed size.

Observing the clusters, accessions grouped in cluster three, resulted with the highest mean values in traits (PH, SL, SpS) that can contribute positively in the wheat yield and breeding programs.

Table 10: Clusters composition with 92 accessions (Code AGB) of bread wheat

Cluster I	0153	0154	0156	0157	0158	0159	0160	0161	0221
0222	0223	0224	0225	0226	0227	0228	0254	0255	0257
0258	0261	0272	0274	0277	0278	0294	0295	0299	0300
0301	0302	0321	0322	0324	0325	0326	0327	0328	0229
0329									
Cluster II	0155	0240	0241	0242	0243	0245	0246	0252	0253
0259	0260	0262	0263	0264	0265	0269	0270	0271	0256
0273	0323	0275	0276	0279	0280	0282	0287	0288	0298
0296	0297	0290							
Cluster III	0239	0244	0247	0248	0249	0250	0251	0266	0281
0283	0284	0285	0286	0289	0291	0292	0293		

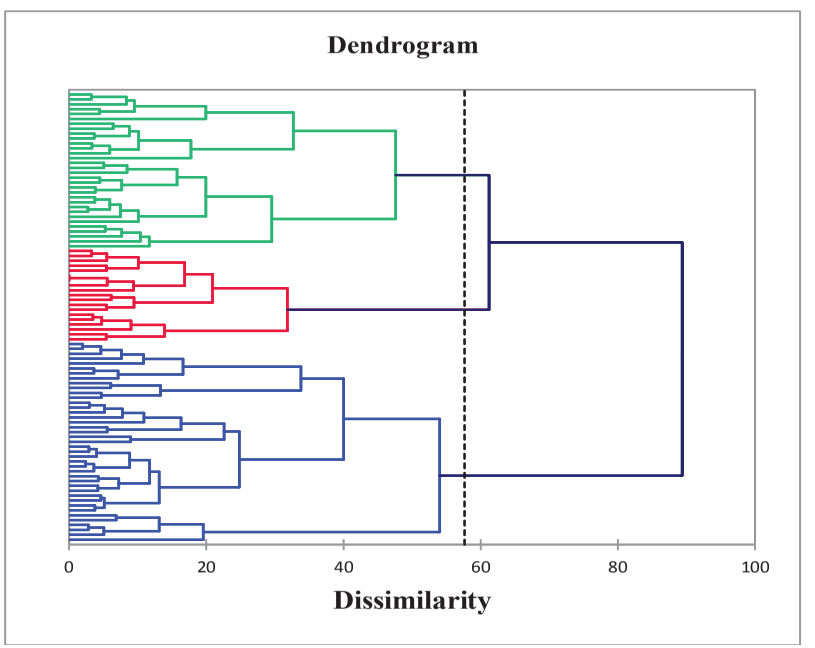

Figure 2: Dendrogram from cluster analysis of 92 bread wheat accessions based on quantitative traits

4 CONCLUSION

Results of this study succeed in obtaining important scientific information on wheat germplasm database stored in the Albanian Gene Bank, and for further wheat breeding programs. The significant differences found in the present study show the existence of a high genetic variability among the 92 bread wheat genotypes and quantitative traits analysed, adequate for selection of desirable traits, and creation of new favourable gene combinations. Among the mean value of genotype for plant height trait, accession AGB 0268 had the highest mean value for SL and the number of spikelets per spike traits, the maximum values were observed in accession AGB 0251 followed by AGB 0268. Regarding number of grains per spikelet character, AGB 0322 had the highest mean value. Data revealed that wheat genotype AGB 0326 presented the highest mean values for GS and seed size trait. AGB 0276 had the lowest values for two traits WGS and seed size followed for this last trait by AGB 0278 and AGB 0221. Three principal components exhibited about 66.42 % of variability where two PCs components influenced mostly the variability (PC1 with 28.1 % and PC2 with 24.43 %). The results suggested that plant height, spike length, number of spikelet per spike were the most important characters in differentiating the genotypes. The use of principal component analysis (showing the largest contributor to the total variance) and correlation coefficient analysis in the wheat germplasm, simplify dependable classification of genotypes, the identification of the superior genotypes (considering the evaluation of mean values) and their relation with morphological traits with possibility expenditure in breeding programs. Identification of the most important quantitative agronomical traits in wheat can facilitate selection of any individual accession and of desirable traits (genes), increasing the information of the wheat germplasm in gene bank.

The traits with more significant weighting on respective PC variance can be utilised successfully as quantitative markers for evaluation, characterization of the wheat germplasm stored in gene bank. Possible parental lines among these bread wheat genotypes that are in conservation in Albanian Gene Bank could be selected and utilised for sustainable field wheat breeding programs.

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Trichopria drosophilae (Diapriidae) and *Leptopilina heterotoma* (Figitidae), native parasitoids of *Drosophila suzukii*, confirmed in Slovenia

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ABSTRACT

The Spotted-wing drosophila (SWD), *Drosophila suzukii* (Matsumura, 1931) (Diptera, Drosophilidae) was recorded for the first time in Slovenia in autumn 2010. Shortly thereafter, it turned out to be one of the most important insect pests of soft and stone fruit in Slovenia and elsewhere. Within the expert work in the field of plant protection, more precisely within task inventarisation of beneficial organisms for biological control, the presence of indigenous *D. suzukii* parasitoids was investigated in 2018. Sentinel traps baited with *D. suzukii* larvae and pupae in banana slices enriched with artificial food medium for drosophilids were used for inventorying *D. suzukii* parasitoids in raspberries. The pupal parasitoid *Trichopria drosophilae* (Perkins, 1910) (Hymenoptera: Diapriidae) and the larval parasitoid *Leptopilina heterotoma* (Thompson, 1862) (Hymenoptera: Figitidae) were recorded parasitizing *D. suzukii* for the first time in Slovenia in August 2018 in Central Slovenia (Ljubljana).

Key words: *Leptopilina heterotoma*; *Trichopria drosophilae;* parasitoids; biological control; natural enemy; *Drosophila suzukii*; spotted wing drosophila

IZVLEČEK

***Trichopria drosophilae* (Diapriidae,) IN *Leptopilina heterotoma* (Figitidae) - PRVI NAJDBI DOMORODNIH PARAZITOIDOV PLODOVE VINSKE MUŠICE (*Drosophila suzukii*) V SLOVENIJI**

Plodova vinska mušica (PVM), *Drosophila suzukii* (Matsumura, 1931) (Diptera, Drosophilidae) je bila prvič ugotovljena v Sloveniji jeseni leta 2010. Kmalu po tem se je izkazalo, da gre za enega najpomembnejših škodljivcev pri pridelavi jagodičastega in koščičastega sadja pri nas in drugod po svetu. V okviru programa strokovnih nalog s področja zdravstvenega varstva rastlin, natančneje v okviru naloge inventarizacija koristnih organizmov za biotično varstvo rastlin, smo v letu 2018 ugotavljali zastopanost domorodnih vrst parazitoidov plodove vinske mušice. Kot vabe za lovljenje parazitoidov PVM smo uporabljali koščke banan okužene z ličinkami in bubami PVM, katerim smo dodali umetni jabolčni medij za vinske mušice. Ugotovili smo, da sta pri nas zastopana larvalni parazitoid *Leptopilina heterotoma* (Thompson, 1862) (Hymenoptera: Figitidae) in parazitoid bub *Trichopria drosophilae* (Perkins, 1910) (Hymenoptera: Diapriidae). Obe vrsti sta bili ugotovljeni avgusta leta 2018 v osrednji Sloveniji z vabami nastavljenimi v grme malin.

Ključne besede: *Leptopilina heterotoma*; *Trichopria drosophilae*; parazitoidi; biotično varstvo; naravni sovražniki; *Drosophila suzukii*; plodova vinska mušica

1 INTRODUCTION

The spotted wing drosophila, *Drosophila suzukii* (Matsumura, 1931), SWD, Diptera, Drosophilidae) originally reported in Japan in 1930, almost at the same time (2008) invaded North America (California) and Europe (Italy, Spain) and emerged as an alien pest of soft fruits (Cini et.al., 2012). While other *Drosophila* species feed on rotten and damaged fruits, *D. suzukii* females possess a serrated ovipositor that allows egg deposition into undamaged fruits causing great harvest losses (Sasaki and Sato, 1995). The fruit fly *D. suzukii* is a highly polyphagous invasive pest with many host plants both cultivated and wild soft-skinned fruits, allowing it to spread rapidly and with high dispersal rate (Cini et. al., 2014). Damage is caused by larvae feeding within the soft tissue of the fruits. Subsequently, secondary fungal or bacterial infections may further

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promote fruit deterioration. Economic losses of fruit production were reported for USA (California) $390 million (Bolda et.al, 2010) and Italy (Trentino) € 3.3 million (Ros et. al., 2013).

Chemical control methods for fruit flies have low efficiency (Bruch et. al., 2011). Therefore, biological control using parasitoids might play an important role as an alternative to synthetic chemical insecticides. For effective use in biological control programmes it is important to promote the use of indigenous natural enemies from the newly invaded areas, also due to strict regulations of European legislation (Barratt et al., 2018; Van Lentern, 2012). Most studied larval parasites of *Drosophila* were of the genera *Leptopilina* and *Asobara* and the pupal parasites *Spalangia*, *Pachycrepoideus* and *Trichopria* (Fleury et. al., 2009). A generalist pupal parasitoid *Pachycrepoideus vindemmiae* (Rondani, 1875) (Hymenoptera Pteromalidae), a major natural enemy of *D. melanogaster* (Martelli), was recently found to be also associated with *D. suzukii* in USA (Brown et al., 2011) and Europe (Rossi Stacconi et al., 2013; Chabert et al., 2012, Knoll et al., 2017). European pupal parasitoid *Trichopria drosophilae* was also found to attack and develop on *D. suzukii* (Mazzetto et al., 2016). The fact that both parasitoids attack the invasive spotted wing drosophila was also reported in Italy (Rossi Stacconi et al., 2013), Spain (Gabarra et al., 2014) and California (Wang et al., 2018). Further, they could be adapted to different climatic conditions. The aim of this survey was to identify the presence of indigenous *D. suzukii* parasitoid species in Slovenia (Central Europe) via field surveys.

2 MATERIALS AND METHODS

2.1 Insect rearing

Studies were conducted at the Agricultural Institute of Slovenia, in Ljubljana, Slovenia. Flies of *D. suzukii* were reared in 30×30×30 cm plastic insect rearing cages (BugDorm-1; Mega View Science, Taiwan) in a growth chamber in D: L cycles of 14:10 h at 21 °C and 77 ± 3 % relative humidity. The flies were provided with tap water and solid artificial food medium (20 g agar, 20 g sugar, 10 g wheat flour, 50 g dry baker's yeast, 500 ml tap water, 400 g grated organic apples, 500 ml organic apple juice, 50 ml apple vinegar and 4 g nipagin (methyl 4-hydroxybenzoate, Sigma-Aldrich).

2.2 Preparation of sentinel traps

Larval and pupal *D. suzukii* parasitoids were sampled using sentinel traps as described elsewhere (Miller et. al., 2015; Rossi Stacconi et. al., 2013) with small modification: Plastic cups (125 ml) containing fresh banana slices (60-70 g) were exposed for 1 to 3 days to *D. suzukii* (Diptera: Drosophilidae) flies in rearing cages. During that time the females laid eggs in banana slices. Once removed from the oviposition, infested banana slices were maintained for 5 to 7 days in the laboratory at room temperature to allow development of larvae and pupae. Afterward the larvae and the remainder of the eaten banana slices were transferred into new 500 ml plastic containers and enriched with (10 g) artificial food medium for drosophilids. In each infested plastic container one dental cotton tampon (Tosama, Domžale, Slovenia) was placed for absorbing excess liquid of contents. At the end containers were covered with mesh dimensions (0.8 × 0.8 mm) through which parasitoids could pass but not the flies. Each container was placed inside a funnel trap (green lid/green funnel/transparent bucket; catalogue number: 30201) from Pherobank, Netherlands and exposed to natural enemies in the environment.

2.3 Laboratory and field observation

Sentinel traps were set to a height of 1 to 1.5 m from the ground into raspberry plants. After 5 to 7 days of field exposure, the containers with the potentially parasitized SWD larvae and pupae were removed from the funnel trap and additionally coated with fine mesh gauze that prevents the passage of the parasitoids. They were transferred to a growth chamber and held at 22 °C and 77 ± 3 % relative humidity, 14 : 10 L : D photoperiod and observed weekly for another eight weeks for emergence of parasitoids. In central Slovenia (Ljubljana) three sentinel traps were field-exposed simultaneously for one week to natural fauna from the second half of June to October in 2018.

3 RESULTS AND DISCUSSION

The pupal parasitoid *Trichopria drosophilae* (Hymenoptera: Diapriidae) and the larval parasitoid *Leptopilina heterotoma* (Hymenoptera: Figitidae) were recorded attacking spotted wing drosophila for the first time in Slovenia in the summer 2018 in Central Slovenia (Ljubljana). Traps baited with *D. suzukii* larvae or pupae exposed in the field were attacked with both species during 23 to 27 July 2018 (30th calendar week). The peak flight of *L. heterotoma* in a growth chamber was recorded one month later 23th August 2018, when more than 30 individual emerged from larvae *D. suzukii*. Only two individuals of *Trichopria drosophilae* were caught on sentinel traps.

Figure 1: *Trichopria drosophila* (Perkins, 1910) (Hymenoptera,Diapriidae)

Figure 2: *Leptopilina heterotoma* (Thompson, 1862) (Hymenoptera,Figitidae)

A lot of parasitoids are reported to attack various Drosophilidae species, and the majority of them are larval parasitoids such as the most generalist parasitoid *Leptopilina heterotoma* (Fleury et al., 2009), which we also found parasitizing *D. suzukii* in our region. It is a solitary koinobiont parasitoid that attacks first and second stages of *Drosophila* larvae (Fleury et al., 2009). The cosmopolitan pupal endoparasitoid *Trichopria drosophilae* attacks many Drosophilidae species, including *D. suzukii*, and could potentially be a good biological control agent for this important pest (Chen et al., 2018). It is idiobiont parasitoid whose host range is known to be limited to Drosophilae (Wang et al., 2016).

4 CONCLUSION

This paper contributes to the knowledge of the wide spread of native beneficial organisms of *D. suzukii* such as the paleartic larval parasitoid *Leptopilina heterotoma* and cosmopolitan pupal endoparasitoid *Trichopria drosophilae*. Results promote awareness of the importance of further field studies to investigate parasitoid adaptation to local agroecosystems and its potential for wider use in biological control.

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Vpliv koristnih talnih mikroorganizmov in endofitov na rastlinsko obrambo pred žuželkami

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IZVLEČEK

Številni talni mikroorganizmi, kot so na primer mikorizne glive in rizobakterije, lahko pomagajo rastlinam premagovati biotični in abiotični stres, in sicer s spodbujanjem rasti rastlin in inducirano obrambo. Koristni talni mikroorganizmi delujejo dvosmerno z nadzemnimi organizmi, kot so herbivori, njihovimi naravnimi sovražniki ter opraševalci. Vloga interakcij med prej omenjenimi organizmi pridobiva v kmetijstvu in naravnih ekosistemih vse več pozornosti. S tem pa tudi narašča zanimanje za razumevanje molekularnih in fizioloških mehanizmov, ki so v ozadju tovrstnih multitrofičnih sistemov.

Ključne besede: talni mikroorganizmi; endofiti; interakcije; herbivori; inducirana sistemska obramba; multitrofični sistemi

ABSTRACT

IMPACT OF BENEFICIAL SOIL MICROORGANISMS AND ENDOPHYTES ON PLANT DEFENSE AGAINST INSECTS

Soil borne microorganisms such as mycorrhizal fungi and plant growth-promoting rhizobacteria help plants to overcome abiotic and biotic stress. Mechanisms used in this situtations are: growth promotion and induced resistance. Beneficial soil microorganisms also interact with foliar insects (herbivores, natural enemies and pollinators). This kind of interactions are getting more and more important in different ecosystems, especially in agriculture. A better knowledege of these systems would certainly help to deepen the understanding of multitrophic interactions.

Key words: soil borne microorganisms; endophytes; herbivores; induced systemic resistance; multitrophic system

1 UVOD

Kmetijstvo se dandanes sooča s številnimi novimi pristopi pri zatiranju gospodarsko pomembnih škodljivih organizmov. Vse več pozornosti se namenja ekološkim pristopom pri varstvu rastlin, prav tako pa je pomembno doseganje večjih pridelkov z omejevanjem negativnih vplivov na okolje.

Rastline so razvile več načinov obrambe pred škodljivimi organizmi. Pri tem ločimo direktne mehanizme, med katere uvrščamo rastlinske značilnosti, kot so rast trnov ali izločanje toksinov, ki neposredno negativno delujejo na škodljive organizme. Razvile so tudi indirektne mehanizme obrambe, ki vključujejo izločanje hlapnih organskih komponent, ki v neposredno bližino privabljajo naravne sovražnike škodljivih organizmov. Zato lahko trdimo, da imajo rastline vlogo posrednika v multitrofičnih interakcijah med številnimi vrstami škodljivih in koristnih organizmov (Schoonhoven et al., 2005). Za rastline je značilno tudi to, da povezujejo talne in nadzemne združbe organizmov.

Raznolike združbe talnih mikroorganizmov (endofitske glive, mikorizne glive, rast spodbujajoče glive ter rizobakterije) pozitivno delujejo na rast rastlin in preživetje prek posrednih in neposrednih obrambnih mehanizmov (Wardle et al., 2004; Bezemer & van Dam, 2005). Dva glavna mehanizma, ki vključujeta spremembe v rastlinski fiziologiji, sta: spodbujanje rastlinske rasti ter inducirana sistemska obramba (v

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nadaljevanju ISO). ISO varuje rastline pred različnimi boleznimi, sprožijo pa jo lahko različne vrste koristnih mikroorganizmov (Sanchez et al., 2005; Van Wees et al., 2008). Prav tako koristni mikroorganizmi vplivajo na nadzemne žuželke (herbivore, naravne sovražnike ter opraševalce) prek rastlinskih posrednikov. Povečana rast rastlin je veljala do nedavnega za enega izmed najpomembnejših mehanizmov interakcij med mikroorganizmi in rastlinami. Zanimanje za ISO je domena novejših raziskav, zato ostaja o tem pojavu še veliko neznanega (Bezemer & van Dam, 2005; Valenzuela-Soto et al., 2010).

2 VPLIV MIKROORGANIZMOV NA RAST RASTLIN

Povečana rast rastlin je eden izmed najpomembnejših pojavov, ki nastane pri simbiozi med rastlinami, mikoriznimi glivami ter dušik fiksirajočimi bakterijami. Tovrsten koristni učinek je še zlasti pomemben v kmetijstvu, saj s tem pripomore k zmanjšani uporabi mineralnih gnojil, kar pa posledično privede do zmanjšanja onesnaženosti kmetijskih zemljišč in voda (Yang et al., 2009; Weyens et al., 2009). Na primer glive iz rodu *Trichoderma* spp., spadajo med rast spodbujajoče glive (v nadaljevanju RSG), ki so komercialno dostopne in zelo pogosto uporabljene v kmetijstvu. *Trichoderma* spp. na rastlino delujejo tako, da spodbudijo sistemsko odpornost (Harman et al., 2004; Trillas et al., 2009; Segarra et al., 2009). Izboljšana rast rastlin pri koristnih simbiozah nastopi zaradi izboljšane prehrane rastlin in zaradi povečane tolerance rastlin na abiotični in biotični stres. Do izboljšane prehrane rastlin pride predvsem zaradi povečanega privzema hranil (npr. fosforja), povečevanja dostopnosti hranil, ki so rastlinam sicer nedostopna in vezave zračnega dušika (Singh et al., 2011; Meena et al., 2017; Felestrino et al., 2017). V zadnjem času se je povečalo zanimanje za tako imenovana mikrobna biognojila, ki poskrbijo za rodovitnost tal, povečajo pridelek in izboljšajo toleranco rastlin na stresne dejavnike (Bhardwaj et al., 2014). Tako so z uporabo tovrstnih biognojil pri pridelavi riža v delti reke Mekong zmanjšali uporabo kemičnih gnojil za kar 52 % (Nguyen et al., 2017). Učinkovitost rast spodbujajočih bakterij (RSRB) so testirali tudi slovenski raziskovalci. Pri svojem poskusu so uporabili mešanico RSRB (*Pseudomonas fluorescens* (Flügge 1886) Migula, 1895 in *Azospirillum brasilense* Tarrand, Krieg & Döbereiner, 1978) in opazovali učinek le-te na pridelek treh različnih sort krompirja. Poskus je bil izveden tekom vročega in zelo sušnega poletja, kljub temu je bil vpliv mešanice na pridelek vseh treh sort krompirja pozitiven (17 %-31 %). Rezultati njihove raziskave dokazujejo, da ima tovrstna bakterijska mešanica pozitiven vpliv na pridelek krompirja v sušnih razmerah in pri majhni okužbi s foliarnimi glivičnimi boleznimi ter napadi listnih žuželk (Trdan et al., 2019).

Nekateri mikroorganizmi lahko sintetizirajo rastlinske rastne regulatorje (citokinine, avksine in giberiline) ter s tem izboljšajo rast rastlin (Baca in Elmerich, 2007; van Loon, 2007; Contreras-Cornejo et al., 2009; Spaepen in Vanderleyden, 2011) in povečajo fotosintezno aktivnost. Znanstveniki so ugotovili, da giberilini, ki jih izločata *Azospirillum brasilense* in *A. lipoferum* (Beijerinck 1925) Tarrand *et al.* 1979 , spodbujata rast poganjkov in povečujeta gostoto korenin pri rižu in koruzi (Baca in Elmerich, 2007). Ugotovljeno je tudi bilo, da mikroorganizmi proizvajajo ACC (1-aminociklopropan-1-karboksilat) deaminazo, ki zmanjšuje raven etilena v rastlinah z namenom ublažitve sekundarnih učinkov stresa (Glick, 2014; Gamalero in Glick, 2015).

Talni mikroorganizmi lahko prispevajo k povečani toleranci rastlin na abiotični stres, kot je suša, zasoljenost tal in prisotnost težkih kovin v tleh (Yang et al., 2009; Weyens et al., 2009; Evelin et al., 2009; Bae et al., 2009). Učinek povečane rasti rastlin zaradi delovanja talnih koristnih mikroorganizmov vpliva tudi na interakcije med rastlinami in žuželkami, zaradi česar imajo slednje tudi več hrane (herbivorija: hranjenje z rastlinskim materialom). Izboljšana sestava hranil v rastlinah tako vpliva na žuželke na različnih trofičnih nivojih (Schoonhoven et al., 2005; Bukovinszky et al., 2008). Večje vsebnosti dušika pozitivno vplivajo na žuželke, ki se hranijo z rastlinskimi tkivi in sesanjem rastlinskih sokov iz floema (Schoonhoven et al., 2005). Nenazadnje so tudi zgledi, ki kažejo, da lahko interakcije med rastlinami in koristnimi talnimi mikroorganizmi privedejo do povečane tolerance rastlin na herbivorijo (Bennett et al., 2006; Vannette & Hunter, 2009). Tako se zaradi izboljšanega prevzema vode in hranil poškodovana tkiva hitreje obnavljajo in nadomestijo manko rastlinske biomase (Herman et al., 2008; Kempel et al., 2009). Kljub temu, da so tovrstni vidiki koristne simbioze med rastlinami in talnimi mikroorganizmi izrednega pomena, še vedno ostajajo slabo preučeni.

3 INDUCIRANA SISTEMSKA ODPORNOST PRI RASTLINAH

Številni talni mikroorganizmi lahko pri rastlinah povzročijo nastanek inducirane obrambe v sistemskih tkivih (ISO). Načeloma ISO sprožijo rast spodbujajoče bakterije (RSRB), kot so *Pseudomonas* in *Bacillus* spp. (Kloepper et al., 2004; Van Wees et al., 2008; Van der Ent et al., 2009), vendar zadnje raziskave kažejo, da lahko ISO spodbudijo tudi mikorizne glive (Pozo & Azcon Aguilar, 2007; Trillas et al., 2009;), endofitske glive (Stein et al., 2008) ter rast spodbujajoče glive (v nadaljevanju RSG) (Harman et al., 2004; Segarra et al., 2009). ISO ima določene lastnosti, po katerih se razlikuje od ostalih sistemskih toleranc. Tako jo spodbudijo nepatogeni organizmi, pri čemer se sproži delovanje obrambnih genov, kar privede do povečane odpornosti na škodljive organizme ter vključuje odzivnost rastline na rastlinske rastne regulatorje, kot sta jasmonska kislina (JA) in etilen (Van Wees et al., 2008; Van der Ent et al., 2009). Pri tem je potrebno poudariti, da je ISO pogojena predvsem s povečano dovzetnostjo rastline za rastlinske rastne regulatorje in ne s povečano produkcijo rastlinskih rastnih regulatorjev (Van der Ent et al., 2009). Mehanizmi, ki uravnavajo ISO ter obrambne strategije pri herbivoriji, se deloma prekrivajo (Pieterse & Dick, 2007; Pieterse et al., 2009,). Odziv rastlin na žuželke, ki povzročajo poškodbe z grizenjem rastlin, je največkrat pogojen s sproščanjem JA (Zheng et al., 2007; Van Oosten et al., 2008;). Enako velja tudi za odziv rastlin na žuželke, ki so škodljive zaradi sesanja (npr. listne uši) (Zhu-Salzman et al., 2005; Zarate et al., 2007), JA je rastlinski rastni regulator, ki ima pomembno vlogo pri ISO in je tudi zelo pomembna pri obrambi rastlin pred napadom škodljivih žuželčjih vrst. Zato lahko rečemo, da koristni talni mikroorganizmi vplivajo na interakcije med rastlinami in žuželkami.

Učinkovitost ISO na različne patogene organizme je bila dokazana na številnih rastlinskih primerih (Van Oosten et al., 2008), pri čemer lahko vzpostavitev obrambnih reakcij poteče po nekoliko različnih poteh. Prvi korak pri aktiviranju ISO, je prepoznavanje molekularnih vzorcev (elicitorjev), ki jih izločajo mikroorganizmi (Fe^{3+}, siderofori, antibiotiki, hlapljive organske snovi) (Bakker et al., 2007; Van Wees et al., 2008; Van der Ent et al., 2009). Ko rastlina prepozna prej navedene spojine, se aktvira gen za transkripcijo MYB72 v koreninah. Mutirane rastline myb72 se na napad žuželk in patogenih organizmov ne odzovejo z ISO tudi, če so kolonizirane s RSRB ali RSG (Van der Ent et al., 2008; Segarra et al., 2009), kar kaže na to, da ima transkripcijski faktor MYB72 ključno vlogo pri nastanku ISO. Tudi transkripcijski faktor MYC2, ki uravnava izločanje JA, je bil v preteklih raziskavah ugotovljen kot pomemben regulator ISO pri navadnem repnjakovcu (*Arabidopsis thaliana* L.) (Pozo et al., 2008; Van der Ent et al., 2009). RSBR bakterija *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 pa je pri paradižniku vplivala na dvig obrambne sposobnosti proti rastlinjakovemu ščitkarju (*Trialeurodes vaporariorum* Westwood,1856) po tako od JA-odvisni poti kot tudi po JA-neodvisni poti (Valenzuela-Soto et al., 2010). V primeru mikoriznih gliv, ko so le-te v začetnih stopnjah kolonizacije prepoznane kot biotrof, kar inducira začetek obrambnih reakcij s spremembami v transkripciji in ravni rastnih regulatorjev. Fiorilli et al. (2011) so opazili znatne spremembe v transkriptomu paradižnika med kolonizacijo z arbuskularno mikorizno glivo *Glomus mosseae* (T.H. Nicolson & Gerd.) Gerd. & Trappe (1974). Pri tem je prišlo do sprememb izražanja genov primarnega in sekundarnega metabolizma v koreninah in poganjkih, vključno z indukcijo obrambnih reakcij poveznih z biotskim stresom. Rizosferni mikroorganizmi in endofiti lahko tako sprožijo različne mehanizme, ki vzpodbudijo obrambo rastline pred herbovornimi žuželkami.

Kljub temu, da je poznavanje molekularnega mehanizma pri ISO izrednega pomena, za samo poznavanje le-te, na to temo še ni bilo izvedenih veliko raziskav. Bodoče raziskave mehanizmov delovanja ISO bi nam vsekakor omogočile globlji vpogled in boljše razumevanje tovrstnih interakcij (Van Oosten et al., 2008; Valenzuela-Soto et al., 2010)

4 VPLIV NA NADZEMSKE ŠKODLJIVE ORGANIZME

Koristni talni mikroorganizmi lahko spodbudijo tudi rastlinsko obrambo pred nadzemskimi herbivori. Tako so v primeru topola *Populus* x *canescens* (Aiton) Sm. ugotovili, da je kolonizacija z ektomikorizno glivo *Laccaria laccata* (Scop.) Cooke zaradi sprememb v transkriptomu rastlinskemu gostitelju omogočila uporabo snovi, ki so bile učinkovitejše pri obrambi pred hroščem *Chrysomela populi*, (L., 1758), kot pa snovi, ki so jih uporabili nekolonizirani topoli (Kaling et al., 2018).

Pri tem pa ne smemo tudi zanemariti učinka številnih abiotskih in biotiskih dejavnikov na interakcije na relaciji mikroorganizem-rastlina-herbivor. Tako lahko na obrambo rastlin pred herbivori vpliva tudi razvojni stadij rastline (Barton & Koricheva, 2010). Upoštevati moramo tudi dejstvo, da so interakcije na relaciji

mikroorganizmi-rastline-herbivori, vrstno specifične, tako pri interakcijah z eno vrsto mikroorganizma kot pri mikrobnih združbah (Goverde et al., 2000; Gange et al., 2005;). Znanstveniki so na zgledu riža (*Oryza sativa* L.) uporabili kombinacijo različnih RSRB sevov, pri čemer so opazili, da je imela kombinacija močnejši negativen učinek na delovanje ličinke *Cnaphalocrocis medinalis* (Guenée, 1854) ter na aktivnost encimov, ki delujejo pri obrambi rastlin (tripsin inhibitorji, polifenol oksidaze), kot pa če bi v poskus bili vključeni samo posamezni sevi (Saravanakumar et al., 2007; Saravanakumar et al., 2008).

Prav tako so bili zabeleženi učinki abiotskega stresa na delovanje koristnih mikroorganizmov (Vidal, 1996; Borowiez, 1997). To so ugotovili na zgledu endofitske glive *Acremonium strictum*, ki je povečala smrtnost rastlinjakovega ščitkarja (*Trialeurodes vaporarium* Westwood, 1856) pri paradižniku, ki je bil izpostavljen suši. Pri optimalni vlagi in preskrbljenosti z vodo gliva ni imela učinka na smrtnost rastlinjakovega ščitkarja (Vidal, 1996).

Izid interakcij med rastlinami, mikrobi in herbivori je pogojen z načinom prehranjevanja škodljivca (Van Oosten et al., 2008; Gehring & Bennett; 2009; Hartley & Gange, 2009; Koricheva et al., 2009). Herbivori, ki spadajo med generaliste, so tako bolj dovzetni na toksične sekundarne metabolite, ki jih izločajo rastline, kot pa specialisti. Slednji tovrstne komponente celo uporabljajo za lociranje svojega gostitelja (Schoonhoven et al., 2005). Pogojenost interakcij z načinom prehranjevanja so dokazali z raziskavo na navadnem repnjakovcu, kjer obramba rastline z ISO, ki jo je vzpodbudila bakterija *Pseudomonas fluorescens*, ni imela učinka pri napadu gosenice repnega belina (*Pieris rapae* L., 1758) (specialist) (Van Oosten et al., 2008).

Ugotovljeno je bilo, da na ISO vpliva tudi oblika ustnega aparata (Gehring & Bennett; 2009, Kempel & al. 2009, Hartley & Gange, 2009, Koricheva & al. 2009). Številni rastlinski sekundarni metaboliti, kot so glukozinolati in cianogeni glikozidi, po hidrolizi z encimi tvorijo toksične produkte (Schoonhoven et al., 2005). Tovrstni encimi so prostorsko ločeni od substratov in pridejo v stik z njimi šele pri poškodbi celic zaradi napada herbivorov. Škodljivi organizmi, ki se prehranjujejo s sesanjem rastlinskih sokov, vstavijo svoje bodalo intercelularno in pri tem ne poškodujejo celičnega tkiva, zato hidrolitski encimi ne pridejo v stik s sekundarnimi metaboliti. Zaradi tega takšen način obrambe na slednje nima učinka (Gehring & Bennett, 2009; Kempel et al., 2009; Hartley & Gange, 2009; Koricheva et al., 2009).

5 VPLIV NA NARAVNE SOVRAŽNIKE HERBIVOROV IN OPRAŠEVALCE

Rastline pri napadu herbivorov izločajo kompleksne mešanice hlapnih organskih snovi, ki privabljajo v svojo bližino naravne sovražnike herbivorov (Vet & Dicke, 1992; Dicke et al., 2009; Zhang et al., 2009; Snoeren et al., 2009). Takšen način obrambe pred herbivori imenujemo posredna obramba. Izločanje JA uvrščamo med posredne načine obrambe in je ena izmed najpomembnejših signalizacijskih poti, ki vplivajo na sproščanje hlapnih organskih snovi. Zato multitrofične interakcije, ki vplivajo na signalizacijske poti JA, posledično vplivajo tudi na sestavo hlapnih snovi (Dicke et al., 2009; Snoeren et al., 2009). Tako lahko pričakujemo, da bodo koristni mikroorganizmi, ki vzpodbudijo JA signalizacijske poti, imeli učinek tudi na izločanje in sestavo hlapnih organskih komponent. Eden izmed zgledov, ki kaže na spremembe v sestavi hlapnih organski snovi pri ISO zaradi koristnih mikroorganizmov, je ta, da so mikorizne rastline izločale manj seskviterpenov, kot nemikorizne rastline. Pri tem je potrebno poudariti, da učinek tovrstnih emisij hlapnih organskih komponent, ki nastanejo pri ISO, zaradi koristnih mikroorganizmov še ni bil natančno preučen (Fontana et al., 2009). Kolonizacija rastlin s koristnimi mikroorganizmi lahko tako vpliva na povečanost napada, zmogljivost in na privabljanje naravnih sovražnikov herbivorov (Guerrieri et al., 2004; Hempel et al., 2009), tudi če je število gostiteljskih herbivorov manjše kot število gostiteljskih herbivorov na nekoloniziranih rastlinah (Saravanakumar et al., 2008). Pri tovrstnih študijah znanstveniki ugotavljajo, da posredna obramba, ki nastopi zaradi koristnih mikroorganizmov, vzpodbudi spremembe pri izločanju hlapnih organskih komponent, ki sprožijo privabljanje naravnih sovražnikov herbivorov.

Zadnje čase znanstveniki vse več pozornosti posvečajo interakcijam med rastlinami, talnimi koristnimi mikroorganizmi ter opraševalci (Gange & Smith, 2005; Cahill et al., 2008). Ugotovili so povečano številčnost opraševalcev na preučevanih rastlinah in večje število semen kot pri kontrolnih rastlinah, ki niso bile kolonizirane (Gange & Smith, 2005; Cahill et al., 2008). Učinek hlapnih organskih snovi v tovrstnih interakcijah je še vedno premalo preučen.

6 VPLIV NADZEMSKIH HERBIVOROV NA KORISTNE TALNE MIKROORGANIZME

Vpliv nadzemske herbivorije na talne koristne mikroorganizme je manj preučevan zgled interakcij (Schoonhoven et al., 2005; Shultz et al., 2009; Sinka et al., 2009; Johnson et al., 2009). Znanstveniki so v nekaterih zgledih odkrili, da lahko hebivorija privede do zmanjšane mikorizne kolonizacije (Gehring in Whitham, 1991; Schoonhoven et al., 2005; Gange, 2007), lahko jo poveča ali pa celo nima učinka (Kosola et al., 2004). V nekaterih zgledih pa so opazili, da sta stopnja mikorizne kolonizacije in herbivorija negativno korelirali (Schoonhoven et al., 2005; Gange, 2007).

Ugotovljeno je bilo, da ima pri takšnih interakcijah zelo pomembno vlogo ogljik, ki ga rastline naložijo v koreninah (Schoonhoven et al., 2005). Nadzemna herbivorija namreč sproži povečano nalaganje ogljika v koreninah, s čimer si rastline zagotovijo obnovo po napadu (Schwachtje et al., 2006; Schwachtje & Baldwin, 2008; Erb et al., 2009; Johnson et al., 2009). Nalaganje ogljika pa je seveda pogojeno tudi s starostjo rastline, pri čemer je najmanjše v stadiju reprodukcije (Wamberg et al., 2003). Sistem je še bolj kompleksen, saj se zaradi nadzemne herbivorije začnejo izločati tudi sekundarni metaboliti in hlapne organske snovi (Soler et al., 2007; Erb et al., 2009). Poleg tega lahko talni mikroorganizmi delujejo tudi škodljivo v določenih razmerah (Jones & Smith, 2004,; Soto et al., 2009), na katere lahko vplivajo obrambni mehanizmi rastline, ki jih vzpostavi pri napadu nadzemnih herbivorov (Heil et al., 2009).

Prav tako nadzemna herbivorija vpliva na sestavo in izločanje koreninskih izločkov (Bezemer & van Dam, 2005), ki imajo pomembno vlogo pri interakcijah rastlina-mikroorganizmi v rizosferi (Rudrappa et al., 2008). Koreninski izločki vsebujejo različne metabolite, kot so ogljikovi hidrati in organske kisline, ki stimulirajo bakterijsko mobilnost in privabljajo bakterije h koreninam rastlin (Rudrappa et al., 2008). Iz zgoraj navedenih zgledov nadzemnih in talnih interakcij lahko povzamemo, da so njihovi učinki dvosmerni ter, da predstavljajo področje, na katerem bi bilo potrebno opraviti še kar nekaj raziskav.

7 ZAKLJUČEK

Mehanizmi, vključeni v interakcije med rastlinami, mikroorganizmi in žuželkami, delujejo predvsem v smeri povečane rasti rastlin in inducirane obrambe pred škodljivimi organizmi. Na končni izid ima vpliv interakcija med samimi obrambnimi mehanizmi, ki jih rastlina uporabi v primeru napada. V znanosti je primer ISO zaradi koristnih talnih mikroorganizmov dobro znan, veliko manj pa je znanega o inducirani obrambi pri napadih škodljivih žuželčjih vrst. Spodbujena odpornost pri herbivoriji pa ni edini mehanizem prek katerega talni koristni mikroorganizmi vplivajo na interakcije med rastlinami in žuželkami. Znano je tudi, da lahko koristni talni mikroorganizmi povečajo učinkovitost naravnih sovražnikov škodljivih žuželk ter s tem zmanjšajo negativni vpliv na rastlino. Tudi v primeru, ko posredni ali neposredni mehanizmi obrambe niso uspešni, lahko talni mikroorganizmi pripomorejo k povečanju rastlinske biomase in pridelka ter na tak način okrepijo toleranco rastline proti škodljivim organizmom. V nadaljnjih raziskavah bi se zagotovo morali osredotočiti na interakcije koloniziranih rastlin s koristnimi mikroorganizmi, opraševalci ter naravnimi sovražniki škodljivih žuželk, saj lahko rezultati tovrstnih raziskav znatno pripomorejo pri optimizaciji uporabe fitofarmacevtskih in biotičnih sredstev za zatiranje škodljivcev v kmetijstvu. Prav tako bi se morali žlahtnitelji gojenih rastlin osredotočati na lastnosti rastlin, ki spodbujajo koristne interakcije med rastlinami in mikroorganizmi.

8 ZAHVALA

Prispevek je nastal v okviru predmeta Ekologija na Oddelku za biologijo Biotehniške fakultete.

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NAVODILA AVTORJEM

UVOD

Acta agriculturae Slovenica je četrtletna odprtodostopna znanstvena revija z recenzentskim sistemom, ki jo izdaja Biotehniška fakulteta Univerze v Ljubljani. Revija sprejema izvirne in še neobjavljene znanstvene članke v slovenskem ali angleškem jeziku, ki se vsebinsko nanašajo na širše področje rastlinske pridelave in živalske prireje in predelave. Pokritost zajema širok razpon tem, kot so agronomija, hortikultura, biotehnologija, fiziologija rastlin in živali, pedologija, ekologija in okoljske študije, agrarna ekonomika in politika, razvoj podeželja, sociologija podeželja, genetika, mikrobiologija, imunologija, etologija, mlekarstvo, živilska tehnologija, prehrana, bioinformatika, informacijske znanosti in ostala področja, povezana s kmetijstvom. Pregledne znanstvene članke sprejemamo v objavo samo po poprejšnjem dogovoru z uredniškim odborom. Objavljamo tudi izbrane razširjene znanstvene prispevke s posvetovanj, vendar morajo taki prispevki zajeti najmanj 30 % dodatnih originalnih vsebin, ki še niso bile objavljene. O tovrstni predhodni objavi mora avtor obvestiti uredniški odbor. Če je prispevek del diplomske naloge, magistrskega ali doktorskega dela, navedemo to in tudi mentorja na dnu prve strani. Navedbe morajo biti v slovenskem in angleškem jeziku. Uredništvo revije zagotovi prevode izbranih bibliografskih elementov (naslova, izvlečka, opomb in ključnih besed) v primeru tujih avtorjev. Prispevke sprejemamo skozi celo leto.

PROCES ODDAJE PRISPEVKA

Avtorji lektorirane prispevke oddajo v elektronski obliki na spletni strani OJS Acta agriculturae Slovenica. Pred oddajo prispevka se mora avtor na spletni strani najprej prijaviti oziroma registrirati, če prvič vstopa v sistem (potrebno je klikniti na Registracija in izpolniti obrazec za registracijo). Bodite pozorni, da na dnu regis-

AUTHOR GUIDELINES

INTRODUCTION

Acta agriculturae Slovenica is an open access peer-reviewed scientific journal published quarterly by the Biotechnical Faculty of the University of Ljubljana, Slovenia. The Journal accepts original scientific articles from the fields of plant production (agronomy, horticulture, plant biotechnology, plant-related food-and-nutrition research, agricultural economics, information-science, ecology, environmental studies, plant physiology & ecology, rural development & sociology, soil sciences, genetics, microbiology, food processing) and animal production (genetics, microbiology, immunology, nutrition, physiology, ecology, ethology, dairy science, economics, bioinformatics, animal production and food processing, technology and information science) in Slovenian or English language. Review articles are published upon agreement with the editor. Reports presented on conferences that were not published entirely in the conference reports can be published. Extended versions of selected proceedings-papers can also be considered for acceptance, provided they include at least 30 % of new original content, but the editorial board must be notified beforehand. If the paper is part of BSc, MSc or PhD thesis, this should be indicated together with the name of the mentor at the bottom of the front page and will appear as foot note. All notes should be written in Slovenian and English language. Slovenian-language translation of selected bibliographic elements, for example the title, abstract, notes and keywords, will be provided by the editorial board. Manuscripts are accepted throughout the year.

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Manuscripts should be submitted to the Acta agriculturae Slovenica OJS site. The submitting author should be registered to the site. Click Register and fill in the registration form. Be sure to check in the Author

tracijskega obrazca ne pozabite odkljukati potrditvenega polja »Avtor«, sicer oddaja prispevka ne bo mogoča.

Proces oddaje prispevka poteka v petih korakih. Priporočljivo je, da se avtor pred oddajo najprej seznani s postopkom in se na oddajo prispevka pripravi:

Korak 1: Začetek oddaje prispevka
- izbrati je potrebno eno od sekcij,
- pri rubriki »Pogoji za oddajo prispevka« morate potrditi vsa potrditvena polja,
- dodatna pojasnila uredniku je mogoče vpisati v ustrezno polje.

Korak 2: Oddaja prispevka
- Naložite prispevek v formatu Microsoft Word (.doc ali .docx).

Korak 3: Vpis metapodatkov
- Podatki o avtorjih: ime, priimek, elektronski naslovi in ustanove vseh avtorjev v ustreznem vrstnem redu. Korespondenčni avtor mora biti posebej označen.
- Vpišite naslov in izvleček prispevka,
- Vpišite ključne besede (največ 8, ločeno s podpičjem) in označite jezik besedila,
- Vnesete lahko tudi podatke o financerjih,
- V ustrezno besedilno polje vnesite reference (med posameznimi referencami naj bo prazna vrstica).

Korak 4: Dodajanje morebitnih dodatnih datotek
- Grafično gradivo naj bo naloženo v eni ZIP datoteki. Grafične slike imenujte Slika1.jpg, Slika2.eps, in podobno,
- Za vsako dodatno naloženo datoteko je potrebno zagotoviti predvidene metapodatke.

Korak 5: Potrditev
- Potrebna je končna potrditev.

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