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Effect of different fertilizing and farming systems in annual medic (*Medicago scutellata* 'Robinson') on soil organic matter and nutrients status

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ABSTRACT

This experiment was conducted to study the effect of different fertilizing and farming systems in annual medic (*Medicago scutellata* 'Robinson') on soil organic matter and nutrients status. Fertilizing systems consisted of control (no fertilizer), chemical fertilizer, biological fertilizer and integrated fertilizers (different combinations of chemical and biological fertilizing systems). The farming systems included irrigated and dry-farming systems. The experiment was conducted in two experimental sites with diverse climatic and soil conditions in Kermanshah province, Iran, during 2009 growing season. The highest amount of soil organic matter of 1.28 % was observed in integrated fertilizing system of nitrogen-fixing bacteria + phosphorus-solubilizing bacteria. Most of the nitrogen applied through chemical fertilizers was leached out of the plant access, however, application of integrated fertilizer resulted in increasing the concentration of nitrogen in soil because of its slow release and efficient utilization by plants. According to the results of this study it was concluded that the integrated fertilizing system was more successful in dry farming compared to other fertilizing systems.

Key words: annual medic; fertilizing system; farming system; soil nutrients; soil organic matter; climatic conditions

IZVLEČEK

UČINEK RAZLIČNIH NAČINOV GNOJENJA IN KMETOVANJA NA VSEBNOST ORGANSKE SNOVI IN HRANIL V TLEH V POSEVKU ŠČITASTOPLODNE METELJKE (*Medicago scutellata* 'Robinson')

V poskusu je bil preučevan učinek različnih načinov gnojenja in kmetovanja na vsebnost organske snovi in hranil v tleh v posevku ščitastoplodne meteljke (*Medicago scutellata* 'Robinson'). Gnojenja so obsegala kontrolo (brez gnojil), mineralna gnojila, biološka gnojila in integrirano gnojenje (različne kombinacije mineralnih in bioloških gnojil). Načina kmetovanja sta bila kmetovanje z namakanjem in brez namakanja. Poskus je potekal na dveh poskusnih mestih, ki sta se razlikovali v talnih in podnebnih razmerah v provinci Kermanshah, Iran, v rastni sezoni 2009. Največja vsebnost organske snovi v tleh (1.28 %) je bila izmerjena pri integriranemu načinu gnojenja, v katerem so uporabili bakterije, ki vežejo zračni dušik in bakterije, ki sproščajo fosfor. Večina dušika, ki je bila dodana v obliki mineralnih gnojil, je bila izprana iz tal, pri integriranem gnojenju pa se je koncentracija dušika v tleh povečala zaradi počasnejšega sproščanja in učinkovitega privzema v rastline. Glede na izsledke te raziskave lahko zaključimo, da je integrirani način gnojenja uspešnejši pri kmetovanju brez namakanja v primerjavi z drugimi načini gnojenja.

Ključne besede: ščitastoplodna meteljka; način gnojenja; način kmetovanja; vsebnost hranil v tleh; organska snov tal; podnebne razmere

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1 INTRODUCTION

Sustainability in agricultural production systems is one of the main management goals. The application of chemical fertilizers has been the best way to increase crop production towards meeting the global demands of food production (Shahoo et al., 2013). Their adverse impacts on soil are not effectively compatible with the goals of the sustainable management of agro-ecosystems (Banerjee et al., 2011 and Garai et al., 2014, Mondal et al., 2015). On the other hand, bio-fertilizers known as the environmental-friendly fertilizers can contribute achieving these goals. However, an economic production, an optimization in fertilizer application, a proper use of pesticides, an increase in soil organic matter, and an environmentally safe production system are required.

It is well known that biofertilizers affect the nutrients uptake by plants in normal and harsh environments (Mal et al., 2013; Hariprasad and Niranjana, 2009; Kim et al. 2009). Employing biological fertilizers, such as symbiotic fungi coexisting with plant roots, phosphate solubilizing micro-organisms and vermicompost in agriculture, not only increase the population and activity of beneficial soil micro-organisms, but also improve the growth and yield of crops (Mondal et al., 2015).

There are also some evidence that dual inoculation of medic under the phosphorus deficiency conditions led to increased mass, consolidation of N_2 and phosphorus content in plants at different levels compared to mono inoculation (Starcheva et al., 2008). Dual inoculation of vesicular-arbuscular mycorrhizal (VAM) and phosphate-solubilizing bacteria (PSB) enhanced plant growth and development compared to sole inoculation with each of these micro-organisms. Increased plant growth and P uptake has been reported by many researchers in several different crop species (Shabani et al., 2015).

With biological fertilizers utilization, it can be postulated that the nitrogen availability for plants will increase providing the suitable growth conditions and so a better quality yields will be expected (Duarah et al., 2011). Combining the biological fertilizer with urea under a suitable irrigation conditions can lead to increased forage quality. Heretofore, mainly the effect of biological fertilizers on the uptake and storage of nitrogen and phosphorus in plants has been studied, however, the effect of biological fertilizers on conservation and utilization of micronutrient elements as well as soil organic matter has been less considered. In general, the results of conducted studies have shown that the application of biological fertilizers had positive effects in terms of quantity, and quality of yield in different crops.

The main goal of this research was to understand how the farming (irrigated and dry farming) and fertilizing systems interaction affect the soil organic matter, micronutrient uptake and annual medic forage yield. We tried to address the following questions:

- What is the best combination of fertilizer and farming system to improve soil organic matter?
- How the soil nutrients are affected by the fertilizer and farming system?
- Does the application of biofertilizers (symbiosis bacteria and mycorrhiza) result in higher yield and quality of annual medic forage?

2 MATERIALS AND METHODS

The experiment was conducted in two locations: 1. Sararood Dryland Farming Research Station with the geographic longitude of 47”, 20’ and latitude of 34’’, 20’ with an elevation of 1351 meters above the sea level, and 2. Mahidasht Soil Fertility Research Station with the geographic longitude of 46”, 50’ and latitude of 24”, 16’ with elevation of 1380 meters above the sea level, during 2009 growing season. Some physical and chemical characteristics of soil and climatic information of two experimental sites are shown in Tables 1 and 2.

Table 1: Selected physical and chemical characteristics of soil (0-30 cm depth) in two experimental sites

Characteristic	Experimental Stations	
	Sararood	Mahidasht
pH	7.68	7.93
Dissolved solids (EC.103)	30.0	55.0
Organic carbon (%)	0.31	0.62
$CaCO_3$ (%)	30	28
Olsen phosphorous (mg kg^{-1})	8.00	9.40
Available potassium (mg kg^{-1})	530	430
DTPA extractable Zn (mg kg^{-1})	0.38	1.56
DTPA extractable Cu (mg kg^{-1})	0.70	1.40
DTPA extractable Fe (mg kg^{-1})	2.00	4.76
DTPA extractable Mn (mg kg^{-1})	2.42	3.78
Soil texture	Loamy silt	Loamy clay

Table 2: Mean precipitation and temperature during annual medic growing season in two experimental sites

Month	Average precipitation (mm)		Average temperature (°C)	
	Sararood	Mahidasht	Sararood	Mahidasht
Feb.	18.3	21.2	7.3	6.4
Mar.	36.1	71.8	9.4	8.0
Apr.	15.2	12.4	16.2	14
May	0.2	0.9	22.7	19.7
Jun.	0	0	26.5	24.0

The experimental sites at both locations were kept as fallow in the year preceding the experiment. The experiment was conducted as randomized complete block design with three replications. Soil samples were collected before the commencement of the experiment from both sites. The experimental treatments consisted of a control (without fertilizer), chemical, biological and integrated fertilizing systems as follows:

T0: control (no fertilizer application)
T1: chemical fertilizer (135 kg/ha urea fertilizer + 185 kg/ha triple superphosphate fertilizer)*
T2: urea chemical fertilizer + phosphorous solubilizing bacteria (*Bacillus coagulans* Hammer, 1915)
T3: urea chemical fertilizer + mycorrhiza (*Glomus intraradices* N.C. Schenck & G.S. Sm.)
T4: urea chemical fertilizer + phosphorous solubilizing bacteria + mycorrhiza
T5: nitrogen fixing bacteria (*Sinorhizobium meliloti* (Dangeard 1926) De Lajudie et al. 1994, comb. nov) + triple superphosphate fertilizer
T6: nitrogen fixing bacteria + phosphorous solubilizing bacteria
T7: nitrogen fixing bacteria + mycorrhiza
T8: nitrogen fixing bacteria + phosphorous solubilizing bacteria + mycorrhiza
* Chemical fertilizers of triple superphosphate and urea were applied according to soil test to fulfill the requirements of the crop in each site.

Land preparation took place before sowing annual medic in early March. Phosphate solubilizing bacteria (*Bacillus coagulans*), nitrogen fixing bacteria (*Sinorhizobium meliloti*), and mycorrhiza (*Glomus intraradices*) solutions were prepared according to Water and Soil Research Institute of Iran, instructions. After calculating the number of seeds per treatment, the seeds were placed into a polyethylene bag (30 mg of each inoculation

substance for 100 g of seed) along with 4 % arabic gum solution. The seed and the adhesive substance were then gently shaken for 30 seconds. One gram of inoculation substance was added to the adhesive seeds and shaken for 45 seconds, ensuring that the inoculation substance was uniformly distributed among the seeds. The inoculated seeds were spread on an aluminum sheet in shade to dry off.

All experimental plots consisted of 6 planting rows of 25 cm apart with 5 meters in length. The annual medic 'Robinson' was planted at a rate of 20 kg of seed per hectare. Seeds were planted in 1 cm of soil depth. A furrow irrigation system was applied in the irrigated site at four stages as follows: irrigation immediately after planting, at four-leaf, at beginning of flowering and at pod formation stages.

Before sowing the seed, based on soil analysis and according to fertilizer recommendations for annual medic, half of urea fertilizer and all phosphorous fertilizer (in treatments containing phosphorous chemical fertilizer) were applied to the soil in bands. The rest of the nitrogen fertilizer was applied on the corresponding plots when plants reached different growing stages according to the treatments.

After harvesting the plants at each treatment, four soil samples were randomly collected from 0-30 cm soil depth to evaluate organic matter percent (Walkley and Black, 1934), as well as macro and micro nutrients contents.

Data were analyzed with a three-way analysis of variance (ANOVA) for three factors of location, production condition (i.e. +- irrigation) and fertilizer treatments. Mean treatment values were compared based on the least significant difference (LSD) at 5 percent probability level. SAS and Excel software were used for statistical analysis of experimental data and drawing graphs, respectively.

3 RESULTS AND DISCUSSION

3.1 Soil organic matter and macronutrients content

The results of ANOVA for soil organic matter content, affected by different fertilizing systems, is shown in Table 3. At both locations, the highest (1.22 %) and lowest (0.99 %) soil organic matter percentage was observed in T6 (nitrogen fixing bacteria + phosphorous solubilizing bacteria) and control treatments, respectively (Table 9). The soil organic matter content was significantly increased in T6 fertilizing system indicating that biological fertilizer treatment compared to other fertilizing treatments, provided with better plant growth and consequently higher biomass and organic matter (OM) in the soil. It seems that the continuous chemical fertilizers application in soil, could decrease the availability of soil organic matter due to increasing salt concentration in the soil solution. These processes could consequently reduce the microorganism population colonies. To support this idea we need to continue the experiment for at least few years.

Table 3: The results of ANOVA for soil elements and organic matter

s.o.v	df	OM	N	P	K	Fe	Mn	Cu	Zn
Location (L)	1	**	**	**	**	ns	**	**	**
Condition (C)	1	**	**	**	**	**	**	**	**
L*C	1	ns	ns	*	**	**	**	**	**
Treatment (T)	8	ns	**	**	**	**	**	**	**
L*T	8	ns	ns	**	**	**	ns	ns	**
C*T	8	ns	ns	**	**	**	**	ns	**
L*C*T	8	ns	ns	**	**	ns	**	ns	**

ns nonsignificant *Significant at $p \leq 0.05$ **Significant at $p \leq 0.01$

The highest contents of soil nitrogen (1.31 g kg^{-1}), phosphorous (18.3 mg kg^{-1}) and potassium (42 mg kg^{-1}) were found in integrated fertilizing treatments of T4, T5 and T6, respectively. However, the lowest amounts of these elements were found in control treatment (Table 9). It seems that the nitrogen generated from sole chemical fertilizer is more vulnerable to leaching leading to a less plant access, while the integrated biological + chemical fertilizing system, could more effectively increase soil nitrogen due to slow releasing of nutrients. These results are supported by the results of Berger et al., (2013) which indicated that the biofertilizer are more efficient because of slowing down the release of nutrients in rhizosphere.

Table 4: Mean soil organic matter and nutrient content in two farming systems

Experimental Condition	OM (%)	N (g/kg)	P (mg/kg)	K (mg/kg)	Fe (mg/kg)	Mn (mg/kg)	Cu (mg/kg)	Zn (mg/kg)
Irrigated	1.07 a	0.102b	8.89b	366 b	4.10 b	18.7a	1.71b	0.606b
Dryland	1.23a	0.136a	17.5a	534 a	4.40 a	17.9a	2.04a	1.06 a
LSD (5 %)	0.226	0.005	0.252	7.66	0.138	1.32	0.101	0.055

Means with the same letter in each column are not significantly different at 5 percent probability level.

Table 5: Mean values of organic matter and macronutrient in soil as affected by different fertilizing systems and experimental research sites

Treatment [a]	OM (%)			N (%)			P (mg/kg)			K (mg/kg)		
	Location		Mean	Location		Mean	Location		Mean	Location		Mean
	L1[b]	L2[c]		L1	L2		L1	L2		L1	L2	
T0	0.855	1.15	1.00b	0.097	0.118	0.107d	9.6	11.9	10.7e	370	417	393c
T1	1.00	1.27	1.03ab	0.110	0.125	0.117bc	12.0	13.0	12.5cd	485	439	462ab
T2	0.963	1.38	1.17ab	0.110	0.123	0.116bcd	11.6	12.3	11.9d	455	471	462ab
T3	0.947	1.28	1.11ab	0.110	0.115	0.112cd	10.5	14.0	12.2cd	420	478	449b
T4	0.967	1.42	1.19ab	0.137	0.127	0.131a	12.5	13.7	13.1c	437	467	451b
T5	1.03	1.35	1.19ab	0.118	0.127	0.122abc	17.7	19.0	18.3a	445	451	447b
T6	1.11	1.44	1.28a	0.118	0.135	0.126ab	11.5	17.4	14.4b	475	471	472a
T7	0.928	1.39	1.16ab	0.110	0.133	0.121abc	13.6	11.8	12.6cd	465	444	454b
T8	1.04	1.16	1.10ab	0.112	0.125	0.118bc	11.2	15.2	13.1c	450	470	460ab
Mean	0.984 a[d]	1.32a		0.113b	0.125a		12.2b	14.2a		444 b	456 a	
LSD local*treat (5 %)	0.313			0.014			1.487			22.2		

[a] T0 to T8: Different fertilizing systems, [b] Sararoud Experimental Site, [c] Mahidash Experimental Site.
[d] Means with the same letter in each column are not significantly different at 5 percent probability level.

The higher organic matter, nitrogen, phosphorus and potassium contents in the soil of Mahydasht station compared to Sararoud station (Table 9) along with better rainfall distribution in this site, could well explain the higher medic forage yield in this site (Table 2).

The maximum phosphorus content in the soil was observed in the T5 (nitrogen fixing bacteria + triple superphosphate fertilizer) treatment (Table 9). It seems that most of the phosphorous applied as chemical fertilizer to the soil was fixed in a non-absorbable form (Rodriguez and Reynaldo, 1999).

Table 6: Mean values of organic matter and micronutrients in soil as affected by different fertilizing systems and Experimental Research Sites

Treatment[a]	Fe (mg/kg)			Mn (mg/kg)			Cu (mg/kg)			Zn (mg/kg)		
	Location		Mean	Location		Mean	Location		Mean	Location		Mean
	L1[b]	L2[c]		L1	L2		L1	L2		L1	L2	
T0	2.87	4.53	3.70d	13.7	19.4	16.5e	1.36	2.00	1.68d	0.367	0.637	0.501f
T1	3.42	5.63	4.52ab	16.1	21.2	18.6c	1.46	2.17	1.81cd	0.645	0.967	0.805cde
T2	3.45	4.87	4.15c	15.6	19.6	17.5d	1.49	2.08	1.78cd	0.563	0.875	0.719e
T3	2.85	4.63	3.74d	15.5	20.6	18.0d	1.79	2.23	2.00a	0.807	0.902	0.854cd
T4	3.47	5.83	4.65a	14.7	20.4	17.5d	1.67	2.12	1.89abc	0.608	0.852	0.730de
T5	3.55	5.77	4.65a	18.0	23.4	20.7a	1.58	2.25	1.91abc	0.677	1.427	1.050b
T6	3.25	5.35	4.30bc	16.0	20.7	18.3cd	1.62	2.05	1.83bc	0.592	0.867	0.729de
T7	3.57	5.17	4.36abc	17.3	21.8	19.5b	1.62	2.28	1.95ab	0.720	1.803	1.260a
T8	2.92	5.42	4.16c	16.3	20.8	18.5c	1.67	2.37	2.01a	0.802	0.972	0.889c
Mean[d]	3.25 b	5.24 a		15.8 b	20.8a		1.58b	2.17a		0.642b	1.03 a	
LSD local*treat (5 %)	0.435			1.273			0.194			0.178		

[a] T0 to T8: Different fertilizing systems, [b] Sararoud Experimental Site, [c] Mahidash Experimental Site.
[d] Means with the same letter in each column are not significantly different at 5 percent probability level.

Table 7: Interaction between farming systems and experimental site (location) on organic matter and macronutrient content in soil

Experimental Condition	OM (%)		N (%)		P (mg/kg)		K (mg/kg)	
	Location		Location		Location		Location	
	L1[a]	L2[b]	L1	L1	L2	L2	L1	L2
D2[c]	0.91b	1.05b	0.09d	0.13b	7.63d	16.84b	331.29d	557.85a
D1[d]	1.23ab[e]	1.41a	0.11c	0.14a	10.15c	18.34a	401.48c	511.29b
LSD Local* Experimental Condition (5 %)	0.32		0.007		0.356		10.83	

[a] Sararoud Experimental Site, [b] Mahidash Experimental Site. [c] Dry farming condition [d] Irrigated system
[e] Means with the same letter in each column are not significantly different at 5 percent probability level.

The interactions between location and production systems showed that the organic matter and macro elements content in the soil under dry farming system were higher than irrigated system (Table 6). The higher yield under the irrigation system could be a good reason of increasing nutrient uptake from the soil (except for potassium) and decreasing the soil nutrients level. The content of extractable potassium before the commencement of the experiment in Sararoud station was more than Mahydasht station, but after annual medic cultivation, the situation was reversed.

Table 8: Interaction between farming systems and experimental site (location) on micronutrient content in soil

Experimental Condition	Fe (mg/kg)		Mn (mg/kg)		Cu (mg/kg)		Zn (mg/kg)	
	Location		Location		Location		Location	
	L1[a]	L2[b]	L1	L2	L1	L2	L1	L2
D2[d]	3.42 b	3.08c	17.84b	13.91c	1.52c	1.63c	0.47c	0.81b
D1[c]	4.77b	5.71a	19.68b	22.06a	1.89b	2.44a	0.73b	1.32a
LSD local* Experimental Condition (5 %)	1.869		1.869		0.143		0.077	

[a] Sararoud Experimental Site, [b] Mahidash Experimental Site. [c] Dry farming condition [d] Irrigated system
Means with the same letter in each column are not significantly different at 5 percent probability level.

Table 9: Interaction of farming systems and fertilizing treatments on organic matter and macronutrient content in soil

Treatments	OM (%)			N (%)			P (mg/kg)			K (mg/kg)		
	Experimental Condition		Mean	Experimental Condition		Mean	Experimental Condition		Mean	Experimental Condition		Mean
	D2[c]	D1[b]		D2	D1		D2	D1		D2	D1	
T0	0.89	1.08	0.99	0.09	0.10	0.09	6.80	8.15	0.09	340.83	375.00	357.91
T1	1.08	1.11	1.10	0.10	0.12	0.11	8.60	14.66	0.11	370.00	445.33	407.66
T2	1.11	1.18	1.14	0.10	0.13	0.11	7.86	16.43	0.11	360.00	554.16	457.08
T3	1.05	1.23	1.14	0.09	0.13	0.11	8.43	16.06	0.11	363.33	565.83	464.58
T4	1.11	1.18	1.14	0.11	0.13	0.12	9.56	16.06	0.12	358.33	535.00	446.66
T5	1.11	1.27	1.19	0.10	0.15	0.12	15.13	16.63	0.13	375.00	545.00	460.00
T6	1.17	1.27	1.22	0.11	0.13	0.12	8.80	21.60	0.12	390.83	520.83	455.83
T7	0.99	1.39	1.19	0.10	0.13	0.11	6.71	20.10	0.11	364.16	555.00	459.58
T8	0.89	1.32	1.10	0.09	0.14	0.11	6.80	18.60	0.11	340.83	545.00	442.91
Mean	1.04	1.22	1.13	0.09	0.12		0.10	0.13		362.59	515.68	
LSD FS*FT (5 %)[d]	0.31			0.01			1.48			22.29		

[a] T0 to T8: Different fertilizing systems. [b] Dry farming condition [c] Irrigated system
[d] Means with the same letter in each column are not significantly different at 5 percent probability level.

Table 10: Interaction of farming systems and fertilizing treatments on micronutrients content in soil

Treatment	Fe (mg/kg)			Mn (mg/kg)			Cu (mg/kg)			Zn (mg/kg)		
	Experimental Condition		Mean	Experimental Condition		Mean	Experimental Condition		Mean	Experimental Condition		Mean
	D2[d]	D1[c]		D2	D1		D2	D1		D2	D1	
T0	3.40	4.46	3.93	17.11	18.76	17.94	1.58	1.91	1.74	0.41	0.62	0.51
T1	4.26	4.00	4.13	19.18	15.95	17.56	1.63	1.77	1.70	0.55	0.59	0.57
T2	4.06	4.78	4.42	18.08	18.05	18.06	1.66	1.98	1.82	0.65	1.06	0.86
T3	3.30	4.25	3.77	17.13	17.06	17.10	1.88	1.91	1.89	0.63	0.78	0.70
T4	4.48	4.18	4.33	18.11	19.00	18.55	1.76	2.13	1.95	0.63	1.07	0.85
T5	4.71	4.81	4.76	22.10	16.93	19.51	1.67	2.01	1.84	0.54	0.82	0.68
T6	3.86	4.60	4.23	18.21	19.33	18.77	1.62	2.15	1.88	0.60	1.56	1.08
T7	4.36	4.73	4.55	20.16	18.45	19.30	1.64	2.04	1.84	0.80	0.85	0.82
T8	3.40	4.36	3.88	17.11	18.88	18.00	1.58	2.25	1.92	0.412	1.722	1.060
Mean	3.98b[d]	4.46a		18.58a	18.04a		1.67b	2.01a		0.58b	1.01a	
LSD FS*FT (5 %)	0.43		1.27		0.19			0.17				

[a] T0 to T8: Different fertilizing systems. [c] Dry farming condition [d] Irrigated system
[d] Means with the same letter in each column are not significantly different at 5 percent probability level.

The result of significant interaction of production system and fertilizing treatments is shown in Table 3. The results indicated that for all fertilizing treatments, the phosphorus and potassium concentrations in the dry farming system were consistently higher than in the irrigated system. The lowest phosphorus and potassium content was observed in control treatment under irrigated system. However, the highest amount of phosphorus and potassium in the soil was obtained in T6D1 and T3D1 fertilizing treatments, respectively. Increasing the soil moisture tension subsequently could have reduced nutrients solubility and availability to the plats root. All these findings would be a good answer of the first and second questions of this study.

3.2 The micronutrients concentration in soil solution

The results of analysis of variance for the soil micronutrients concentration is shown in Table 3. The effect of location, production systems and their interaction for micronutrients were significant. The results of the interaction of fertilizing system × location are shown in Table 10. The lowest concentration of micronutrients in both experimental sites was observed in control (no fertilizing treatment). However, the maximum iron content of 4.65 mg kg^{-1} was observed in T4 and T5 fertilizing treatments. The maximum Mg content of 20.7 ppm, copper of 2.01 ppm, and Zn of 1.26 ppm were measured in T5, T8, and T7 treatments, respectively.

Overall concentration of the soil micronutrients in Mahydasht was significantly higher than in Sararoud. It should be mentioned that preceding the experiment commencement, the concentration of soil micronutrients in Mahydasht site was already higher than in the Sararoud station (Table 1). The interaction between location and production systems (Table 8) showed a significantly higher concentration of the micronutrients in D1L2 fertilizing treatment.

According to Table 6, fertilizing treatments of T4 and T5 provided significantly higher iron content compared to other treatments. Application of biological fertilizers along with optimum utilization of chemical fertilizers, played a positive role in maintaining the soil fertility. Our results was supported by other literature reports on the synergistic effects of integrated application of chemical, organic and biological fertilizers on crop yields (Patidar and Mali, 2001; Hariprasad and Niranjana, 2009; Kim et al. 2009). Accordingly the findings of Duarah et al (2011) indicated that the application of biofertilizers (phosphorus solubilize bacteria) can significantly increase the plant biomass compare to application of NPK. Integrated fertilizing systems not only reduces the environmental pollution through reduction in chemical fertilizer consumption, but also improves forage quality features. This is demonstrated that the application of integrated chemical and bio-fertilizer can improve the soil function to better support the plant nutrition (Mondal et al., 2015; Mal et al., 2013). So the third questions of this study was answered and our finding indicated that the application of biofertilizers (symbiotic bacteria and mycorrhiza) could result in higher yield and quality of annual medic forage. On the other hand this situation would result in the soil nutrients availability and their uptake by plants and that supports the answer of the second question of this study.

According to the results of this study integrated fertilizing system would result in better performance both in soil nutrient and crop yield especially in dry farming conditions. The finding of this research emphasize that the integration of fertilizing systems improved the soil chemical properties.

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Yield and its attributes responses of drought tolerant upland 'NERICA' rice to different nutrient supplying treatments in rainforest transitory agroecology

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ABSTRACT

A screen house trial was conducted to evaluate reproductive growth responses of drought tolerant upland rice cultivars (NERICAs 1-4, WAB 56-104 and Moroberekan) to arbuscular mycorrhizal (AMF) inoculation under water deficit. A field trial was organized in randomized complete block test with three replicates, conducted in the late cropping season of 2012. We evaluated upland rice cultivars to different nutrient sources (AMF, AMF + 60 kg N ha^{-1} + 30 kg K ha^{-1}, 60 kg N ha^{-1}+ 30 kg K ha^{-1} and control). In the screen house inoculated rice had higher ($P < 0.05$) grain yield $plant^{-1}$ (19.29 g $plant^{-1}$) and its attributes than non-inoculated, except number of grain per panicle (108). On the field combination of AMF + 60 kg N ha^{-1} + 30 kg K ha^{-1} produced higher ($P < 0.05$) reproductive growth. Varietal variability ($P < 0.05$) was observed on AM colonisation and reproductive growth in both trials, with 'NERICA 2'was the most promising cultivar under tested agroecology condition.

Key words: arbuscular mycorrhizae; rainforest transitory agroecology; root colonization; reproductive growth

IZVLEČEK

ODZIV PRIDELKA NA SUŠO ODPORNIH SORT NENAMAKANEGA RIŽA 'NERICA' IN PARAMETROV PRIDELKA NA RAZLIČNA OBRAVNAVANJA S HRANILI V AGROEKOLOŠKIH RAZMERAH PREHODA V DEŽEVNI GOZD

Za ovrednotenje odziva na inokulacijo z arbuskolarnimi mikoriznimi glivami (AMF) na izbrane sorte nenamakanega, na sušo odpornega riža ('NERICAs 1-4', 'WAB 56-104' in 'Moroberekan') v reproduktivni rastni fazi, sta bila izvedena lončni poskus v rastlinjaku in poljski poskus v razmerah pomanjkanja vode. Poljski poskus je bil izveden kot popolni bločni poskus s tremi ponovitvami v pozni rastni sezoni 2012. Sorte nenamakanega riža so bile ovrednotene glede na različna obravnavanja s hranili (AMF, AMF + 60 kg N ha^{-1} + 30 kg K ha^{-1}, 60 kg N ha^{-1}+ 30 kg K ha^{-1} in kontrola). V lončnem poskusu v rastlinjaku je imel inokuliran riž večji pridelek zrnja na rastlino ($P < 0.05$,19.29 g $rastlino^{-1}$), večji so bili tudi drugi parametri pridelka kot pri neinokuliranem, z izjemo števila zrn na lat (108). V poljskem poskusu je obravnavanje AMF + 60 kg N ha^{-1} + 30 kg K ha^{-1}vzpobudilo večjo ($P < 0.05$) reproduktivno rast. Razlika med sortami ($P < 0.05$) je bila opažena tako v kolonizaciji z arbuskularnimi glivami kot v reproduktivni rasti v obeh poskusih. Sorta NERICA 2 se je izkazala kot najobetavnejša v preiskušenih agroekoloških razmerah.

Ključne besede: arbuskularna mikoriza; agroekološke razmere; prehod v deževni gozd; kolonizacija korenin; reproduktivna rast

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1 INTRODUCTION

The production of the upland rice in the tropics has been on the rise in terms of area under cultivation in recent years. Area under upland rice production in Nigeria according to Oikeh et al.(2008) constituted only 25 % of the total area under rice production. Though comparatively, grain yield of upland rice is lesser than that of the lowland rice, however the issue of sustainability in the face of climate change has necessitated a rethink in the production technology of rice in particular. Conventionally, lowland rice production demands usage of fresh water. With uneven rainfall pattern occasioned by climate change limiting the availability of fresh water (Fjelde and von Uexkull, 2012), there is a need for more sustainable water management. This is where upland rice production technology provides an alternative perspective.

Exploitation of the upland rice production ecology could only be feasible if the problem of soil moisture deficit is adequately addressed, especially at the reproductive growth stage. Improvement of rice performance could be done through genetic improvements, cultural management or both approaches. In most rice growing areas of sub-Sahara Africa, *Oryza sativa* L. cultivars are very popular due to their high productivity. However, they are highly susceptible to a wide range of deteriorating abiotic and biotic environmental factors (Jones, 1997; Jones et al., 1997). The native *Oryza glaberrima* Steud. is more resistant to a wide range of abiotic and biotic environmental factors than *O. sativa* but with lesser performance (Linares, 2002). It was in the light of this that AfricaRice, the umbrella research and development organisation mandated to research into rice value chain in Africa introduced interspecific varieties of rice (NERICA), combining high productivity of *O. sativa* and the hardiness of *O. glaberrima* (Defoer et al., 2004). Arbuscular mycorrhizae fungi (AMF) form a symbiotic relationship with most agricultural crops. Extensive report had been made on their nutritional effect on most crops especially phosphorus uptake in marginal soils (Smith and Read, 2008). Other reports had equally cited their effect on plant water status (Augé, 2001;Augé, 2004). The confounding effect of nutrient uptake on water status of inoculated crops though difficult to disentangle, the positive effect of AMF inoculation on crop water status remains a subject of intensive debate in the literature (Boyeret al., 2014; Jayne and Quigley, 2013; Rapparini and Peñuelas, 2014). Inoculation with AMF was reported to have significant effect on both soil (Augé, 2004) and water status (Augé, 2001). It was indicated that improved soil water potential could have been mediated through its effect on the formation of glomalin (Rillig, 2004) and stability of soil aggregates (Miller and Jastrow, 1994). On the crop the possibilities of AMF colonisation altering hydraulic properties of the root and leaf have been reported (Koide et al., 1989), though the results were inconsistent over several crop species. Other non-hydraulic effects had also been reported. These responses could affect leaf gas exchange parameters (Awotoye et al., 1992), nutrient uptake (Franson et al., 1991) and consequently grain yield.

Most reports on the positive effect of AMF colonisation were cited on marginal soil fertility (Hayman et al., 1975). There were conflicting perspectives on the combination of nutrient sources on the activity of AMF in the literature. Root colonisation of the host plant was reported to be a function of nutrient status of the soil (Liu et al., 2000) and that of the host *(*Ratnayake et al., 1978). Abbott et al. (1984) indicated that a high P status of the soil reduced host root colonisation by AMF. A converse pattern was reported for N and K (Furlan and Bernier-Cardou, 1989; Sylvia and Neal, 1990). Different combinations of N, P and K were investigated in the literature on root colonisation by AMF (Azcón et al., 1978; Sumner and Farina, 1986) with inconsistent results depending on the host and location where the investigation was conducted. There is also paucity of information on the genotypic differences of some upland rice cultivars on percentage AMF colonisation at different growth stages in the rainforest transitory ecology. Since rice crop is susceptible mostly to water deficit at reproductive growth stage, we hypothesised that increased root colonisation at such a stage would confer some level of drought tolerance on 'NERICA' rice.

This investigation was conducted to investigate the effect of AMF inoculation of some upland NERICA rice cultivars under soil water stress on AMF root colonisation and reproductive growth. We equally aimed to explicate the effect of

combination of different nutrient sources on AMF root colonisation and reproductive growth of these upland rice cultivars in the transitory rainforest.

2 MATERIALS AND METHODS

Two experiments (pot and field) were conducted.

2.1 Description of pot experiment

A pot experiment was conducted in the screen house of the College of Plant Science and Crop Production, FUNAAB in May, 2012. The pot experiment had a 6 × 2 × 2 factorial treatment structure, which was laid out in a complete randomised design (CRD) with three replicates. Six varieties of upland rice were used ('NERICA 1', 'NERICA 2', 'NERICA 3', 'NERICA 4', 'WAB 56-104' and 'Moroberekan'), inoculated and un-inoculated with AMF with two stress status (water stressed and control).

Before planting, the soil was maintained to 100 % field capacity using the gravimetric method. Seeds were planted in pots (10 kg capacity), filled to three quarter with sandy loam soil. Two to three seeds of each variety were planted per hole to a depth of about 2-3 cm and 50 g of AMF inoculum was applied at the base of the seeds during planting. The plants were thinned to one plant per stand ten days after sowing (DAS). The pots were maintained to field capacity for 21 days after which moisture stress was imposed. At the seedling stage (sowing to mid-tillering), the amount of water supplied to the pots daily was determined through differences in mass at full field capacity and water loss to evapotranspiration, while at full canopy (vegetative to reproductive growth stage), supply of water was based on the degree of soil surface dryness as reported by Yoshida and Hasegawa, (1982). Soil moisture stress was imposed on all the six varieties except control, at 21 (DAS) (vegetative growth), 50 DAS (reproductive growth) and 70 DAS (grain filling). The duration of soil water deficit was 20 days at each growth stage. There after water was restored untill harvest maturity.

2.2 Mycorrhizal inoculation

The mycorrhizal fungi used in this study were *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schuessler strains (previously *Glomus mosseae* (T.H. Nicolson & Gerd.) Gerd. & Trappe) isolated from rice farmers' field sampled across different agroecological zones of Nigeria. The inoculum was multiplied in the screen house for inoculum production using method described by Noyd, (1965). Pots were filled with 5 kg soil, with 5 cm hole made in the centre. A 30 g of pure inoculum of AMF species (*Glomus mosseae)* was applied to 2 seeds of maize per hole during planting, watered with sterilized H_2O and placed in the screen house for four months. Sterilized H_2O with 5 ml of Hoagland's solution (Hoagland and Arnon, 1950) was applied every 2 days as source of irrigation. Watering of maize ceased just before commencement of heading. The maize plants were removed from the pot. The remaining root from severed crop and soil were carefully mixed and used as inoculum. Root samples were collected at 8 weeks after planting (WAP) to establish AMF colonization count. Modified wet sieving method of Giovannetti and Mosse, (1980) was used to extract spores from Quartz sand used for multiplication of spore while spores were counted using the dissecting microscope.

2.3 Location and soil properties of the field experiment

A field trial was conducted at the Teaching and Research Farm of FUNAAB, Alabata, Ogun State (07^o 20' N; 03^o 23' E) in the rainforest transitory agroecology of Nigeria. Soil particle size distribution was determined using the hydrometer method (Bouyoucos, 1962). The pH was determined (in 1: 1 soil: water) using a pH meter (glass electrode) (McLean, 1982). The organic content of the samples was determined using wet – oxidation method Walkley and Black (1934) modified by Allison et al. (1965). Total nitrogen was determined using modified micro Kjeldahl digestion technique (Jackson, 1962). Available phosphorus was determined using Bray-1 (Olsen and Dean, 1965) and determined colometrically using the method of (Murphy and Riley, 1952). K^+ in the extract was determined by flame photometry. The textural class of experimental site was sandy loam, with slightly acidic pH (6.4), of 0.52 % organic matter content. The total N in the

soil was 0.05 %, while that of K was 1.63 cmol kg^{-1}. Available P in the soil was 38.8 mg kg^{-1}.

2.4 Treatments and design

The field experiment was 6 × 4 factorial treatment structure, laid out in randomised complete block design (RCBD), with three replicates. Six upland rice varieties and four nutrient supplying treatments were used (+ AMF, AMF + 60 kg N ha^{-1} + 30 kg K ha^{-1}, 60 kg N ha^{-1} + 30 kg K ha^{-1} and a control). Three viable seeds were planted per hole and later thinned to one at the spacing of 0.2 m × 0.2 m, with a gross plot of 3 m × 3 m (9 m^2) and a net plot of 2 m × 3 m (6 m^2).

2.5 Cultural practice

The site of the field experiment was cleared, ploughed once and harrowed. The field was laid out into plot sizes of 3 m × 3 m. Planting was done in August/September, 2012, at seeding rate of 3seeds per hole, in 0.2 m × 0.2 m grid. AMF was applied at 50 g per hole at planting. Nitrogen and potassium fertiliser in the form urea (60 kg N ha^{-1}) and muriate of potash (MOP) (30 kg K ha^{-1}), respectively was applied. Split application of nitrogen was conducted (½ at planting and ½ at panicle initiation).

2.6 Sampling and measurements

Root sampling for AMF infection

Fine root samples of rice cultivars were collected to estimate % AMF colonisation. The root samples were washed with tap water, and cut into 1 cm length and 0.25 g of the selected fresh fine roots were taken, cleaned in 10 % potassium hydroxide (KOH) in water bath for 20-30 minutes at 80 ^{0}C, then rinsed with water and stained with staining solution [methyl blue in lactoglycerol (1:1:1 lactic acid, glycerol and water)]placed in water bath for 2 minutes at 90 ^{0}C (Phillips and Hayman, 1970). The stained solution was rinsed off the roots and preserved with 50 % glycerol solution for further observation under the compound microscope. Mycorrhizal infection was quantified using the magnified intersection method as described by (McGonigle et al. (1990). Five hills of rice plant per net plot were used for the determination of reproductive growth parameters. Yield and its components were determined according to Standard Evaluation System (International Rice Research Institute, 2002).

2.7 Statistical analysis

Data collected were subjected to Analysis of Variance (ANOVA), fixed model at 5 % probability level. Discrete data were transformed using square root transformation before subjecting them to ANOVA. Other variables were checked for the violation of ANOVA assumptions prior to analysis. Significant means were separated using Standard Error of Differences (SED) and Duncan Multiple Range Test (DMRT). GenStat statistical package 12th Edition was used for all the analysis.

3 RESULTS

At all growth stages, moisture stress status had no significant (P > 0.05) effect on percentage AM colonisation (Table 1). All crops inoculated with AMF had significantly (P < 0.05) higher percentage AM colonisation than those without inoculation across all growth stages. Significant (P < 0.05) varietal variability was observed on percentage AM colonisation at all growth stages. At all growth stages ‘Moroberekan’ had the least percentage AM colonisation except at reproductive, where ‘NERICA 3’ had the least AM colonisation (18.83 %). Conversely at vegetative growth stage ‘NERICA 3’ had significantly higher percentage infection (45.3 %) than other varieties. At reproductive growth stage ‘NERICA 4’ had significantly (P < 0.05) higher percentage infection (35.29 %) compared to others. At grain filling stage, it was observed that ‘NERICA 1’ had significantly (P < 0.05) higher percentage AM colonisation (31.55 %) relative to other varieties.

Table 1: Effect of moisture stress at different growth stages on % AM colonization of upland 'NERICA' rice

Treatments	Vegetative phase	Reproductive phase	Grain-filling phase
		% AM colonisation	
Stress status			
unstressed	31.9	23.13	25.68
stressed	34.3	26.98	25.38
	NS	NS	NS
SED±	2.20	1.70	1.76
AMF			
Without AMF	29.4	17.05	19.52
With AMF	36.8	33.06	31.55
	**	**	**
SED±	2.20	1.70	1.76
Varieties			
NERICA 1	25.7cd	22.36bc	31.55a
NERICA 2	31.5bc	25.79b	27.58ab
NERICA 3	45.3a	18.83c	22.89bc
NERICA 4	39.1ab	35.29a	28.00ab
WAB 56-104	35.6b	25.97b	24.29bc
Moroberekan	21.3d	22.08bc	18.88c
	**	**	*
Stress status × AMF	NS	NS	NS
Stress status × Varieties	NS	NS	NS
AMF × Varieties	NS	NS	NS
Stress status × AMF× Varieties	NS	NS	NS

Means with the same alphabets are non-significantly different using Duncan Multiple Range Test (DMRT) at 5 % level of significant. * means significant at 5 % probability level, **means significant at 1 % probability level.

Table 2 indicated that percentage AM colonisation (39.87 %) was significantly ($P < 0.05$) higher in crops inoculated with AMF alone than others, the least ($P < 0.05$) was observed in crops with no nutrient source (16.26 %). 'NERICA 4' had the highest percentage AM colonisation (49.35 %), with the least significant observed in 'Moroberekan' (19.95 %).

Table 2: Effect of nutrient sources on % AM colonisation of upland 'NERICA' rice.

Treatments	% AM colonisation
Nutrient Sources	
AMF	39.87
AMF + 60 kg N + 30 kg K	36.91
60 kg N + 30 kg K	26.10
Control	16.26
	**
SED±	2.233
Varieties	
NERICA 1	22.38c
NERICA 2	31.91b
NERICA 3	21.48c
NERICA 4	49.35a
WAB 56-104	33.63b
Moroberekan	19.95c
	**
Varieties × Nutrient sources	*

Means with the same alphabets are non-significantly different using Duncan Multiple Range Test (DMRT) at 5 % level of significant. * means significant at 5 % probability level, **means significant at 1 % probability level.

In the screen house, across all growth stages all upland rice grown in unstressed pot had significantly ($P < 0.05$) longer panicle (Table 3). Similar pattern was observed on number of grains per panicle, 100 seed mass and yield per plant across all growth stages (Tables 4, 5 and 6). Inoculation of upland rice with AMF gave significantly ($P < 0.05$) longer panicle than without AMF. This was observed across all growth stages except at vegetative stage, where there was no significant effect of AMF inoculation on panicle length. Significant varietal variability on panicle length was observed at vegetative and grain filling stage, except at reproductive stage, where no significant effect was observed. At vegetative growth stage, 'NERICA 2' had significantly longer panicle (29.87 cm), however most other varieties had similar panicle length. It was observed that at grain filling stage, 'NERICA 4' had significantly longer panicle with most varieties having no significant differences among them.

Table 3: Effect of moisture stress at different growth stages on panicle length (cm) of upland 'NERICA' rice inoculated with AMF

Treatments	Vegetative phase	Reproductive phase	Grain-filling phase
Stress status			
unstressed	26.20	25.66	26.18
stressed	23.05	12.22	10.54
	**	**	**
SED±	0.76	1.25	0.85
AMF			
Without AMF	24.90	14.96	13.68
With AMF	24.35	22.92	23.03
	NS	**	**
SED±	0.76	1.25	0.85
Varieties			
NERICA 1	23.93bc	19.37	16.18c
NERICA 2	29.87a	19.74	20.49ab
NERICA 3	23.79bc	17.02	16.34c
NERICA 4	21.29c	20.51	22.10a
WAB 56-104	23.06bc	19.84	17.46bc
Moroberekan	25.80b	17.15	17.58bc
	**	NS	**
Stress status × AMF	**	**	**
Stress status × Varieties	NS	NS	**
AMF × Varieties	**	*	**
Stress status × AMF × Varieties	NS	NS	NS

Means with the same alphabets are non-significantly different using Duncan Multiple Range Test (DMRT) at 5 % level of significant. * means significant at 5 % probability level, ** means significant at 1 % probability level.

Inoculated upland 'NERICA' rice grown in the screen house had significantly higher 100 seed mass, higher yield per plant across all growth stages than non-inoculated except number of seeds per panicle, where a conserve pattern was observed with non-inoculated crops having a significantly higher number of seeds per panicle (Tables 4, 5 and 6). On the field significant difference among the nutrient sources were observed in terms of panicle, grains per panicle, 100 grain mass and grain yield ha^{-1} (Table 7). Combination of AMF + 60 kg N ha^{-1} + 30 Kg K ha^{-1} produced significantly higher grain yield and its components compared to other nutrient sources. Conversely, control had significantly the least panicle length (17.48 cm) and grain yield ha^{-1} (1.53 t ha^{-1}).

Table 4: Effect of moisture stress at different growth stages on grains per panicle of upland 'NERICA' rice inoculated with AMF

Treatments	Vegetative phase	Reproductive phase	Grain-filling phase
Stress status			
unstressed	298	292	290
stressed	221	70	48
	*	**	**
SED±	32.80	27.90	25.80
AMF			
Without AMF	394	243	231
With AMF	125	120	108
	**	**	**
SED±	32.80	27.90	25.80
Varieties			
NERICA 1	248b	186	170b
NERICA 2	373a	283	275a
NERICA 3	292ab	169	158b
NERICA 4	176b	135	126b
WAB 56-104	237b	167	136b
Moroberekan	229b	148	149b
	**	NS	*
Stress status × AMF	NS	**	**
Stress status × Varieties	NS	*	*
AMF × Varieties	NS	NS	NS
Stress status × AMF × Varieties	NS	NS	NS

Means with the same alphabets are non-significantly different using Duncan Multiple Range Test (DMRT) at 5 % level of significant. * means significant at 5 % probability level, ** means significant at 1 % probability level.

Table 5: Effect of moisture stress at different growth stages on 100 grain mass (g) of upland 'NERICA' rice inoculated with AMF

Treatments	Vegetative phase	Reproductive phase	Grain-filling phase
Stress status			
unstressed	3.22	4.47	3.37
stressed	2.63	1.82	1.56
	**	**	**
SED±	0.11	1.19	0.11
AMF			
Without AMF	2.50	1.68	1.45
With AMF	3.34	4.61	3.48
	**	*	**
SED±	0.11	1.19	0.11
Varieties			
NERICA 1	3.23a	2.40	2.44bc
NERICA 2	2.88a	2.46	2.57b
NERICA 3	3.22a	2.28	2.18bc
NERICA 4	3.02a	3.43	3.08a
WAB 56-104	2.82a	6.12	2.42bc
Moroberekan	2.36b	2.16	2.13c
	**	NS	**
Stress status × AMF	**	NS	*
Stress status × Varieties	NS	NS	NS
AMF × Varieties	*	NS	NS
Stress status ×AMF × Varieties	NS	NS	NS

Means with the same alphabets are non-significantly different using Duncan Multiple Range Test (DMRT) at 5 % level of significant. * means significant at 5 % probability level, ** means significant at 1 % probability level.

Table 6: Effect of moisture stress at different growth stages on yield per plant (g/plant) of upland 'NERICA' rice inoculated with AMF

Treatments	Vegetative phase	Reproductive phase	Grain-filling phase
Stress status			
unstressed	18.32	18.60	18.24
stressed	13.56	8.47	7.01
	**	**	**
SED±	0.99	0.91	1.05
AMF			
Without AMF	10.29	6.13	5.96
With AMF	21.60	20.94	19.29
	**	**	**
SED±	0.99	0.91	1.05
Varieties			
NERICA 1	12.66	15.46ab	14.11b
NERICA 2	16.71	14.30b	18.50a
NERICA 3	17.44	8.90c	8.47d
NERICA 4	17.17	18.14a	11.62bcd
WAB 56-104	16.02	14.16b	12.97bc
Moroberekan	15.65	10.24c	10.07cd
	NS	*	NS
Stress status × AMF	NS	NS	NS
Stress status × Varieties	NS	*	**
AMF × Varieties	**	*	NS
Stress status × AMF × Varieties	NS	*	NS

Means with the same alphabets are non-significantly different using Duncan Multiple Range Test (DMRT) at 5 % level of significant. * means significant at 5 % probability level, ** means significant at 1 % probability level.

Significant varietal variability was observed on all the yield components and grain yield ha^{-1} on the field (Table 7). Most 'NERICA' rice together with 'WAB 56-104' had no significant differences on panicle length except 'NERICA 4' that had the least panicle length (19.66 cm) that was not significantly different from 'Moroberekan'. 'Moroberekan' had significantly the least grain per panicle (74.70), 100 grain mass (2.50 g) and grain yield ha^{-1} (1.22 t ha^{-1}). 'NERICA 1' and 'NERICA 2' had significantly higher grain per panicle, 100 grain mass and grain yield ha^{-1} than other varieties. Other 'NERICAs' occupied intermediate position for yield and components.

Table 7: Effect of nutrient sources on yield and its components of upland NERICA rice

Treatments	Panicle length (cm)	Grain/panicle	100 grain mass (g)	Grain Yield (t/ha)
Nutrient Sources				
AMF	21.46c	80.70b	2.63b	2.51a
AMF+60 kg N + 30 kg K	25.92a	106.60a	3.07a	2.54a
60 kg N + 30 kg K	23.82b	81.30b	3.07a	2.41a
CONTROL	17.48d	91.20b	2.69b	1.53b
	*	*	**	**
Varieties				
NERICA 1	22.64ab	102.9a	3.07a	2.89a
NERICA 2	24.09a	98.40ab	2.72ab	2.90a
NERICA 3	22.66ab	92.30abc	2.83ab	2.53b
NERICA 4	19.66c	86.40bcd	2.83ab	2.19c
WAB 56-104	23.13a	85.00cd	2.83ab	1.68d
Moroberekan	20.83bc	74.70d	2.50b	1.22e
	**	**	*	**
Varieties × Nutrient sources	**	**	NS	NS

Means with the same alphabets are non-significantly different using Duncan Multiple Range Test (DMRT) at 5 % level of significant. * means significant at 5 % probability level, **means significant at 1 % probability level.

4 DISCUSSION

Results obtained in this investigation indicated a reduction in yield attributes of lowland rice cultivars subjected to soil moisture stress at different growth stages. Response of crop to stress is dependent on its intensity, timing and duration (Bray, 1997; Robertson and Holland, 2004). The pattern observed in the yield attributes investigated indicated that the most pronounced depression was noticed when soil moisture stress was imposed at the reproductive growth stage. This observation is consistent with earlier observations (Liu et al., 2006), where it was noticed that the most susceptible growth stage of rice to abiotic stressor is at this stage. AMF colonisation of the host rice plant under soil moisture deficit at all growth stages was similar with unstressed rice. This pattern of response to water deficit among all the cultivars of rice investigated could have suggested that they must have devised other mechanisms to ameliorate the negative impact of water deficit on them. Most crops were able to avoid soil moisture stress by increasing the volume of soil they could capture water from through increased root volume. Hyphae of AMF had been reported to increase root volume of crops under moisture deficit (Koide, 1993). Similar pattern was also observed here albeit not significant. Other reasons could be the condition where the AMF inoculation was carried out in the pot which could not preclude the possibility of confounding effects of other microbes in the screen house. Significant reduction in grain yield per plant under soil moisture deficit could have been attributed to the reduced yield components of upland rice cultivars.

All the yield attributes of upland rice were significantly affected by inoculation with AMF when stressed at different growth stages except panicle length when soil moisture stress was imposed at vegetative growth stage. This positive effect of AM inoculation on all the yield attributes of upland rice cultivars subjected to soil moisture stress at different growth stages could have contributed to the significant effect of AMF inoculation on grain yield per plant. Earlier reports had indicated a compensatory relationship between number and mass of grains under abiotic stressors (Squire, 1990). The mass of kernel is mostly conserved at the expense of the reduction in its number. This experiment was able to indicate that percentage reduction in number of grains per panicle was more pronounced when upland rice cultivars were subjected to soil moisture stress at vegetative growth stage than subsequent growth stages irrespective of inoculation status. It is possible that vegetative growth stage is a period when the reproductive structures were established or alternatively incidence of water stress at vegetative growth stage would compromise dry matter (Boonjungand & Fukai, 1996) that would later be remobilized when reproductive structures would be established. Other explanation could be that moisture stress at this growth stage would reduce amount of assimilate that would be sufficient for the host plant and the fungi. In the presence of competition for assimilates at this growth stage establishment of reproductive growth structure would be grossly compromised subsequently. These speculations need further empirical evidences to establish its validity. Increase in grain yield per plant when upland rice cultivars were subjected to soil moisture stress at reproductive and grain filling growth stages were similar in treatments with and without the AMF inoculation. This is consistent with our earlier suggested argument on the availability of assimilates for host-fungi interaction and colonisation. Subsequent incidence of water deficit would be offset by the exploration of soil volume by the fungi hyphae to improve crop water status. AMF response to soil fertilization is dependent on the soil nutrient gradient and host nutrient status (Treseder and Allen, 2002). Several studies had been conducted in the past on the effect of different combinations, especially NPK on AMF colonization (Hepper, 1983; Rajeshkannan et al., 2009; Treseder and Allen, 2002). One common trend was that increased P fertiliser depresses AMF root colonisation (Jensen and Jakobsen, 1980). Conversely N and K fertilization induces inoculation potential (Furlan and Bernier-Cardou, 1989). However, combination of N and K on inoculation potential varies with the host plant and fertility status of the soil (Treseder and Allen, 2002). The fertility status of the soil under which the investigation was conducted would be referred to as low according to the fertility classification criteria for Nigerian soils proposed by (Adepeju et al., 2015). The only nutrient that was in sufficient quantity among the macronutrients was P, which

could have justified the choice of treatment combinations. In this trial on the field, it was observed that combination of AMF + 60 kg N + 30 kg K resulted in significantly higher yield attributes of upland rice cultivars than other combinations in this trial. This was subsequently reflected in the grain yield ha^{-1}. Report had earlier indicated that symbiotic association of AMF with host plant is capable of increasing sink strength of the host through simulation of assimilate export to the fungi and increased carbon assimilation (Kaschuk et al., 2009). K was also cited in the literature to be involved in the production (Liebhardt, 1968; Trolldenier, 1972) and transportation (Epstein, 1972) of the assimilate to the fungus. This could increase root colonisation and alter other physiological processes of the inoculated crop positively. N was implicated in increasing reducing sugar, amino acid composition and infectivity by the fungi (Ratnayake et al., 1978). This combination of AMF with inorganic N and K could have acted synergistically in this agroecology to simulate yield attributes of upland rice cultivars. However, significant differences in grain yield were not observed among the treatments except control with significantly lower grain yield than others. From a sustainable production of lowland rice perspective, it would appear justifiable to apply only AMF to increase grain yield in this agroecology. Since a comparable yield could be obtained when other more economically and environmentally unsustainable nutrient combinations are used.

Significant varietal differences were observed on the percentage root colonization when upland rice cultivars were subjected to soil moisture stress at all growth stages in the screen house. Similar pattern was observed on the field. 'NERICA 4' displayed significantly higher root colonisation than other upland rice cultivars when subjected to soil moisture stress at all growth stages in both trials. This response pattern in 'NERICA 4' could have predisposes it towards better acclimation to soil moisture stress at all growth stages. Root colonization by AM could extend the root volume and increase the uptake of available water at lower soil depth (Osonubi et al., 1992). This observation could be comparatively advantageous at the most sensitive period to water deficit (reproductive and grain filling growth stages), as reflected in the significantly longer panicle and 100 grain mass observed in 'NERICA 4' than other upland rice cultivars when subjected to soil moisture stress at grain filling stage. However this improved yield attributes could only translate to significantly higher grain yield per plant observed in NERICA 4 cultivar than others when subjected to soil moisture deficit at the reproductive growth stage. It could be hypothesized that other factors could be limiting grain yield per plant when soil moisture stress was imposed at other growth stages. A significantly higher grain yield per plant observed in 'NERICA 2' when subjected to soil moisture stress at grain filling stage could have been attributed to higher number of grains per panicle when stressed at vegetative and grain filling stages and significantly longer panicle when stressed at vegetative growth stage compared to other upland rice cultivars. This finding was validated on the field despite the similarities in the yield components among the upland rice varieties except number of grains per panicle, which was observed to be significantly higher in 'NERICA 1' than others.

5 CONCLUSION

Across all growth stages, yield and its components were higher ($P< 0.05$) in upland rice sown in unstressed than water stressed condition. Conversely % AM colonisation of upland rice experienced a non-significant increase under water stress condition. In both trials AM colonisation was higher ($P< 0.05$) in inoculated upland rice than non-inoculated ones. In the screen house inoculated upland rice had higher ($P< 0.05$) grain yield per plant and its components than non-inoculated, except number of grains per panicle when stressed across all growth stages. On the field combination of AMF + 60 kg N ha^{-1} + 30 kg K ha^{-1} produced higher ($P< 0.05$) reproductive growth. Varietal variability ($P< 0.05$) was observed on AM colonisation and reproductive growth in both trials, with 'NERICA 2' been most promising in terms of grain yield ha^{-1}.

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Effect of irrigation with nutrient solutions mixed with treated wastewater on Asiatic lily 'Brunello' grown in a closed soilless culture

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ABSTRACT

A plastic greenhouse study was implemented to evaluate the potential use of treated wastewater for irrigation of Asiatic lily 'Brunello' grown in zeolite. Plants received the following treatments: a nutrient solution (N) alone, (N) mixed with treated wastewater (W) at rates of 3N:1W, 1N:1W and 1N:3W respectively. A closed system was used in which the drain solution was circulated for several days until its electrical conductivity reached 2.3 dS m^{-1}, after which fresh irrigation solutions were used to start a second cycle of circulation. The results indicated that plants irrigated with (N) or 3N:1W solution had the longest stems (34.4 and 36.2 cm) respectively, peduncles and buds (about 4.5 cm), and the greatest shoot (3.4 and 3.8 cm) and bud mass (14.95 and 17.6 g) respectively. Plants irrigated with 1N:3W solution had the highest dry mass tissue content of K (3.06 g kg^{-1}) and B (35.5 mg kg^{-1}). Plants irrigated with 1N:1W or 1N:3W were inferior to other plants. It can be concluded that 1N:3W mixture may be used for production of high quality cut flowers of lily. Moreover, it is expected to save 3.4 litres m^{-2} of a nutrient solution and 1850, 347 and 1870 mg m^{-2} day^{-1} for N, P, and K respectively.

Key words: *Lilium*; irrigation; wastewater; zeolite; nutrient solution; drainage solution recycling; JUST

IZVLEČEK

UČINKI NAMAKANJA Z MEŠANICO HRANILNE RAZTOPINE IN ODPADNE VODE NA AZIJSKO LILIJO 'Brunello' GOJENO V ZAPRTEM BREZTALNEM SISTEMU

V raziskavi, izvedeni v plastenjaku, je bila ovrednotena potencialna uporaba odpadne vode za gojenje azijskih lilij 'Brunello' gojenih na zeolitu. Opravljena so bila naslednja obravnavanja: uporaba čiste hranilne raztopin (N), mešanica hranilne raztopine (N) z odpadno vodo (W) v razmerjih 3N:1W, 1N:1W in 1N:3W. Uporabljen je bil zaprt sistem, v katerem je raztopina krožila več dni, dokler njena električna prevodnost ni dosegla 2,3 dS m^{-1}, potem je bila uporabljena sveža raztopina za začetek drugega kroga poskusa. Rezultati so pokazali, da so imele rastline, ki so bile namakane samo s hranilno raztopino (N) ali z mešanico 3N:1W, najdaljša stebla (34,4 in 36,2 cm), cvetne peclje in popke (okrog 4,5 cm), največjo maso poganjkov (3,4 in 3,8 cm) in popkov (14,95 in 17,6 g). Rastline, ki so bile zalivane z raztopino 1N:3W, so imele največjo suho maso tkiv, največjo vsebnost K (3,06 g kg^{-1}) in B (35,5 mg kg^{-1}). Rastline, ki so bile namakane z mešanicami raztopin 1N:1W in 1N:3W so bile slabše v primerjavi s prejšnjimi. Zaključili bi lahko, da bi za pridelavo zelo kvalitetnega rezanega cvetja azijskih lilij lahko uporabili mešanico raztopin 1N:3W in s tem prihranili 3,4 l m^{-2} hranilne raztopine in 1850, 347 in 1870 mg m^{-2} dan^{-1} N, P in K.

Ključne besede: *Lilium*; namakanje; odpadna voda; zeolit; hranilna raztopina; recikliranje raztopine; JUST

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1 INTRODUCTION

Closed soilless culture is used to conserve water, increase fertilizer use efficiency, and reduce nutrient leaching into the environment. However, continual use of the drain solution in closed cultures may result in increased salinity in the circulating solution (Baas et al., 1995; Karam and Al-Daood, 2005; Sonneveld and Van der Burg 1991; Sonneveld et al., 1999) and nutritional imbalances (Savvas, 2001). So, management of agricultural water becomes very important (Maryam et al., 2015; Mohammad, 2012a; Mohammad, 2012b; Stavros et al., 2015). To reduce consumption of potable water in agriculture, treated wastewater has been used as an alternative source of irrigation water which may also compensate for partial fertilizer requirements (Karam et al.,2009) and provide adequate amount of nutrients (Khan et al., 2009) for some plants. Treated wastewater has shown to have no adverse effect on rose (*Rosa hybrida*) (Bernstein *et al.*, 2006) and to improve quality and yield of ornamental plants including croton (*Codiaeum variegatum* Blume) (Karam et al., 2006), lily (*Lilium '*Aziatische Group ') (Safi et al., 2007a) and gerbera *(Gerbera jamesonii* Bolus ex Hooker f.*)* (Damasceno et al., 2010). However, treated wastewater was found to increase salinity of soil (Hussain et al., 2002; Safi et al., 2007b).

Salinity affects plant growth through osmotic potential of the soil solution, nutritional imbalance and/or specific ion effects (Shannon, 1998). Nutritional imbalances are related to nutrient availability, competitive uptake, and transport or partitioning within the plant (Grattan and Grieve, 1994). In fact, salinity was reported to reduce growth and yield of cut flowers (Baas et al., 1995; Sonneveld and Van der Burg, 1991) including lily (Daood and Karam, 2007; Karam and Al-Daood, 2005; Morgan, 2006; Sonneveld et al., 1999).

To overcome salinity issues, zeolites, a microporous aluminosilicate mineral, has been commonly used as growing medium in plant production. Zeolites have the ability to mitigate the salt damage of plants by exchanging Na in water for Ca of the zeolite (Yasuda et al., 1998). So, they may be used when saline water is used for irrigation. Occlusion of NaCl from aqueous solutions occurs in zeolite since the surface conductivity of zeolite increases in salt solutions due to the ion pairs of salt adsorbed onto the hydroxyl groups of the zeolite surface (Breck, 1974), consequently, preventing excessive Na uptake by the plant and reducing its toxicity (Qian et al., 2001). Incorporation of zeolitic tuff into peat-perlite was recommended to reduce substrate EC and to offset the adverse effect of salinity associated with treated wastewater, rendering the substrate more favourable for growth of croton (Karam et al., 2006; Karam et al., 2009).

Closed soilless culture was reported to reduce water and fertilizer use for Asiatic lily 'Zsa Zsa' in zeollitic tuff without affecting plant performance (Karam and Al-Daood, 2005). Treated wastewater has also been used for cut flowers in open soilless culture (Safi et al., 2007b) and in soil (Safi et al., 2005; Safi et al., 2007b). However, there are no documented studies on irrigation of hybrid lily with treated wastewater in a closed soilless culture. Using treated wastewater as an alternative source of irrigation water would minimize the use of fresh water. The objective of the current study is to evaluate the potential use of treated wastewater for irrigation of Asiatic lily 'Brunello' in a closed soilless culture.

2 MATERIALS AND METHODS

The experiment was conducted in Jordan University of Science and Technology-Jordan (JUST), inside a plastic greenhouse with average daily temperature of 25±3 ºC, relative humidity (RH) of 35 %, photosynthetic photon flux density (PPFD) of 97 μmol m^{-2} s^{-1}, and natural photoperiod.

Sixteen growing beds (3 m length x 0.30 m width x 0.25 m depth each) were constructed from polyethylene (1000 μ) and were placed on the ground at a slope of 1-2 %. A screen filter was fitted at the end of each bed to prevent passage of the substrate particles out of the bed. Each bed was filled with two layers of black zeolitic tuff, which

consisted of 5 % zeolite minerals, 70 % basalt volcanic glass and 25 % silicate minerals, with 15 meq 100 g^{-1} CEC, 6.8-7.2 pH and 0.8-1.2 dS m^{-1} EC. The lower layer (5 cm depth) consisted of coarse particles (8-16 mm), which contained (%) 1.0 NH_4, 1.5 P_2O_5, 2.0 K_2O, 7.0 CaO, 5.0 MgO, 1.0 SO_3, 0.1 Na_2O, 14.1 Al_2O_3, 43.3 SiO_2, 2.0 TiO_2, 12.0 H_3O^+, 9.9 FeO, 0.9 MnO and (ppm) 150 Zn, 200 Cu, 100 Mo, 40 B and 0.2 As. The upper layer (15 cm depth) consisted of fine particles (< 3 mm), which contained (%) 0.31 P_2O_5, 0.81 K_2O, 11.44 CaO, 7.08 MgO, 0.8 Na_2O, 13.17 Al_2O_3, 46.98 SiO_2, 1.95 TiO_2, 12.64 Fe_2O_3 and 0.15 MnO. One layer of plastic mesh (10 x 10 cm) was laid on the bed surface before planting to support the plants and prevent lodging.

Asiatic lily 'Brunello' bulbs (12-14 cm circumference) were planted 10 cm deep in each growing bed at 25 x 25 cm planting space. The bulbs were irrigated with tap water for two weeks before starting the treatments. The experiment was conducted in a completely randomized design with four replicates (growing beds) per treatment and 24 plants per bed.

A nutrient solution (N) was prepared by diluting 2 l of each of three stock nutrient solutions in 400 l tap water. The first stock solution contained (per liter) 112.5 g KNO_3, 31.5 g $(NH_4)_2SO_4$, 0.49 g $ZnSO_4$, 0.48 g $MnCl_2$ and 0.035 g $CuSO_4$, the second contained 112.5 g KNO_3 and 6.1 g $Fe(NO_3)_3$. $9H_2O$ and the third contained 30.12 ml H_3PO_4 and 116.65 g $Ca(NO_3)_2$. The treatments consisted of four irrigation solutions: the nutrient solution (N), singly or mixed with secondary treated wastewater (W) at rates of 3N:1W, 1N:1W or 1N:3W. Tap water had EC of 1.27 dS m^{-1}, pH 8.2 and treated wastewater had EC of 2.48 dS m^{-1} and pH 7.9. Chemical analysis of the irrigation solutions is presented in Table 1.

The irrigation solutions were injected separately using a submerged pump in 400 l tank per treatment into a GR drip irrigation system with 8 drippers per growing bed and 4 l h^{-1} dripper discharge. The plants were automatically irrigated with the irrigation solutions three times a day at 8.00, 12.00 and 18.00 o'clock for 15 min each. At the end of the day, the drain solutions from the four replicate beds of each treatment were collected, mixed and the volume was measured. The drain solution was then returned to the corresponding tank to be mixed with the irrigation solution to form the supply solution which was circulated the next day. The EC and pH of the supply and drain solutions were measured once daily using a manual EC meter (Omega TDH-5031, USA) and pH meter (Omega PHH-5012, USA). Irrigation scheduling and drainage management were repeated daily until the drain solution reached a threshold EC (2.3 dS m^{-1}), after which the supply solution in the tank was replaced with a fresh irrigation solution to start a new cycle of circulation. This strategy was adopted until the end of the experiment. The volume of the circulating solution supplied to each treatment / day / m^2 was calculated as follows: [4 growing beds * 8 drippers * 4 l h^{-1} dripper discharge * 45 min irrigation duration] / [4 growing beds * 3 m long * 0.3 m width] = 26.7 l.

At the end of the experiment (40 days after planting), the plants were harvested when the first flower was fully coloured, but not yet open. The plants were dug out and the tuff particles around the roots were carefully removed keeping the root system intact. Data were recorded on stem length (from bulb tip to peduncle base) and diameter, leaf number, root length (from bulb base to the longest root tip), shoot (stem and leaves) and root fresh and dry (oven dried at 70 ºC for 48 h) mass, peduncle length and bud number, length and mass. Tissue analysis was performed for determination of concentrations of macro, micro and heavy metal elements.

Table 1: Chemical analysis of the irrigation solutions prepared from a nutrient solution (N), singly or mixed with treated wastewater (W) at 3:1, 1:1 or 1:3

Variables	Irrigation solution treatments			
	N	3N:1W	1N:1W	1N:3W
EC (ds m^{-1})	1.65	1.73	1.91	2.12
pH	6.58	7.72	7.92	8.01
N (ppm)	184	144	105	65
P (ppm)	34.5	26.6	18.7	10.8
K (ppm)	186	154	100	79
Ca (ppm)	126	113	99	86
Mg (ppm)	61	57	53	48
Na (ppm)	137	211	284	358
Cl (ppm)	230	316	403	489
S (ppm)	181	212	242	272
Fe (ppm)	1.69	1.27	0.85	0.42
Zn (ppm)	0.22	0.17	0.11	0.06
Mn (ppm)	0.27	0.2	0.14	0.07
Cu (ppm)	0.02	0.015	0.01	0.005
B (ppm)	0.35	0.44	0.54	0.63

The leaves were dried and ground using a laboratory mill (Thomas Scientific, USA) to pass through a 0.5-mm sieve. The leaves were analysed for total nitrogen using Kjeldahl digestion (Nelson and Sommers, 1982). The remaining ground leaves were subjected to dry ash digestion at 550 °C for 2 h. The cooled ash was moisturized in 3-4 drops of diluted HNO_3 (1:1) on a heating plate until HNO_3 completely evaporated. The aliquot was used to determine concentration of P by ammonium molybdate-vandate method (Chapman and Pratt, 1961) using a spectrophotometer (Genesys 10, USA). Analysis of K, Ca, Mg, Na, Cl, Fe, Zn, Mn, Cu and B was performed as described by the Association of Official Analytical Chemists (Horwitz, 2000). The remaining ash was dissolved in diluted HCl (1:1) and put in lanthanum and lithium solutions. The aliquots were analysed for K and Na using a flame photometer (Sherwood Scientific Ltd 410, UK) and Ca, Mg, Fe, Zn, Mn, Cu and B by atomic absorption spectroscopy (Varian model, Agilent Technologies Co., USA). Chloride was determined by titration using 0.05N $AgNO_3$ (Richards, 1954.). Concentrations of Cd, Cr, Ni and Pb were determined using microwave digestion system (Microwave Digestion Lab Station MD 01, Milestone S.r.L., Italy) and Solar 969 Atomic Absorption spectroscopy (TJA Solution co., UK) (Rechcigl and Payne, 1990).

Net daily water consumption was calculated according to the following equations:

First equation: $A = B - D$
Where A is the net water consumption (m^3), B is the quantity of applied water (m^3), and D is the quantity of drain water (m^3).

Second equation: $Y = A \times P \times C$
Where Y is the quantity of the nutrient taken by plant from treated wastewater (g), A is the net water consumption (m^3), P is the percentage of wastewater in nutrient solution, and C is the concentration of a certain nutrient (N, P, and/or K) (g m^{-3}).

Third equation: $WUE = BY / A$
Where WUE is water use efficiency (kg m^{-3}), BY is the biological yield (shoots and flowers) (kg), and A is the net water consumption (m^{-3}). In all calculations, the volume of discharged solutions after being replaced with fresh ones was not considered.

In addition, biomass water use efficiency (WUE) was calculated for shoot fresh mass and flower mass (bud number x bud mass) for each treatment as follows: average shoot fresh mass or flower mass per plant at harvest / total volume of irrigation solution supplied per plant from the first day of treatment application until harvest.

2.4 Statistical analysis

Data were subjected to analysis of variance by the General Linear Models procedure using SAS (Statistical Analysis System, version 9.1, 2002). Mean comparison was performed using the Least Significant Difference (LSD) method at $P \leq 0.05$.

3 RESULTS AND DISCUSSION

3.1 Plant growth and flowering

Stem diameter, leaf number, root length or bud number was not affected by the irrigation solution (Table 2). With the exception of root mass, all other parameters were significantly greater in plants irrigated with N or 3N:1W solution than in plants irrigated with 1N:1W or 1N:3W solution (Table 2, Fig. 1). Plants irrigated with 1N:3W solution had the least root fresh and dry mass.

Table 2: Growth and flowering of Asiatic lily 'Brunello' as influenced by irrigation with different ratios of a nutrient solution (N) to treated wastewater (W) in a closed soilless culture

Variables	Irrigation solution treatments [z]			
	N	3N:1W	1N:1W	1N:3W
Stem length (cm)	34.4 ab	36.2 a	32.7 b	32.9 b
Stem diameter (mm)	9.88NS	9.13 NS	9.5 NS	9.48 NS
Leaf number	109 NS	107 NS	109 NS	109 NS
Shoot fresh mass (g)	30.1 a	32.4 a	27.0 b	26.5 b
Shoot dry mass (g)	3.4 ab	3.82 a	3.14 b	3.05 b
Root length (cm)	12.53	12.19	12.34	12.29
Root fresh mass (g)	3.4 a	3.6 a	3.2 a	2.8 b
Root dry mass (g)	0.208 b	0.251 a	0.237 ab	0.150 c
Peduncle length (cm)	4.25 ab	4.82 a	3.52 c	3.85 bc
Bud number	3.4 NS	3.5 NS	3.3 NS	3.3 NS
Bud length (cm)	4.27 ab	4.42 a	3.48 c	3.68 bc
Bud mass (g)	14.95 a	17.62 a	10.93 b	11.83 b

[z] Means within rows having different letters are significantly different according to LSD ($P \leq 0.05$). Values are average of 10 plants. NS means non-significant within rows.

Figure 1: Growth of Asiatic lily 'Brunello' as influenced by irrigation with different ratios of a nutrient solution (N) and treated wastewater (W).

3.2 Plant tissue analysis

Leaf analysis revealed that concentrations of only K, Na, Fe and B were affected by the irrigation solution (Table 3). Plants receiving N, 3N:1W or 1N:3W solution had the highest concentrations of K (3.28, 3.23 or 3.06 g kg^{-1} dry mass, respectively) and B (75.4, 64.4 or 53.5 mg kg^{-1} dry mass, respectively). Furthermore, the highest level of Na (0.90, 0.94 or 0.99 g kg^{-1} dry mass) was detected in plants irrigated with 3N:1W, 1N:1W or 1N:3W, respectively. Plants irrigated with 3N:1W or 1N:3W had the highest level of Fe (862 or 694 mg kg^{-1} dry mass). There was no significant effect of the irrigation solution on tissue content of the heavy metal Cd, Cr, Ni or Pb (Table 3).

Table 3: Concentrations of elements in the leaves of Asiatic lily 'Brunello' as influenced by irrigation with different ratios of a nutrient solution (N) to treated wastewater (W) in a closed soilless culture.

Variables	Units	Irrigation solution treatments [z]			
		N	3N:1W	1N:1W	1N:3W
N	(g kg^{-1} dry mass)	2.64	2.53	2.62	2.71
P		0.36 NS	0.32 NS	0.29 NS	0.25 NS
K		3.28 a	3.23 a	2.87 b	3.06 ab
Ca		0.89 NS	1.23 NS	0.98 NS	0.98 NS
Mg		0.35 NS	0.39 NS	0.35 NS	0.38 NS
Na		0.78 b	0.90 ab	0.94 a	0.99 a
Cl		0.58 NS	0.7 NS	0.55 NS	0.58 NS
Fe	(mg kg^{-1} dry mass)	564 b	862 a	542 b	694 ab
Zn		56.2 NS	50.1 NS	54.3 NS	57.8 NS
Mn		28.6 NS	28.3 NS	25.3 NS	29.1NS
Cu		9.97 NS	8.36 NS	8.15 NS	8.72 NS
B		75.4 a	64.4 a	31.7 b	53.5 ab
Cd		0.02 NS	0 NS	0 NS	0 NS
Cr		0.3 NS	0.35 NS	0.46 NS	0.35 NS
Ni		0.65 NS	0.73 NS	0.7 NS	0.68 NS
Pb		0.007 NS	0.007 NS	0.003 NS	0.002 NS

[z] Means within rows having different letters are significantly different according to LSD ($P \leq 0.05$). NS means non-significant within rows.

3.3 Electrical conductivity and pH of the supply and drain solutions

Two cycles of recirculation of the irrigation solutions were performed throughout the experiment (Fig. 2-5). At the start of the first cycle, the solutions N, 3N:1W, 1N:1W and 1N:3W had EC of 1.65, 1.73, 1.91 and 2.12 dS m^{-1} and pH of 6.58, 7.72, 7.92 and 8.01, respectively (Table 1). At the start of circulation, the drain solutions from beds receiving N, 3N:1W, 1N:1W or 1N:3W had EC of 1.62, 1.68, 1.87 or 2.09 dS m^{-1} and pH of 6.65, 7.79, 7.98 or 8.07, respectively. The threshold EC (2.3 dS m^{-1}) of the drain solutions for the treatments N, 3N:1W, 1N:1W and 1N:3W was attained after 18, 17, 15 and 12 days of circulation, respectively. In the second cycle, the threshold EC was attained (after 13 days) only when 1N:3W was used because the experiment was terminated, thus was not long enough to reach the target EC when the other irrigation solutions were used. Throughout the experiment, the average EC of the supply solution was 1.76, 1.81, 1.96 and 2.09 dS m^{-1} (Fig. 2) and of the drain solution was 1.77, 1.82, 1.97 and 2.11 dS m^{-1} for the treatments N, 3N:1W, 1N:1W and 1N:3W, respectively (Fig. 3).

In both cycles, EC of all supply (circulating) and drain solutions decreased in the first few days of circulation, then increased and the solution 1N:3W and its drain had the highest EC followed by 1N:1W (Fig. 2 and 3). Moreover, EC values of the supply solution 3N:1W or its drain were not different from those of the solution N or its drain in the first cycle, but were higher in the second cycle. pH of supply (Fig. 4) and drain (Fig. 5) solutions increased in both cycles. At the end of the experiment, pH of the supply solutions N, 3N:1W, 1N:1W and 1N:3W was 7.22, 8.44, 8.52 and 8.97 and of the drain solutions was 7.29, 8.49, 8.57 and 9.05, respectively. In both cycles, the supply solution N and its drain had the lowest pH.

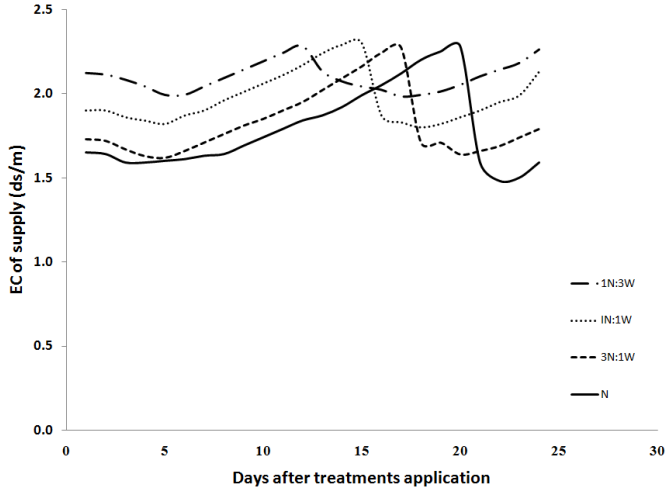

Figure 2: Changes in EC of the supply solution with time of circulation as influenced by irrigation Asiatic lily 'Brunello' with different ratios of a nutrient solution (N) and treated wastewater (W).

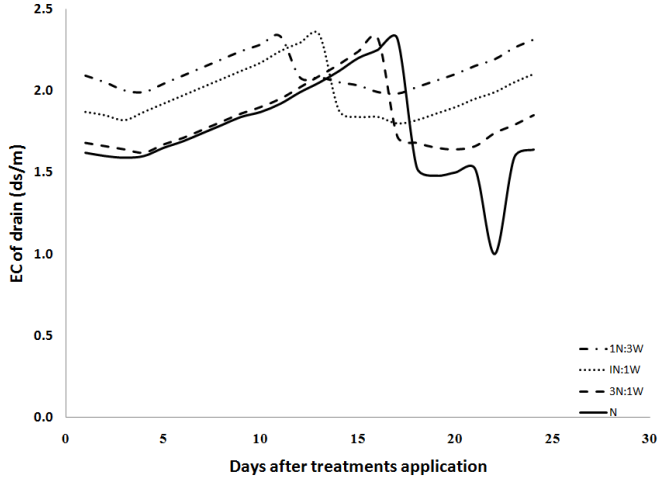

Figure 3 and Figure 2: Changes in EC of the drain solution with time of circulation as influenced by irrigation Asiatic lily 'Brunello' with different ratios of a nutrient solution (N) and treated wastewater (W).

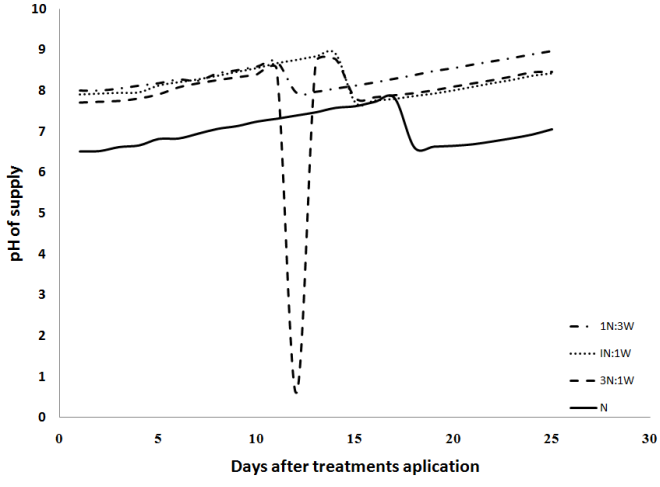

Figure 4: Changes in pH of the supply solution with time of circulation as influenced by irrigation Asiatic lily 'Brunello' with different ratios of a nutrient solution (N) and treated wastewater (W).

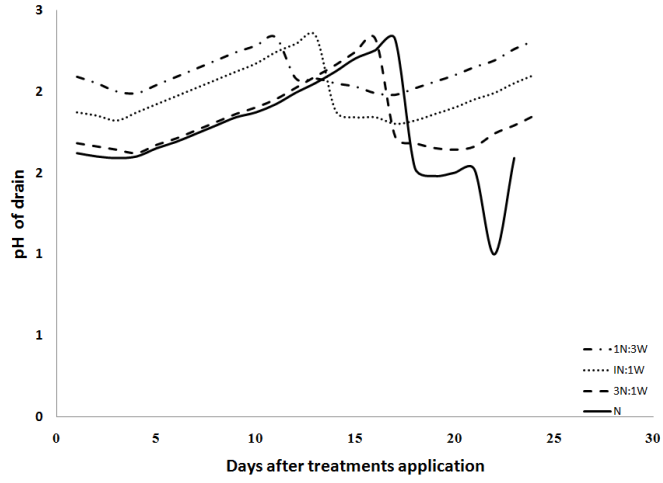

Figure 5: Changes in pH of the drain solution with time of circulation as influenced by irrigation Asiatic lily 'Brunello' with different ratios of a nutrient solution (N) and treated wastewater (W).

3.4 Savings in irrigation solutions and biomass water use efficiency

Savings in the nutrient solution N were 10.1 l m^{-2} day^{-1} and in the nutrients N, P and K were 1850, 347 and 1870 mg m^{-2} day^{-1}respectively. The highest savings were recorded when 1N:3W solution was used (Table 4). It was observed that biomass WUE was 1.16, 1.25, 1.04 or 1.06 kg m^{-3} for shoot fresh mass and 2.0, 2.37, 1.39 or 1.56 kg m^{-3} for flower mass, when N, 3N:1W, 1N:1W or 1N:3W solutions were used, respectively.

Table 4: Effect of irrigation of Asiatic lily 'Brunello' with different ratios of a nutrient solution (N) to treated wastewater (W) on average savings in the irrigation solutions and the nutrients N, P and K due to the use of (W)

Variables	Irrigation solution treatments [z]			
	N	3N:1W	1N:1W	1N:3W
Supplied irrigation solution (l m^{-2} day^{-1})	26.7 NS	26.7 NS	26.7 NS	26.7NS
Drainage (l m^{-2} day^{-1})	12.9	13.2	13.2	13.3
Consumed irrigation solution [u](l m^{-2} day^{-1})	13.7 NS	13.5 NS	13.4 NS	13.4NS
Consumed N solution [w](l m^{-2} day^{-1})	13.7 a	10.1 b	6.7 c	3.3 d
Saved (N) solution [x](l m^{-2} day^{-1})	0 d	3.4 c	6.7 b	10.1 a
Saved N [y] (mg m^{-2} day^{-1})	0 d	621 c	1236 b	1850 a
Saved P [y] (mg m^{-2} day^{-1})	0 d	116 c	232 b	347 a
Saved K [y] (mg m^{-2} day^{-1})	0 d	628 c	1249 b	1870 a

[u]Volume of supplied irrigation solution - volume of drainage.
[w]Volume of consumed irrigation solution * % (N) solution in irrigation solution.
[x]Volume of consumed irrigation solution * % (W) in irrigation solution.
[y]Amount of nutrient consumed using (N) solution - amount of nutrient consumed using the considered irrigation solution, which = consumed irrigation solution * proportion of (N) solution in the considered irrigation solution * concentration of the nutrient in (N) solution.
[z]Means within rows having different letters are significantly different according to LSD ($P \leq 0.05$).
NS means non-significant within rows.

Treated wastewater had potential as irrigation water for production of Asiatic lily, when mixed with potable water. However, high percentage of treated wastewater was a limiting factor. Mixing a nutrient solution, usually recommended for Asiatic lily, with treated wastewater at a ratio of 1:1 or 1:3 did not prove to be feasible since the plants exhibited growth retardation (Table 2). This may be attributed to exposure of the plants to salinity stress because of the high EC of the circulating solution (Fig. 2) and the drain solution (Fig. 3) around the roots. This is in agreement with studies which indicated that continual reuse of the drain solution in closed soilless culture usually increases EC of the circulating solution (Baas and Van den Berg, 1999; Bar-Yosef et al., 2001; Daood and Karam, 2007; Karam and Al-Daood, 2005). This effect was evident in the current study when the nutrient solution N was used alone for irrigation (Fig. 2). However, the problem of elevated EC was aggravated when 1N:1W or 1N:3W was used. This may be explained by the high concentrations of Na, Cl and HCO_3 in treated wastewater which rendered the initial EC of 1N:1W and 1N:3W high (1.91 and 2.12 dS m^{-1}, respectively) (Table 1) and may have resulted in accumulation of Na and Cl in the root zone (Karam et al., 2006; Karam et al., 2009). Plant growth is adversely affected under salt stress due to osmotically induced water stress, specific ion toxicity due to high concentration of Na^+ and Cl^-, nutrient ion imbalance due to high levels of Na^+ and Cl^- which reduce uptake of K^+, NO_3^- and PO_4^{3-} (Greenway and Munns, 1980).

Accumulation of Na in the circulating solution in closed soilless culture may lead to reduced level of K in the circulating solution (Karam and Al-Daood, 2005; Savvas and Manos, 1999; Sonneveld, 1981) and the plant (Savvas et al., 2009). Accumulation of Na is expected to be more significant when zeolite is used as a substrate due to adsorption of K ions by zeolite after replacing Na ions resulting in less availability of K and higher concentration of Na in the substrate (Williams and Nelson, 1997). The problem of Na accumulation is aggravated when saline water is used in closed cultures (Pardossi et al., 2006). Reduced K uptake by the plant is attributed to the competition between K and Na for the absorption sites of the roots (Rusan et al., 2003). In the current study, the suppressive effect of Na on K uptake was evident from the lower K and higher Na contents in plants irrigated with 1N:1W or 1N:3W compared to those irrigated with N or 3N:1W (Table 3). In fact, K concentration in plants irrigated with the former solutions was less than the sufficiency range reported for Easter lily (3.3-5.0 %) (Jones et al., 1991). It is obvious that the competition was advantageous to Na when 1N:1W or 1N:3W solution was used due to the high initial Na and low initial K contents in those solutions (Table 1).

In addition to the negative effect of Na on K acquisition by the roots, high levels of Na under saline-sodic conditions may disrupt the integrity of root membranes and alter their selectivity from K to Na (Grattan and Grieve, 1999). According to the authors, the selectivity of the root system for K over Na must be sufficient to meet the levels of K required for metabolic processes, ion transport regulation and osmotic adjustment which affect water uptake. Fulfilling the need of plants for K may be achieved by increasing concentration of K in the irrigation solution. In the present study, the high initial content of K in the irrigation solutions (N) and 3N:1W (Table 1) was apparently sufficient to increase selectivity of the roots for K over Na, resulting in increased K and reduced Na contents in plants irrigated with those solutions (Table 3).

Only the plants that were irrigated with 1N:3W exhibited reduced root fresh mass, implying reduced water content, probably due to elevated EC (2.31 dS m^{-1}) of the drain solution at the end of the experiment (Fig. 3). Those plants also exhibited reduced root dry mass implying reduced root mass, which occurs under salinity conditions (Shannon and Grieve, 1999), thus reduced water uptake. Results of the present study confirm findings of Karam and Al-Daood (2005) who reported reduction in root fresh mass of Asiatic lily 'Zsa Zsa' only when EC of the drain solution reached 2.2 dS m^{-1} and in root dry mass as EC increased up to 2.0 dS m^{-1}. Reduced water uptake by the plant may reduce Ca transport in the plant since the rate of transpiration is regarded as the main force for Ca transport to the leaves (Clarkson, 1984). However, retardation in growth of plants irrigated with 1N:1W or 1N:3W is not likely to be due limited Ca uptake since the plants were not significantly different from those irrigated with N

or 3N:1W solution with respect to Ca content in the leaves (Table 3).

Electrical conductivity of the supply and drain solutions for all treatments decreased in the first 6 days of circulation (Fig. 2 and 3), which may be attributed to the high ion adsorption and cation exchange capacity of zeolitic tuff (Mumpton, 1983). The pH of all supply and drain solutions increased with increasing EC (Fig. 4 and 5), which confirms findings of Karam and Al-Daood (2005). Increased pH in the root zone usually limits absorption of micronutrients. In the present study, pH of the drain solution increased above 8 for all treatments except for the solution (N), yet there was no effect of pH or EC on tissue contents of micronutrients other than Fe and B (Table 3), which may be due to the relatively short duration of circulation in each cycle. Our findings are in agreement with those reported for Asiatic lily 'Zsa Zsa' which revealed no effect of EC on tissue content of micronutrients except Mo (Karam and Al-Daood, 2005).

Tissue contents of only K, Na, Fe and B were significantly affected by the irrigation solution (Table 3). Similarity in tissue N content at all EC values implies lack of effect of EC on N uptake as was reported for Asiatic lily 'Zsa Zsa' (Karam and Al-Daood, 2005) with EC of 2.2 dS m^{-1} and petunia (*Petunia* × *hybrid* (Hook) Vilm.) and begonia (*Begonia* 'Semperflorens Cultorum Group') (James and van Iersel, 2001) even with EC of 3 dS m^{-1}. Tissue content of Fe for all treatments (Table 3) was substantially higher than the upper level of sufficiency range for Easter lily (60-200 ppm) (Jones et al., 1991), suggesting luxury consumption. Tissue contents of P, Ca, Mg, Zn, Cu and B for all treatments were within the sufficiency range for Easter lily (0.25-0.7 %, 0.6-1.5 %, 0.2-0.7 %, 20-200 ppm, 8-50 ppm and 25-75 ppm, respectively), whereas contents of N and Mn were lower than the sufficiency range (3.3-4.8 % N and 35-200 ppm Mn) (Jones et al., 1991).

Only plants irrigated with 3N:1W were comparable to those irrigated with (N) solution (Table 2). This may be explained by the similar EC conditions that the plants were exposed to for most part of the experiment (Fig. 2 and 3). Although the first cycle of circulation lasted for 18-19 days, the difference in EC between the two solutions or their drains was slight. In the second cycle, the difference in EC between the two solutions was large, but the cycle lasted for only 7-8 days. The threshold EC (2.3 dS m^{-1}) of the drain solution of the treatments N, 3N:1W, 1N:1W and 1N:3W was attained after 18, 17, 15 and 12 days of circulation, respectively (Fig. 3). Accordingly, using the solution 1N:3W the replacement with a fresh solution every 12 days was necessary, whereas using N or 3N:1W solution required replacement with new solutions after 17-18 days. Karam and Al-Daood (2005) were able to achieve a target EC of 2.2 dS m^{-1} after 20 days of circulation of a nutrient solution in a closed soilless culture of Asiatic lily 'Zsa Zsa'.

Stem diameter, leaf number, root length or bud number was not affected by the treatment (Table 2), indicating that EC up to 2.3 dS m^{-1} did not affect those parameters. This confirms results obtained by Karam and Al-Daood (2005) which revealed no change in leaf number in Asiatic lily 'Zsa Zsa' at EC 1.6-2.2 dS m^{-1} or in stem diameter or bud number at EC 1.8-2.2 dS m^{-1}. In the current study, the plants irrigated with 1N:1W or 1N:3W were exposed to EC above 1.8 dS m^{-1} throughout the experiment (Fig. 2 and 3) and exhibited reduced stem length, shoot fresh mass and bud mass (Table 2), and those irrigated with 1N:3W exhibited reduced root fresh and dry mass. This implies that 'Brunello' cultivar is quite sensitive to salinity as was reported for other cultivars of Asiatic lily. For example, Karam and Al-Daood (2005) reported reduced stem length and diameter, shoot fresh and dry mass, peduncle length and bud number in 'Zsa Zsa' lily as EC of the drain solution increased from 1.6 to 1.8 dS m^{-1} and reduced stem length, shoot dry mass and peduncle length as EC increased from 1.8 to 2.0 dS m^{-1}. Daood and Karam (2007) also demonstrated that a rise of 0.48 dS m^{-1} in EC was accompanied with reductions of 10 % in stem length, 22 % in shoot mass, 32 % in root or bud mass, 13 % in peduncle length and 18 % in bud length in 'Zsa Zsa' lily. Furthermore, Morgan (2006) reported that the ideal EC for lily production in hydroponic cultures is 1.2-1.8 dS m^{-1} and that higher EC levels resulted in slow growth, stunted plants and small flowers. Sonneveld et al. (1999) also reported that EC higher than 2.0 dS m^{-1} caused growth retardation in lily.

The results of this study can be expanded to other regions where ornamental plants are grown. This can be achieved when using soilless culture in a closed system and where treated wastewater is available. Since wastewater treatment plants exist almost everywhere, and plastic greenhouses are constructed widely in agricultural areas. Also, the soilless culture using zeolite has become very common. All these conditions make this study applicable to different regions and cultures. And good results seem to be achievable.

4 CONCLUSION

A nutrient solution mixed with treated wastewater at a ratio of 3:1 may be used to produce Asiatic lily 'Brunello' cut flowers in a closed soilless culture in which the plants are grown in zeolitic tuff and the drain solution is recycled until its EC reaches 2.3 dS m^{-1}. By using such a strategy, it is expected to save daily 3.4 l m^{-2} of the nutrient solution and achieve the highest biomass water use efficiency for shoot fresh mass and flower mass. This translates into savings in fresh water and fertilizers. This research is unique since it investigated for the first time the effect of using mixed treated wastewater on cut flowers. Also, the growing system was closed soilless culture using zeolite. And the most common growing activities in the study area use treated wastewater in open fields to grow forage crops not cut flowers. The advantages of this research can be summarized in using water efficiently in regions where scarcity of water is a major problem such as Jordan. Moreover, nutrients can also be saved while producing high quality cut flowers. Future studies are needed to navigate the efficiency of using similar irrigation management practice in open fields. Furthermore, research can be made to grow different cut flowers and other ornamental plants.

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Response of lowland rice-ratooned rice-fluted pumpkin sequence to fertilizer in rainfed inland valley in derived savannah of Nigeria

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ABSTRACT

Trial was carried out at Federal University of Agriculture, Abeokuta, Nigeria between 2010 and 2012 to determine response of lowland rice-ratooned rice-fluted pumpkin sequence to fertilizer. Experiment was laid out in Randomized Complete Block Design arranged in split-split plot in three replicates. Three rice genotypes constituted the main treatments sown in May and harvested in August. Split fertilizers application constituted sub-plot treatments were 90:45:45 (single dose), 45:22.5:22.5 & 45:22.5:22.5 (1:1), 30:15:15 & 60:30:30 (1:2) and 60:30:30 & 30:15:15 (2:1) NPK ha^{-1} at tillering and heading. N-fertilizer rates were sub-sub plot treatment and applied to ratooned 'NERICA L-42' had the tallest plants compare to others. 'Ofada' had the lowest number of days to 50 % flowering for main and ratooned rice, while 'NERICA L-42' had the highest number of days to 50 % heading. 'NERICA L-41' variety had the highest grain yield in main and ratooned rice. Based on this study, 'NERICA L-41' plus its ratooned rice obtained from single dose NPK and zero N-fertilizer plots produced grain yield of 4.69 t ha^{-1}.

Key words: rice; ratooned; triple cropping sequence; fertilizer; inland valley; Nigeria

IZVLEČEK

ODZIV NIŽINSKEGA KOLOBARJA RATONIRANEGA RIŽA IN KRILATE BUČKE NA GNOJILA V NENAMAKANEM NIŽAVJU ANTROPOGENE SAVANE V NIGERIJI

Poskus je potekal na Federal University of Agriculture, Abeokuta, Nigeria v rastnih sezonah med 2010 in 2012 z namenom ugotoviti odziv kolobarja ratoniranega riža in krilate bučke na gnojenje. Poskus je bil zasnovan kot popoln naključni bločni poskus, na ploskvah s tremi ponovitvami. Glavna obravnavanja so obsegala tri genotipe riža, ki so bili posejani maja in požeti avgusta. Gnojenje z enkratnim odmerkom NPK gnojil 90:45:45 je potekalo na podploskvah, ostala obravnavanja pa v kombinacijah 45:22.5:22.5 & 45:22.5:22.5 (1:1), 30:15:15 & 60:30:30 (1:2) in 60:30:30 & 30:15:15 (2:1) NPK ha^{-1} v fazi bilčenja in latenja riža. Gnojenja z dušikom pri obravnavanjih na podploskvah z ratoniranim rižem 'NERICA L-42' so dala najvišje rastline v primerjavi z drugimi. Sorta Ofada je potrebovala najmanjše število dni do 50 % cvetenja, sejanega in ratoniranega riža, 'NERICA L-42' pa je potrebovala največ dni do 50 % latenja. Sorta NERICA L-41 je imela največji pridelek zrnja pri sejanem in ratoniranem načinu pridelave. V raziskavi je bilo ugotovljeno, da je sorta NERICA L-41 pri običajni setvi in sledečem ratoniranem posevku pri enkratnem dodatku gnojil in brez dodatnega dognojevanja z N dosegla pridelek zrnja 4.69 t ha^{-1}

Ključne besede: riž; ratonirani posevek; trisetveni kolobar; gnojilo; notranje nižine; Nigeria

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1 INTRODUCTION

Rice is a staple food consumed by more than 3.5 billion people of the world based on the fact that it contains more energy (kcal/kg) than wheat (Muthayya et al., 2014). The straw serves as fodder for livestock, while rice bran is a good component in the preparation of poultry feed (FAO, 2003). Globally, rice production ranks third after wheat and maize whereas in Nigeria, it is the sixth major crop following sorghum, millet, cowpea, cassava and yam in terms of land area under cultivation (Akinbile, 2010). Rice represents a symbol of equally cultural identity and global unity, a main source of employment and livelihoods and also an increasingly important cereal crop with world annual production estimated at 738.2 million tonnes in 2015 during which Africa recorded an average of 28.5 million tonnes (FAO, 2003; FAO, 2016).

Rice production in Nigeria in 2006 was estimated at 2.10 million tonnes while consumption was 3.71 million mega grams. The balance of 1.60 million mega grams was obtained by importation (Africa Rice Center, 2008). Nigeria imports one million tonnes of rice, valued at $700 million or about N106 billion, from the Peoples Republic of Thailand every year (Sams, 2010). Currently, Nigeria is the second largest importer of rice (N356 billion per year i.e. approximately N1 billion per day) after Philippines in the World (ATA, 2011).

Ironically, Nigeria has the resource (abundant rainfed upland and inland valley) and management potential to produce enough rice to meet local and as well as for exportation (FAOSTAT, 2008). Lançon and Erenstein (2002) reported that out the total land area of 1.6 million ha devoted to rice cultivation, rainfed upland accounted for 492, 600 ha (30 %), rainfed lowland (wetland i.e. inland valleys and flood plains) for 788, 860 ha (48 %), irrigated lowland for 262, 720 ha (16 %), deep water floating for 82, 100 ha (5 %) and mangrove for 16,420 ha (1.0 %). Whereas World Bank (2006) reported that Nigeria has 3 million ha of inland valleys and flood plain (FADAMA) suitable for rice production. Consequently, with efficient utilization of land resources, there are two options available in order to reduce rice importation via; land intensification and extensification of the naturally abundant fadama.

Adigbo et al. (2007) examine the intensification of inland valley via triple cropping of lowland rice-upland rice-vegetable) within 3 year and reported that it was possible to grow 3 crops without reducing the yield of lowland rice. However, the upland rice component in the sequence decreased the overall benefit/cost ratio of triple cropping rather than increasing it. Hence, Adigbo et al. (2012a) report that ratooned rice was technically feasible technology to fit into the niche between lowland rice and fluted pumpkin. Rice ratooning is the practice of harvesting grain from tillers originating from the stubble of previously harvested crop and it enhances rice grain yield without increasing land area because it provides higher resources use efficiency per unit land area per unit of time (Jason et al., 2005). Ratooned rice crop was reported to be an economically viable technology capable of boosting rice production and consequently increasing the overall productivity of the inland valley in a lowland rice–rice–vegetable cropping sequence (Adigbo et al., 2012b).

The assertion that the continual inflow of nutrients from the adjacent uplands guarantees the inland valley sustainability (Mitsch and Gosselink, 1993) perhaps for one crop per annual but that may not be true for triple crops. Adigbo (2008) was of the opinion that there was the need for additional fertilizer to supplement the geological fertilization from the adjacent uplands. But how much of fertilizer to be applied at the critical growth stages to complement the geological fertilization need to be evaluated. Beside, how much residual fertilizer previously applied to lowland rice was available to ratooned rice. What quantity of N-fertilizer should be added to ratooned rice? Earlier studies reveal that judicious and proper use of fertilizers can markedly increase the yield and improve the quality of rice (Place et al., 1970). The profitability of rice production system depends on yields and inputs quantities (Moya et al., 2004). So, the appropriate fertilizer input that is not only for getting high grain yield but also for attaining maximum profitability. Earlier study by Adigbo et al. (2013) on fertilizer requirement for ratooned rice in lowland rice-ratooned rice-vegetable was in

sawah rice based technology. Sawah is leveled, bunded, puddled rice field with inlets and outlets to water control. Simple irrigation is involved and fixation of nitrogen by soil microbes under a submerged sawah systems could reach 20 – 200 kg/ha/year (Kyuma, 2003; Hirose & Wakatsuki, 2002). However, the current study was based solely on rainfall where water is not controlled and without power tiller for puddling. Resource constraint farmers who accounted for 75 % of food production in Nigeria may not be able to afford the cost of power tiller and labour to construct bunds required for sawah. The objectives of the study were to: (1) evaluate the yield and yield component of lowland rice in inland valley (2) evaluate the effects of split fertilizer application on grain yield and yield component of lowland rice (3) effects of the preceding lowland rice, split application of fertilizer and urea fertilizer on ratooned rice.

2 MATERIALS AND METHODS

The experiment was conducted in 2010/2011 and 2011/2012 cropping seasons at the bottom of the inland valley of the Federal University of Agriculture, Abeokuta. The top 20 cm soil layer was taken using soil auger. The pre-planting sampled soil was air dried before determining following: total nitrogen (using Macro-Kjedahl method), available phosphorus (Bray extractable P), exchangeable potassium (extracted with 1 M ammonium acetate and measured using Flame photometry), Organic matter (Walkley–Black method), cation exchange capacity (CEC), pH (1:2, soil/water) and the textural class (Table 1). The soil series of the experimental site was Ikire (Aiboni, 2001). This is equivalent of Aquic Ustifluvents according to Aiboni (2001).

The available long-term climatic data are: precipitation (1148 mm/annum) and mean temperature (28 ^{o}C). At first peak of the bimodal rainfall during the raining season, the water table was above the soil surface. This receded to the soil level but remains saturated in August and became flooded, again at the second peak of rainfall in September. The major part of the experimental soil remains saturated throughout the dry seasons.

The 3 x 4 x 4 experiment was laid out in a Randomized Complete block Design (RCBD) in split split-plot with three replicates on the same site for two years. The size of the main, sub and sub sub-plots were 15 m x 11 m, 11 m x 3 m and 3 m x 2 m, respectively. Three selected varieties of lowland rice; NERICA L-41, NERICA L-42 (NERICA = New Rice for Africa) and Ofada (control variety) constituted the main treatment assigned to main plot. Each of the variety was dry dibbled at the spacing of 20 cm x 20 cm by chipping 4 to 6 seeds into 16 plots of 6 m^{-2} raised beds on 14th and 17th May 2010 and 2011, respectively. They were harvested in 27 and 30th September 2010 and 2011, respectively. After harvesting of the lowland rice, the straws were cut to about 5 to 10 cm above the soil surface with aid of secateurs on 4th and 7th of October 2010 and 2011, respectively.

The sub plot treatments were split fertilizer applications at the ratio of 1:0 (i.e. single dose of 90 kg N, 45 kg P ha^{-1} and 45 kg K^{-1} was applied at 3 three weeks after planting (WAP), 1:1 (45 kg N ha^{-1}, 22.5 kg P ha^{-1}, 22.5 kg K ha^{-1} each applied at 3 and 11 WAP), 1:2 (30 kg N ha^{-1}, 15 kg P ha^{-1} and 15 kg K ha^{-1} was applied at 3 WAP while 60 N kg ha^{-1}, 30 kg P ha^{-1} and 30 kg K ha^{-1} at 11 WAP) and 2:1 (60 N kg ha^{-1}, 30 kg P ha^{-1} and 30 kg K ha^{-1} at 3 WAP while 30 kg N ha^{-1}, 15 kg P ha^{-1} and 15 kg K ha^{-1} 11 WAP in the form of NPK 20:10:10 compound fertilizer). The split applications at 3 and 11 WAP were targeted to enhance tillering (prior to tillering stage) and grain filling (50 % heading stage). The sub sub-plot treatments were N-fertilizer rates applied to ratooned crop (0, 40, 80 and 120 kg N ha^{-1}) in the form of urea applied at 1 week after cutting (WAC) the main rice straw.

During the dry season, pre-germinated fluted pumpkins [(*Telfairia occidentalis* Hook f.) is an important cucurbitaceous leafy vegetable rich in Fe, protein, minerals, vitamins and oil which nourishes the body], was planted at spacing of 1 m x 0.5 m on the entire experimental plots in late December as the third crop in the sequence. The seeds were pre-germinated in moist saw dust to enhance germination given the fact that the soil was wet.

Contact herbicide was sprayed on field on the fifth day after planting rice but prior to lowland rice emergence to keep the field free of weeds. Other supplementary weeding were done at 3, 6 and 9 WAP for main rice crop whereas ratooned crop was weeded at 1 and 4 WAP while vegetable was weeded at 3, 6 and 9 WAP.

2.1 Data collected on rice crops

Chlorophyll content (greenness of rice leave): The leaf chlorophyll content was determined by using chlorophyll meter (model SPAD 502) to measure the average greenness of lower, middle and upper leaves of rice plant. This was done for 5 randomly selected plants.

Number of days to 50 % flowering: The day at which 50 % of the panicles of stands emerged.

Panicles m^{-2}: With the aid of the quadrat of 1 m X 1 m, the total number of panicles enclosed within quadrat was recorded.

Panicle mass (g): Five panicles were randomly selected from each plot and their lenghts were taken with the aid of ruler in the lab which was divided by 5.

Number of grains/panicle: Five panicles were randomly selected from each plot, threshed and grains were counted which was divided by 5.

Grain yield: The brown panicles were harvested with the aid of a harvesting knife. The harvested panicles were sun dried, threshed and weighed. This was converted to t ha^{-1}.

3 RESULTS AND DISCUSSION

3.1 Pre-planting soil chemical analysis of the inland valley in 2010/2011

The soil physico-chemical properties prior to planting in 2010/2011 experiment are shown in Table 1. The total nitrogen, available P, exchangeable K and organic matter of the soil level were below the critical levels according to Enwezor et al. (2002). However, sulphur level of the soil appeared to be adequate enough. The textural class of the soil justified the split fertilizer application as a factor because of its porousness associated with less colloids which will encourage leaching. The soil pH of 6.00 recorded was slightly acidic but could likely be increased by anaerobic condition of the soil. According to Adigbo et al. (2013), the pH of both acid and alkaline of paddy soil tend to converge on a pH of 7 soon after flooding when he reviewed the potentials of inland valley for poverty alleviation in Nigeria. Furthermore, the process of anaerobiosis in paddy soils, iron phosphate tends to be reduced, with a release of some of the P in available forms. Moreover, reduction of iron oxides releases some of the occluded P into the soil. Thus, raising the availability of P in paddy soils (FFTC, 2007).

Table 1: Soil physico-chemical properties before the commencement of the study in 2010/2011 cropping season

Properties	Values
pH	6.00
Sand (g kg^{-1})	788
Silt (g kg^{-1})	116
Clay (g kg^{-1})	97
Textural Class	Sandy loam
Available P mg kg^{-1}	7.89
Exchangeable Na (c mol kg^{-1})	2.23
Exchangeable K (c mol kg^{-1})	0.15
Exchangeable Ca (c mol kg^{-1})	1.09
Exchangeable Mg (c mol kg^{-1})	0.49
Exchangeable H^{+} (c mol kg^{-1})	0.11
CEC (c mol kg^{-1})	4.07
Organic matter	0.91
Total nitrogen	0.05
Sulphur mg kg^{-1}	8.49

3.2 Response of lowland rice to split fertilizer application

There was significant difference among the varieties in the all parameter considered except chlorophyll content of the leaves (Table 2). 'NERICA L-41' had significantly highest number of panicles m^{-2} while 'Ofada' was the lowest. The panicle length observed in 'NERICA L-41' and 'NERICA L-42' were similar but significantly longer than that of 'Ofada' variety in 2010/2011. 'NERICA L-42' consistently had significantly the highest number of days to 50 % flowering while 'OFADA' had the lowest in both cropping seasons. The biomass obtained from the 3 varieties was similar in 2010/2011 but 'NERICA L-41' had 1.60 and 2.25 times higher grain yield than 'NERICA L-42' and Ofada', respectively in 2011/2012. The grain yields of lowland rice ranged between 3.21 and 1.87 t ha^{-1}.

The chlorophyll content of the leaves which is a measure of leaf greenness of the rice plant arising from the nutrient uptake monitored throughout life cycle of the lowland rice was influenced by split fertilizer application at heading stage in both cropping seasons. Plots treated with 30:15:15 at 3 WAP and 60: 30:30 at 11 WAP had significantly greener leaves compared plots that received single dose NPK 90:45:45 at 3 WAP in 2010/2011 whereas plots that received single dose NPK 90:45:45 at 3 WAP application had significantly greener leaves than those plots treated with split application of 60:30:30 at 3 WAP and 30:15:15 11 WAP in 2011/2012. However, the differences in the greenness in the various split fertilizer application did influence rice straw (biomass) in 2010/2011. Split fertilizer application of ratio 1:1 had significantly higher biomass compared to others but this difference could not be translated into higher grain yield. Adigbo et al. (2013) reported similar difference in greenness of rice leaves which did not translate to increase in grain yield.

Table 2: Effects of fertilizer on the chlorophyll content, yield and yield component of lowland rice

Treatments	Chlorophyll content @ heading		Panicles m^{-2}		Panicle length (cm)		Days to 50 % flowering		Grain yield (t ha^{-1})	
	2010/2011	2011/2012	2010/2011	2011/2012	2010/2011	2011/2012	2010/2011	2011/2012	*2010/2011	2011/2012
Variety (V)										
NERICA L-41	39.71	35.70	-	164.0	-	24.8	90	86	20.6	3.20
NERICA L-42	40.66	36.60	-	130.8	-	26.2	100	105	22.6	2.61
Ofada	39.91	35.63	-	119.9	-	20.9	80	79	20.1	1.87
LSD	NS	NS	-	25.95	-	2.21	8.5	2.32	NS	0.47
Fertilizer split application (F)										
1:0 (90:0)	38.93	37.22	-	138.9	-	24.4	89	90.1	19.1	2.79
1:1(45:45)	40.26	35.92	-	148.0	-	24.0	91	89.5	23.4	2.94
1:2 (30:60)	41.28	35.79	-	129.1	-	23.9	89	89.8	22.4	2.88
2:1 (60:30)	39.90	34.98	-	136.8	-	23.7	91	90.3	19.5	2.91
LSD	1.63	2.24	-	NS	-	NS	NS	NS	3.58	NS
V x F	NS	NS		NS	-	NS	NS	NS	NS	NS

Data were unavoidably lost, * Biomass and NS Not significant

3.3 Response of ratooned rice to preceding lowland rice, split fertilizer application and N-fertilizer rates

There were no variations in the chlorophyll content among the leaves of ratooned lowland rice varieties obtained from the preceding lowland rice in 2010/2011 and 2011/2012. However, the chlorophyll content of the leaves of ratooned lowland rice in the preceding plot treated with single dose of 90:45:45 fertilizer had significantly greener leaves than the others in 2011/2012. The observed greener leaves in the preceding plots of single dose of fertilizer application may not only imply cost and labour saved but suggests that higher residual fertilizer was available to the succeeding ratooned rice. The ratooned rice obtained from the preceding plots of Ofada and NERICA L-41 varieties had significantly higher

panicle length in 2010/2011 and 2011/2012 cropping systems, respectively than the others.

Ratooned rice obtained from preceding plots of Ofada variety significantly flowered earlier than the two improved varieties in both cropping seasons. The earlier attainment of flowering of Ofada ratooned rice compared to the two improved varieties contradicted the report of Africa rice center (2008a) that the improved varieties mature earlier than the local ones. The same trend of observed earliness in flowering in the main rice crop of 'NERICA L-42' > 'NERICA l-41' > 'Ofada' was genetically transferred to their ratooned counterpart. However, the number of days to flowering in the main crop was 3.0 times higher than in the ratooned rice crop for all the three lines. This also agrees with the findings of Oad et al. (2002); Rehman et al. (2007) and Adigbo et al. (2012) who reported that the ratooned crop matures earlier than the main crop. The number of days to 50 % flowering was not influenced by the preceding split fertilizer application but the interaction of variety x split fertilizer application and variety x N-fertilizer were significant in 2011/2012 (Figs. 1 and 2). 'NERICA L-42' consistently had highest number of days to flowering irrespective of the split fertilizer application ratio and N-fertilizer while 'Ofada' also constantly had the lowest number of days to flowering across the split fertilizer ratio. This is a pointer to the fact that the numbers of days to flowering among the three cultivars were genetically inherent and cannot be influenced by fertilizer application.

The grain yield obtained from 'NERICA L-41' was superior to the other varieties in 2011/2012 cropping season (Table 3). However, it is pertinent to note that the preceding plots of split fertilizer application did not influence the overall performances of the ratooned rice. The grain yield of ratooned rice variety ranged between 1.39 and 1.79 t h^{-1} with grand mean of 1.64 t ha^{-1} in 2010/2011 while those of 2011/2012 cropping season was 1.02 and 1.49 t ha^{-1} with grand mean of 1.2 t ha^{-1}. The range of the mean yield obtained in 2010/2011 was not substantially different from the results of Oad et al. (2001) and Adigbo et al. (2013) who reported 1.68-1.83 and 1.39 – 1.62 t ha^{-1}. However, the range of the mean yield got in 2011/2012 was slightly lower than those of Oad et al. (2011) and Adigbo et al. (2013). The lower grain yield obtained in this report compared to the earlier reports of Adigbo et al. (2013) and Oad et al. (2001) could be attributed to water control and other facilities used that had additional cost of operations to the farmers. The grain yield of ratooned rice obtained from the niche in the inland valley was similar to the obtainable yield from one cropping season of the upland ecology according the following researchers IITA (1990), Adigbo et al. (2003) and Africa Rice Center (2008b) who reported 1.5, 1.2 and 1.4 t ha^{-1}, respectively. The ratooned rice of Ofada, NERICA L 41 and NERICA L 42 varieties contributed about 58.3, 46.5 and 39.1 %, respectively to their corresponding grain yield of the lowland rice and indicates that 'Ofada' has better ratoonability than the others. This corroborate the findings of Stansel (1997), Oad et al. (2002) and Adigbo et al. (2012) who reported 30, 50 and 43 % of the total yield. Furthermore, it is pertinent to note that the grand mean grain yield (1.2 t ha^{-1}) obtained from ratooned rice in 2011/2012 was about 59.1, 63.2, 72.7 and 43.6 % of the national average rice paddy yield of Nigeria according to FAO reports of 2011, 2012, 2013 and 2014, respectively (http://faostat3.fao.org/download/Qov/E).

Generally, the non-significant grain yield and yield components observed among the split fertilizer and N-fertilizer application levels could be attributed the loose texture of the soil, lack of water control and nutrients solubility which favoured the free movement of soil nutrient elements along the flow of water occasioned by excess water within and between the plots.

The total grain yield obtained from the main lowland rice varieties and their ratooned rice counterpart were 4.69, 3.63 and 2.96 t $ha^{-1}yr^{-1}$ in 2011/2012 for NERICA L-41, NERICA L-42 and Ofada, respectively.

Table 3: Effects of preceding lowland rice and fertilizer on chlorophyll content, yield and yield components of ratooned rice

Treatments	Chlorophyll content		Panicles m^{-2}		Panicle length (cm)		Days to 50 % flowering		Grain yield (t ha^{-1})	
	2010/ 2011	2011/ 2012	2010/ 2011	2011/2012	2010/ 2011	2011/2012	2010/ 2011	2011/ 2012	2010/ 2011	2011/ 2012
Variety (V)										
NERICA L-41	37.73	35.70	133	122	29.9	30.5	30	29	1.73	1.49
NERICA L-42	39.08	36.60	136	124	28.4	23.4	31	34	1.79	1.02
Ofada	37.73	35.63	102	122	33.3	23.2	28	23	1.39	1.09
LSD	NS	NS	NS	NS	3.65	4.59	1.86	4.10	NS	0.30
Fertilizer split application (F)										
1:0 (90:0)	38.23	37.22	121	123	30.72	26.86	30	29	1.65	1.24
1:1(45:45)	36.69	35.92	131	123	30.81	23.75	30	29	1.62	1.09
1:2 (30:60)	38.47	35.79	126	123	29.44	24.06	30	29	1.59	1.14
2:1 (60:30)	39.31	34.98	118	123	31.14	28.14	30	29	1.68	1.32
LSD	NS	2.24	NS	NS	NS	NS	NS	NS	NS	NS
V x F	NS	NS	NS	NS	NS	NS	NS	S	NS	NS
N-fertilizer (N)										
0	37.64	34.98	122	122	31.58	24.81	30	29	1.69	1.16
40	38.31	35.79	132	123	31.25	25.08	29	29	1.67	1.13
80	38.56	35.92	120	124	29.58	26.69	30	28	1.57	1.21
120	38.22	37.22	122	123	29.69	26.22	30	29	1.61	1.30
LSD	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
N x V	NS	NS	NS	NS	NS	NS	NS	S	NS	NS
N x F	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
N x V x F	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

NS = Not significant

The pumpkin planted in December was adversely affected by anaerobic condition of the soil. The leaves had yellow colorations which were likely to be the symptoms of sulphur, nitrogen and other nutrients interaction as well as the overall effects of anaerobic conditions which resulted in wilting off. This chlorosis, symptom of sulphur and nitrogen deficiency, could not have been associated with lack of these elements in the soil. The pre-planting soil analysis showed that there was sufficient S supply. Although N content was below the critical level, but the residue from the previous application of NPK (90:45:45) and urea (0, 40, 80 and 120 kg N ha^{-1}) to the preceding crops of lowland and ratooned rice, respectively were expected to have corrected the deficiency symptoms if it was not caused by the anaerobic soil conditions. Consequently, the symptoms could be attributed to excess water which reduced nitrate to ammonium and sulphate to sulphid. These reduced forms of S and N in anaerobic soil conditions were not available to fluted pumpkin. This agrees with the opinion of George et al. (1992) that, under anaerobic conditions the N in the form of nitrate are reduced to ammonium (NH_4) and will not be available to upland crop except rice. As the soil becomes even more reductive, sulphate reducers, which are strict anaerobes, produce sulphides; and methanobacteria, also strict anaerobes, produce methane (FFTC, 2007). However, in the opinion of Setter et al. (2009) who reviewed the importance of anaerobiosis and element toxicities associated with different soils in Australia and India with respect to wheat improvement for waterlogging tolerance documented as follows that waterlogging alters the cation exchange capacity of soil particles and valency of nutrient elements (more reduced forms), making them toxic or unavailable for plant uptake. Hypoxia-induced nutrient deficiency/toxicity interferes with a range of shoot physiological processes such as photosynthesis, respiration and growth, causing chlorosis and necrosis and ultimately, plant death (Dodd et al., 2013; Bailey-Serres and Colmer 2014). These explained why pumpkin vegetable could not thrive in saturated inland valley suggesting that another rice crop would be more appropriate to be planted instead of fluted pumpkin.

The successful management of inland valley for triple of rice-rice-vegetable, rice-rice-cowpea have been reported (Adigbo et al., 2007, 2010, 2012a, b and 2013) but this study was a deviation from the previous ones. This observed deviation in inland valley was buttressed by FAO (http://www.fao.org/docrep/003/x6611e/x6611e03a.htm) who are of the opinion that inland valleys varied: internally, where they comprise such different elements as valley bottoms, slopes and crests, as well as externally where they have a characteristically high spatial variability due to differences in parent material, physiography and climate and, as a resultant thereof, hydrology and soils. Inland valleys, therefore, do have very high variability in actual and potential uses. Consequently, the correct use of this particular inland valley should be rice-rice-rice because of its uniqueness in water availability to support three crops of rice in year.

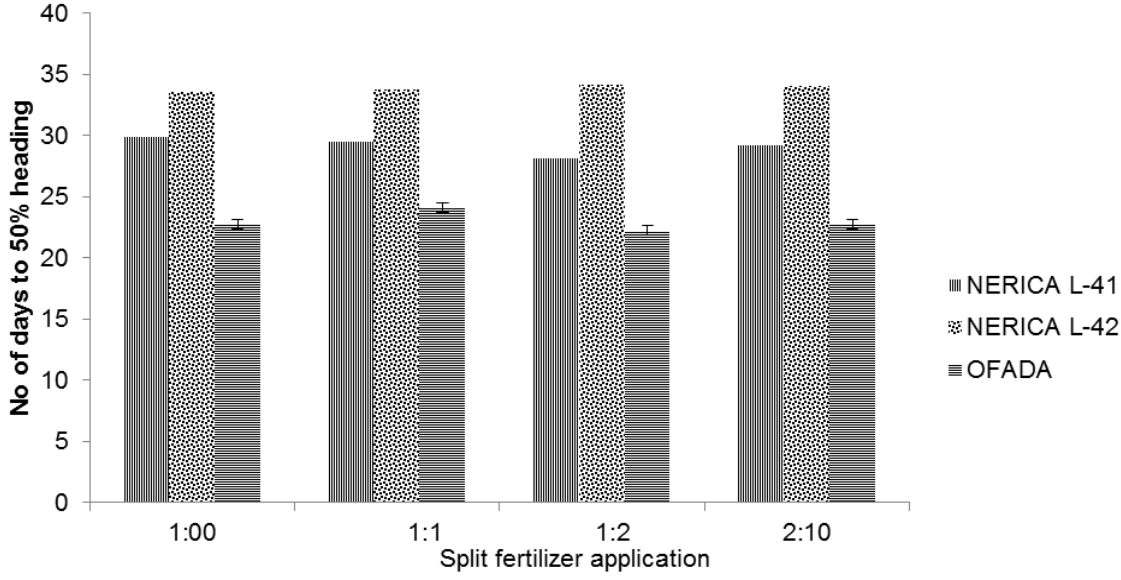

Figure 1: Interaction of variety X split fertilizer application on number of days to 50 % heading

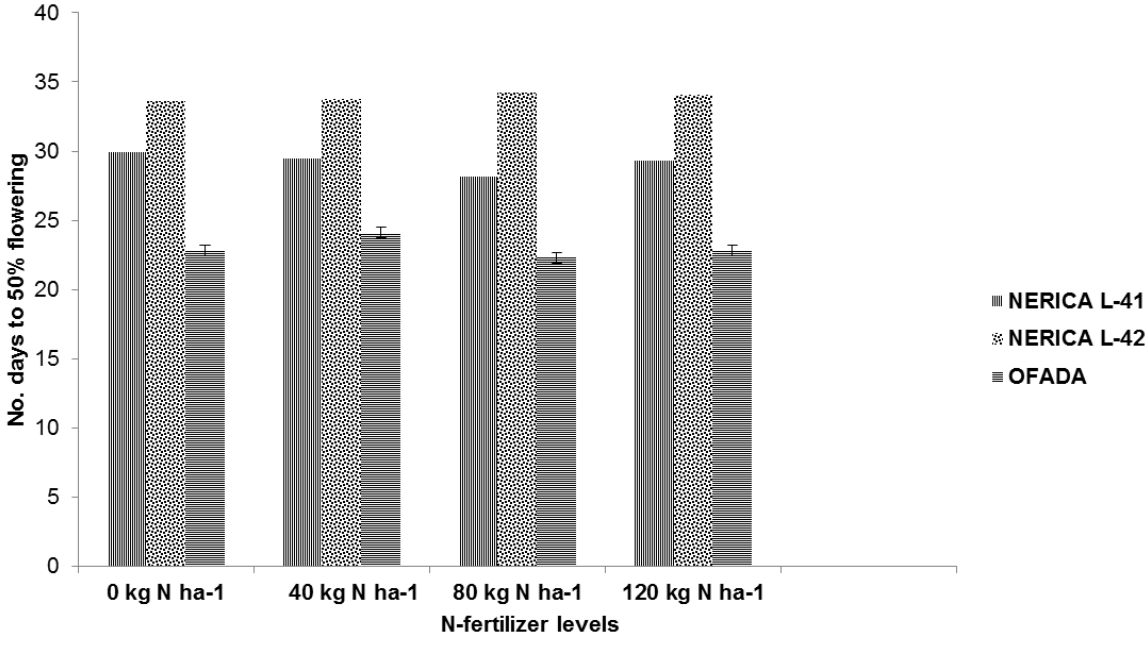

Figure 2: Interaction of variety and N-fertilizer on days to 50 % flowering of ratooned rice

4 CONCLUSION

'NERICA L-42' lowland rice had the highest grain yield. Single dose of NPK 90:45:45 could conveniently be applied to the main lowland rice. The panicle length and grain yield of ratooned rice from 'NERICA L-41' plot were the highest. Consequently, 'NERICA L-41' and its ratooned rice were the best combination. The combination of single dose of NPK (90:45:45) in the form of 20:10:10 and urea were sufficient for the crops of rice. The ratooned rice of 'Ofada', 'NERICA L 41' and 'NERICA L 42' varieties contributed about 58.3, 46.5 and 39.1 %, respectively to their corresponding grain yield of the lowland rice. The total grain yield obtained from the main lowland rice varieties and their ratooned rice counterpart were 4.69, 3.63 and 2.96 t $ha^{-1}yr^{-1}$ for 'NERICA L-41', 'NERICA L-42' and 'Ofada', respectively. The study also showed that this particular inland valley used in this trial should be planted to three crops of rice rather than two rice crops and vegetable or legume.

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Effects of increased concentrations of chloride on the expression of Mn-SOD enzyme in tobacco

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ABSTRACT

Chlorine is one of the ions contributing to salinity, despite being an essential micronutrient. Cl^- absorption takes place more easily than other nutrients so, the toxic effects of chlorine on the growth has considered rather than its scarcity. Salt stress can ultimately leads to oxidative stress through ROS increase and antioxidant defense system is induced. Therefore, in this study the effect of different concentration of chlorine in irrigation water on the expression of manganese superoxide dismutase was investigated as an indicator of antioxidant defense system activation. Seedlings of tobacco were treated with different concentrations, i.e. 2, 4, 8 mM of $CaCl_2$. Evaluation of Mn-SOD isoenzyme gene expression was performed using RT-qPCR (quantitative reverse transcription PCR) at 0, 3, 6 and 12 hours after treatment. The results showed Mn-SOD gene transcription increased after 3 h treatment with 8 mM $CaCl_2$ and peaked at 6 hours. Based on the observed changes, concentrations of calcium chloride greater than 8 mM in water used for irrigation of tobacco causes stress that results in activation of antioxidant response.

Key words: chlorine; Mn-SOD; RT-qPCR (quantitative reverse transcription PCR); salt stress

IZVLEČEK

UČINKI POVEČANIH KONCENTRACIJ KLORIDA NA IZRAŽANJE GENA ZA ENCIM Mn-SOD PRI TOBAKU

Klor je escencialno mikrohranilo, ki znantno prispeva k slanosti talne raztopine. Privzem Cl^- poteka lažje kot drugih hranil zato so toksični učinki na rast pogostejši kot njegovo pomanjkanje. Solni stres vodi v oksidacijski stres preko tvorbe reaktivnih zvrsti kisika (ROS) in posledično v indukcijo antioksidativnega obrambnega sistema. V ta namen je bil v tej raziskavi preučevan učinek različnih koncentracij klora v vodi za namakanje na izražanje gena za mangan superoksid dizmutazo kot indikatorja aktivacije antioksidativnega sistema. Sadike tobaka so bile izpostavljene 2, 4, 8 mM koncentracijam $CaCl_2$. Ovrednotenje izražanja gena za izoencim Mn-SOD je bilo opravljeno z RT-qPCR metodo (kvantitativni PCR z reverzno transkripcijo) 0, 3, 6 in 12 ur po obravnavanju. Rezultati so pokazali, da se je transkripcija gena za Mn-SOD povečala po treh urah obravnavanja z 8 mM $CaCl_2$ in je dosegla višek po šestih urah. Na osnovi teh sprememb lahko zaključimo, da večje koncentracije kalcijevega klorida kot je 8 mM v vodi za namakanje tobaka povzročijo stres, ki vodi v aktivacijo antioksidacijskega odziva.

Ključne besede: klor; Mn-SOD; kvantitativni PCR z reverzno transkripcijo; solni stres

1 INTRODUCTION

Abiotic stresses including drought, salinity, cooling, heating and heavy metal exposure are the major threats to plants and, thus to sustainable agriculture. Together, they decrease cereal production by more than 50 % across the world (Tuteja, 2007). Salinity is one of the key stressors in the water or soil of arid and semi-arid regions and is able to limit growth and productivity of plants (Koca et al., 2007; Allakhverdiev et al., 2000). The rate of water evaporation and precipitation of salt are determinants of soil salinity. The process of water absorption by plant roots is impacted by high salinity via reduction in soil water osmotic potential, the outcome of which

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is a physiological drought in plants (Mahajan and Tuteja, 2005). Although chloride ion is an essential micronutrient, it is also one of the ions contributing to salinity via osmotic stress induction, ion toxicity and nutrient imbalance. High concentrations of the ion adjoin to the active sites of many enzymes and disrupt cell function (Teakle and Tyerman, 2010). Salt stress, like other abiotic stresses, can lead to oxidative stress by the production of increased ROS (such as superoxide, hydrogen peroxide and hydroxyl radicals), which in turn leads to cell injuries due to the oxidation of lipids, proteins and nucleic acids (Esfandiari et al., 2007). Chloroplasts, mitochondria and peroxisomes are the major centers of ROS production (Fridovich, 1986). To reduce the effects of oxidative stress, plant cells have a complex antioxidant defense system. Superoxide dismutase is the first line of defense against ROS (Alscher and Hess, 1993). In eukaryotic cells SODs are the only enzymes that can catalyze the reduction of superoxide radicals to H_2O_2 and O_2. SODs are metal-ubiquitin enzymes which exist in eukaryotic and prokaryotic cells with aerobic metabolism (Luis et al., 2002). Comparison of amino acid sequences of three isoforms of SOD indicate that Mn-SOD and Fe-SOD are ancient forms of the enzymes and probably came from the same ancestral enzyme. Cu-Zn-SOD, on the other hand, is a eukaryotic enzyme that has no sequence homology to Mn-SOD and Fe-SOD and must, therefore, have evolved separately. The fourth group of SOD isoforms, which exists in *Streptomyces* sp., is Ni-SOD (II/III); 2 Ni^+ are located in the active site of the enzyme (Bowler et al., 1992). Mn-SOD is located in mitochondria and peroxisomes. Studies show that the high production of Mn-SOD in mitochondria is associated with increased resistance to stress (Shah and Nahakpam, 2012). Many successful attempts have been made to produce transgenic plants with each of the three isoforms of the SOD enzymes (Faize et al. 2011). However, only in transgenic plants expressing introduced Mn-SOD protection against stress-induced damage was manifested – e.g., as mitigation of biomass reduction and leaf damage (Samis et al., 2002). These findings are consistent also with numerous studies investigating cold stress which have linked Mn-SOD to the plants responses in pea (Palma et al., 1998, Sevilla et al., 1980), corn (Baum and Scandalios, 1981), pine (Streller et al., 1994) and tea (Vyas and Kumar, 2005). Although it seems clear that manganese superoxide dismutase is an essential enzyme for the elimination of free radicals in plant cells under environmental stress (Baek and Skinner, 2003), it is also able to enhance salt stress tolerance in transgenic *Arabidopsis* overexpressing Mn-SOD (Wang et al., 2004). Its role in plant cells has not been clearly identified under salt stress. We have therefore investigated the effect of increased concentrations of $CaCl_2$ in irrigation water on the expression of manganese superoxide dismutase (Mn-SOD) in tobacco plants (*Nicotiana tabacum* L.) using the RT-qPCR method.

2 MATERIALS AND METHODS

2.1 The plant cultivation in hydroponic condition and sampling

Seeds of tobacco ('Coker 347') were hydroponically fed in Hoagland solution for 2 weeks and, after germination, were moved to 10 cm diameter pots filled with perlite. Seedlings were grown on a 16 h light, 8 h dark schedule, at 60-80 % humidity, and at a temperature of 25-30 °C, with light intensity of ~90 µmol photons m^{-2} $s^{-1}$for two months. There are three vegetative growth phases in tobacco plants, including root development, fast growth phase and leaf ripening. Naturally, the fast growth phase is usually the most sensitive. Selected samples with highly similar vegetative growth in the fast growth phase (8 leaf stage, 70-80 cm tall) were treated for 0, 3, 6 and 12 hours with concentrations of 2, 4 and 8 mM $CaCl_2$ before sampling. Leaf discs were prepared from young leaves (second and third leaves from above) at 0, 3, 6 and 12 hours after treatment then transferred to liquid nitrogen and stored in a freezer at -70 °C.

2.2 RNA extraction

RNA was extracted from 2 leaf discs from the same plant for each replicate by grinding them in liquid nitrogen. All extraction procedures were performed using Accuzol buffer from BIONEER

Company, in accordance with their instructions. Extracted RNA was dissolved in 50 µl DEPC-treated water. Electrophoresis on 1 % agarose gels and determination absorption of the band on the gel at 280/260 nm was used to evaluate the quality and concentration of the extracted RNA. RNA concentration in µg/µl was calculated from the absorption at 260 nm using an extinction coefficient of 40 mM^{-1} cm^{-1} and 1 µg RNA was used for cDNA synthesis using the Accupower RT premix kit according to the instructions provided by the BIONEER Company.

2.3 cDNA synthesis and Primer design

1 µg of RNA mixed with 0.5 µg Oligo (dT) primer and was placed at 70 °C for 5 min for primer annealing. The material was then transferred to micro-tubes containing AccuPower RT PreMix and brought to a final volume of 20 µl with DEPC water. The resulting solution was then vortexed for a few seconds then incubated at 42 °C for approximately 60 minutes. Synthesized cDNA was then incubated again at 94 °C for 5 minutes and stored at -20 °C. PCR primers were designed to amplify Mn-SOD gene as a master gene and Ef-1a as reference gene and synthesized based on Oligo 7 software. Primers (Table 1) amplified a 155 base pair (bp) fragment of EF-1a gene cDNA, as well as a144 base pair (bp) cDNA fragment of the gene for Mn-SOD.

Table 1: The sequences of the primers used for Real-Time PCR Analysis

Accession number	Putative function	Tm (°c)	Primer sequence (5´-3´)
BAC75399.1	Superoxide dismutase	57.6	F: CGACACTAACTTTGGCTCCCTAGA R: GGTTCCTCTTCTGGGAATAGACGT
D63396.1	Ef-1a	53.5	F: AAGCCCATGGTTGTTGAGAC R: GTCAACGTTCTTGATAACAC

2.4 RT-qPCR

RT-qPCR reactions were performed to measure Mn-SOD gene expression in treated and control samples. The reaction mixture was prepared in 25 µl volumes consisting of:

1) 12.5 µl of Maxima®SYBR Green/ROX qPCR Master Mix (2X) (Fermentas)
2) 3µl of Forward and Reverse Primer
3) 2.5µl of Template cDNA,
4) 7µl of Sterile distilled water

These reactions were performed for as three technical replicates of samples from three biological replicates to measure the expression of target genes. Expression of Ef-1a, a housekeeping gene, was measured for the standardization of the Real-time PCR reactions. After standardizing the data to the expression of the housekeeping gene, the amount of target gene (Mn-SOD) mRNA expression was determined using the comparative ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). Statistical analysis was performed using One Way ANOVA and Duncan's multiple range test using the SPSS 18 software package and diagrams related to changes in gene expression were plotted in Excel.

3 RESULTS

As seen in Figure 1, RNA bands, later extracted, are visible as bright spots on a dark background of the gel. The quality of the RNA is very good, as indicated by the very clearly demarcated 18s, 28s and 5/8s rRNA bands and by the fact that the intensity of the 28s rRNA band is substantially greater than that of the other bands (Figure 1A).

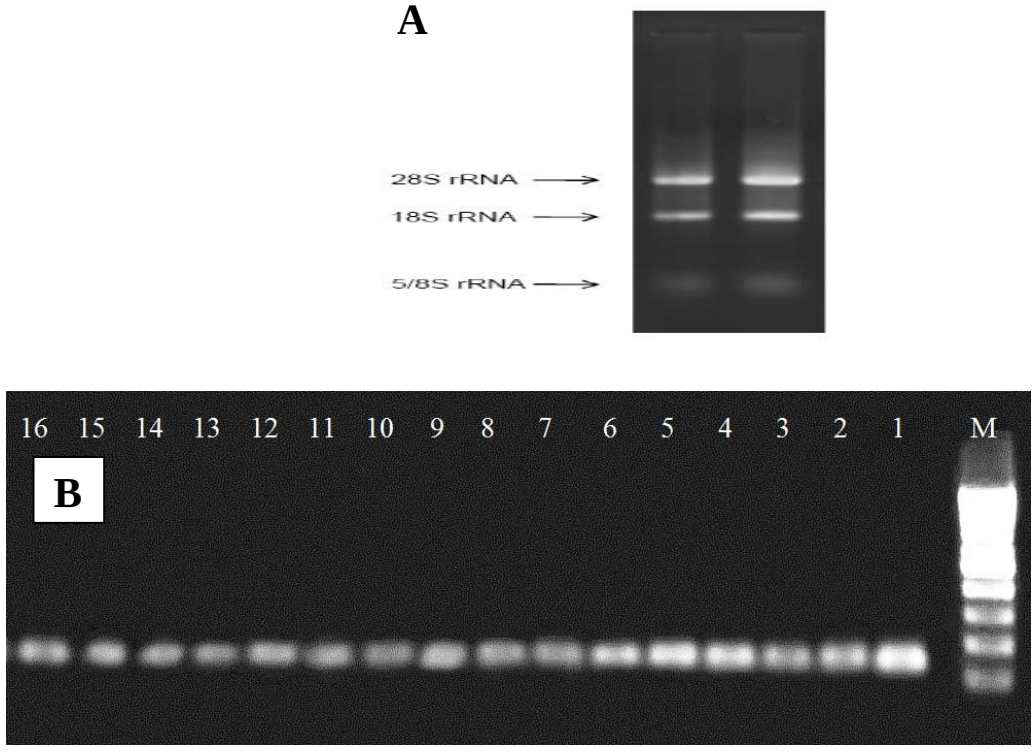

Figure 1: (A) Electrophoretic bands of the 18s and 28s RNAs related to the total RNA on 1 % agarose gel extracted from tobacco leaves. The bands show proper quantity, lack of RNA degradation and no evidence of protein or DNA contamination in the samples. (B) 1 % agarose gel electrophoresis for PCR products of the Mn-SOD gene in tobacco leaves. Bands 1 to 16 are related to 0 (1-4), 3 (5-8), 6 (9-12) and 12 (13-16) hours after treatment with 0, 2, 4 and 8 mM $CaCl_2$ treatments, respectively.

Fragment sizes of 144 bp (Mn-SOD) and 155 bp (Ef-1a) were amplified by the Real-Time PCR (Figure 1B). The measurement of manganese superoxide dismutase gene expression yielded different results in tobacco leaf at different hourly periods. Changes in the expression of the gene at 0, 3, 6 and 12 hours after treatment are shown in Figure 1B. According to the results, superoxide dismutase gene expression was the same in the all treatments at time of zero, immediately before chloride stress was initiated. There were also no significant differences between treated samples exposed to concentrations of 2, 4 and 8 mM calcium chloride. In other words, the Ef-1a gene is expressed in cells consistently in small amounts and remains in a base level under all conditions that we tested. The results for Mn-SOD were strikingly different. Three hours after the initiation of Cl^- stress, a significant increase in Mn-SOD mRNA expression was observed in plants treated with 8 mM calcium chloride. In contrast, expression decreased in plants treated with the lower concentrations (Figure 2B). An extremely significant change was seen only at 8 mM chloride relative to the control, six hours after initiation of the treatment (Figure 2C). By 12 hours exposure to 8 mM chloride, Mn-SOD gene expression declined relative to levels after 3 hours exposure; Mn-SOD expression also remained constant in plants exposed to the lower concentrations (Figure 2D).

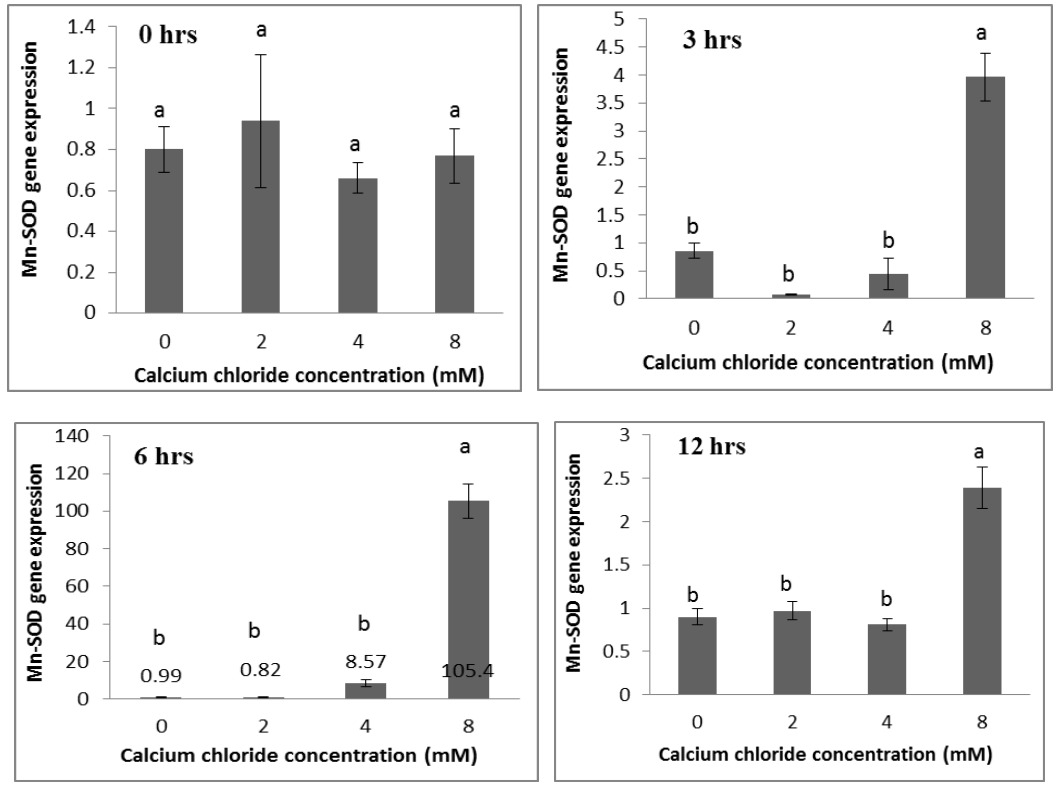

Figure 2: The expression of Mn-SOD at the time of zero, 3, 6 and 12 hours after stress at different concentration of calcium chloride. Data is average of three replicates ± standard error (SE) respectively. Different letters indicate significant differences between treatments according to Duncan's test with $P < 0.05$

4 DISCUSSION

Research has shown that the expression of various proteins is different under stress. In many plants, expression of some logically relevant soluble proteins, such as antioxidant enzymes, significantly increase or diminish in response to stress. Salinity also reduces synthesis of some proteins in certain plants and increases the hydrolysis of those proteins, leading to increasing of free amino acids (Kozlowski, 1997). According to the research conducted by Brou et al. (2007), superoxide dimutases are among several enzimes whose gene expression is upregulated in response to stress. This enzyme has multiple isoforms and differing expression can be seen among the isoforms in response to stress conditions. Therefore, these enzymes have been called biochemical markers for oxidative stress (Brou et al., 2007). In this study, increasing of expression of Mn-SOD was observed to be dependent on calcium chloride concentration and to the length of exposure of the plant to that stressor. While, as was noted in the results section, stressing the tobacco plants with 2 and 4 mM calcium chloride did not cause significant changes in the expression of Mn-SOD. This is not entirely surprising, since it has been reported that concentrations of about 1 mM are optimal for tobacco seedling growth (Norastehnia et al., 2014). In contrast, 8 mM calcium chloride resulted in a strong stress response, as indicated by the dramatic increase in the expression of Mn-SOD. This very different response, extending for at least 12 hours, of increased expression of Mn-SOD indicates that chloride at this concentration is stressful to the plants. From the evidence our study has obtained, it can be suggested that Mn-SOD gene expression, like many of the genes involved in stress tolerance in plants, has a biphasic function. Basal gene expression is low. Upon exposure to Cl^- stress, like other stresses, a rapid and significant increase occurs in gene expression (Sohani et al., 2009). This increase in the expression of Mn-SOD was similar to that also observed in other short-term oxidative stress studies, whereas long-term

oxidative stress has been shown to reduce Mn-SOD expression, resulting in the accumulation of O_2^- - particularly in chloroplasts and mitochondria (Liu and Huang, 2000). This decrease in the activity of an isoform of antioxidant enzymes alone does not indicate an inability of the plant to cope with stressful situations. There are many other enzymes that may also be involved in stress response. However, that is outside the scope of this particular study, but very relevant. Different isoforms of an enzyme, even, often exhibit their maximum activities in differing conditions or over differing time frames (Brou et al., 2007).

As was observed in the research of Brou et al. (2007), there are three isoforms of SOD in beans, including Mn-SOD, Fe-SOD Cu/Zn-SOD; in drought stress conditions their intensities and time frames of action are quite different. While Mn-SOD and Fe-SOD expression increases during stress, the activity of Cu/Zn-SOD is reduced. Other studies have shown that in *Pisum sativum* L., increased expression of Mn-SOD occurs within 2-96 hours of oxidative stress (Malecka et al., 2012). Many other researchers such as studying wheat (Keunen et al., 2011) and *Brassica napus* L. (Basu et al., 2001), have shown that increased expression of Mn-SOD is a good indicator of stress. Based on these studies, it can be said that plants deal with stress via increased expression and activity of antioxidant enzymes. That said, the specific type of stress, stress intensity and stress period has significantly different effects on gene behavior and impacts on the expression of many different proteins. Since, the first line of defense against reactive oxygen species are the superoxide dismutase (SOD), increasing the amount of SOD under stress can be considered as an indicator for the formation of oxidative stress. Therefore, that Mn-SOD expression is increased under stress from concentrations of chlorine more than 4 mM strongly indicates that oxidative stress is induced by excess chloride in tobacco plants. Future work to determine the mechanism by which this oxidative stress is generated will be of great interest.

5 CONCLUSION

According to this study seems stressing the tobacco plants with 2 and 4 mM calcium chloride did not cause significant changes in the expression of Mn-SOD, while the concentration of 8 mM calcium chloride acts as a severe stress for samples, so that the expression of Mn-SOD significantly increase. Irrespective of the impact of stress on expression of Mn-SOD, it can be expected that 8 mM concentration of chlorine, is in the critical range in irrigation water for tobacco plants.

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Utility of some floral characters in the assessment of genetic diversity in sesame (*Sesamum indicum* L.)

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ABSTRACT

Sesame collections were evaluated for quantitative floral characters and data obtained were subjected to various statistical analyses. Result showed narrow diversity in most of the quantitative floral characters with moderate variability in length of flower (2.03-3.27 cm), length of style (1.10-1.40 cm), length of capsule (2.33-2.98 cm) and number of seeds per capsule (38.67 – 57.67). Correlation study revealed significantly ($p < 0.01$) positive correlations for length of ovary versus length of flower (r= 0.70) and length of capsule versus length of style (r= 0.77). The first two principal components accounted for 61.59 % of which the first component had 34.13 % and the second was 27.46 %. Dendrogram divided the seventeen accessions/landraces into two major groups (A and B). Group A had only one cluster with five members whilegroup B had three clusters (Cluster II, III and IV) with seven, three and two members respectively. Each accession within a cluster could be employed as baseline parent in crossbreeding for improvement of yield in Nigerian sesame.

Key words: *Sesamum indicum;* accessions; multivariate analysis; floral characters; principal components; clusters; genetic diversity; dendrogram

IZVLEČEK

UPORABNOST NEKATERIH LASTNOSTI CVETOV PRI VREDNOTENJU GENETSKE RAZNOLIKOSTI SEZAMA (*Sesamum indicum* L.)

Zbirke sezamovih genotipov so bile ovrednotene po nekaterih kvantitativnih lastnostih cveta in nato podvržene različnim statističnim analizam. Rezultati so pokazali majhno variabilnost pri večini vključenih lastnosti, z zmerno raznolikostjo v dolžini cveta (2.03-3.27 cm), dolžini vratu pestiča (1.10-1.40 cm), dolžini glavice (2.33-2.98 cm) in v številu semen na glavico (38.67 – 57.67). Korelacijske raziskave so pokazale značilno ($p < 0.01$) pozitivno korelacijo med dolžino plodnice in dolžino cveta (r= 0.70) ter dolžino glavice in dolžino vratu pestiča (r= 0.77). Prvi dve glavni komponenti variabilnosti sta znašali 61.59 %, kjer je prva komponenta obsegala 34.13 % in druga 27.46 %. Dendrogram je razdelil 17 akcesij v dve glavni skupini (A in B). Skupina A je imela samo en klaster s petimi akcesijami, medtem, ko je skupina B obsegala tri klastre (klaster II, III in IV) s sedmimi, tremi in dvema akcesijama. Vsako od akcesij v navedenih klastrih bi lahko uporabili kot izhodiščno starševsko linijo pri hibridizaciji, s ciljem izboljšanja produktivnosti sezama v Nigeriji.

Ključne besede: *Sesamum indicum;* akcesije; multivariatna analiza; cvetne lastnosti; PCA; klastri; genetska raznolikost; dendrogram

1 INTRODUCTION

Sesame (*Sesamum indicum* L.) is a flowering plant belonging to the genus *Sesamum* with numerous wild relatives occurring in Africa. It is believed to have originated either around the Fertile Crescent or the Indian subcontinent or Iran-Afghanistan region (Ashri, 1989; Mohamed and Awatif, 1998; Pathak et al., 2014). The prediction of Africa as probably the primary centre of origin of cultivated sesame because of the preponderance of the wild species of the plant in the region was defeated by lack of genetic variability. However, genetic variability for cultivated sesame has been found to

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be abundant in India subcontinent. Moreover, sesame seed and oil are well referenced in several Hindus scriptures as far back as 1500 BC and have since become important components of Hindus rituals and offerings (Bhat et al. 1999). Scientific evidences of successful crosses and production of fertile hybrid between *S. malabaricum* Burm. ,(a wild form), only reported from Malabar in the west coast of India and *S. orientale* L. (Hiremath and Patil, 1999) suggested the origin of cultivated sesame from wild populations native to India. Close phylogenetic relationship between the two taxa based on RAPD markers (Bhat et al., 1999; Nanthakumar et al., 2000), chemical data and existence of enormous genetic variability further supported the view that sesame was domesticated in the Indian subcontinent (Bedigian, 2003).

Sesame is cultivated for its edible seeds which are produced in capsules. Sesame seeds come in variety of colors from cream to white to charcoal black (Bedigian, 2006). In general, the paler varieties of sesame seem to be more valued in the West and Middle East, while the black varieties are prized in the Far East. Sesame seeds and oil are put to great variety of uses. Sesame seeds are primarily a source of oil for cooking in India subcontinent and African countries (Bhat et al., 1999). The seeds, hulled or unhulled, roasted or raw are widely used in European and North American bakery industry as a garnish on bread products. About one third of the sesame crops imported by the United States from Mexico are purchased by McDonalds for their sesame seeds buns (Anon, 2002). In Nigeria, the crop, often referred to as beniseed is widely used and very popular in parts of the central, north western and north eastern zones where it is usually grown (Falusi and Salako, 2001). The most popular species in cultivation is *Sesamum indicum*, which has hundreds of varieties and strains with considerable variation in size, seed, color and composition. Some wild varieties are also cultivated to some extent for their leaves used as vegetable and medicinal decoctions (Uzo et al., 1985).

The inflorescence type in sesame is spike with flowers that are zygomorphic, and located in leaf axil (Ruhi et al., 2015). Each of the flowers has five petals with white and pink color, and the lower petal is longer with lip folded over the top, keeping it closed to around sunrise; when it opens to form a running strip for bees (Langham, 2007). The stamens are didynamous and the ovary is superior (as in hypogynous flower), bi/tetracarpellate and each carpel has two locules. Its fruit is capsule consisting of oleaginous seeds, of which most of the capsules are dehiscent (Kumar and Hiremath, 2008). Sesame is known to possess greater genetic variability than most of the self-pollinated crops. Kobayashi (1981) considered morphological data such as branching habits, number of flower per axil, capsule type and seed coat color, and employed them to study genetic diversity in which some sesame genotypes were closely grouped in sub clusters. Similar results indicating diversity among varieties of different clusters using morphological characters have been reported by Furini and Wunder (2004). Zhigila et al. (2015) used morphometric characters to delimit some accessions of *S. indicum* collected from Nigeria.

The study of floral development is important in helping to understand phylogenetic relationships among plants (Buzgo et al., 2004). Suarez-Cervera et al. (1992) studied the pollen morphology in the Pedaliaceae family, while Ruhi et al. (2015) studied anatomical structure of vegetative organs, floral meristem and pollen development in sesame. Azeez and Morakinyo (2011a, 2011b) inferred genetic diversity among the cultivated and wild accessions of sesame using seed physical dimensions, seed oil and fatty acid profile. In another study, Azeez, et al. (2013) established genetic diversity in sesame and its crosses using crude seed protein while Alege (2015) also used this same technique to investigate genetic diversity in some Nigerian sesame. Several studies have reported the use of isozymes (Isshiki and Umezaki, 1997; Nyongesa et al., 2014) and DNA markers (Akbar et al., 2011; Wei et al., 2011; Adeoti et al., 2011) in the analyses of genetic diversity in sesame. However, there is dearth of knowledge on the usefulness of quantitative reproductive characters in the assessment of genetic diversity in sesame. As a result, this study was designed and aimed at assessing the variability among seventeen accessions of sesame using floral characters with a view to identify promising accessions for future breeding programs.

2 MATERIALS AND METHODS

Seventeen accessions/landraces of sesame seeds used in this study were collected from National Cereal Research Institute (NCRI), Badegi Niger State and across other five States (i.e. Oyo, Kwara, Kogi, Jigawa and Katsina) in Nigeria. The seeds were sown in Nursery bags on the research field of the Department of Pure and Applied Biology, LAUTECH, Ogbomoso in January 2009. The seedlings emerged 3 to 4 days after sowing and were constantly watered for two weeks before the seedlings from each Nursery bag were transplanted 2 plant stands per bag. Each accession was replicated in 18 bags out of which nine bags were later selected and were divided into three plots with arrangement following complete randomization pattern. Inter-row and within row spacing of 0.5 m was maintained throughout. Identities of the accessions used and their seed color are given in Table 1.

A total of 10 floral characters were recorded in this study. They include, length of flower, length of ovary, length of two upper anthers, length of two lower anthers, length of two upper filaments, length of two lower filaments, length of style, length of capsule, breadth of capsule, and number of seeds per capsule. Means and standard error of means were used to determine central tendency and dispersion for the reproductive characters recorded. Estimate of analyses of genetic diversity was executed using SPSS for window 7.0, version 16 (Norusis, Munich, Germany). Degree of association among the various floral characters was assessed using Pearson's correlation. Principal component analyses were performed to evaluate the contribution of each character to genetic diversity and the total variation was calculated as the sum of extracted eigenvalues. Grouping of accessions/landraces into similar categories was performed by estimates for Euclidean dissimilarity coefficients for floral data while hierarchical cluster analysis was carried out and dendrogram of relationship based on Ward's method was constructed using SPSS version 16 (Azeez et al., 2013).

3 RESULTS

Most of the floral characters measured exhibited narrow variability except for length of flower, length of style, length of capsule and number of seeds per capsule. Most accessions/landraces used in the study were white seeded (64.71 %), while others with brown and black seeds were equally represented. Majority of the accessions/landraces evaluated produced white flowers at flowering stage (82.35 % of total collections), while others which were black seeded produced purple colored flowers. Mean values calculated for all the floral characters' studied revealed moderate variability in length of flower (2.03 – 3.27 cm), length of style (1.10 – 1.40 cm), length of capsule (2.33 – 2.98 cm) and number of seeds per capsule (38.67 – 57.67) as shown in Table 2. Eight of the correlation coefficients were positive and significant (Table 3), out of which only 3 were highly significantly correlated ($p<0.01$). The highest positive correlation was between length of the two lower filaments and length of the two upper filaments (r = 0.78), followed by length of capsule versus length of style (r = 0.77) and length of ovary versus length of flower (r = 0.70). Length of ovary was negatively correlated with length of the two lower anthers (r = -0.55) and length of the two upper anthers (r = -0.50) (Table 3).

The principal components analyses (Table 4) revealed that the first two principal components accounted for 61.59 % of the total variability among the accessions/landraces. Most variation was explained by the first component (34.13 %), followed by the second (27.46 %). The first component had high positive loadings from length of ovary (0.75), length of style (0.74), length of flower (0.71) and length of capsule (0.66) while it had high negative loading from length of two lower anthers (-0.69). The second component also had high positive loadings from length of the two upper anthers (0.75), length of the two upper filaments (0.70), length of the two lower filaments (0.66) while it recorded high negative loadings for breadth of capsule (-0.63) and number of seeds per capsule (-0.61). However, none of the characters was redundant.

Table 1: List of studied accessions/landraces of sesame and their seed color

Accession No.	Code	Source/Origin	Seed Color
1.	69B-882	NCRI, Badeji, Niger State	Brown
2.	AYK	Kabah, Kogi State	Black
3.	EVA	NCRI Badeji, Niger State/FAO Italy	Dirty white
4.	65-8B	NCRI, Badeji, Niger State	Brown
5.	C-K2-1	NCRI, Badeji, Niger State	Light brown
6.	IBS	Bode Saadu, Kwara State	Black
7.	PACH	NCRI, Badeji, Niger State /FAO Italy	Dirty white
8.	GUMEL Local	Jigawa State	Dirty white
9.	DANEKA I	Katsina State	Dirty white
10.	C-K2-2	NCRI, Badeji, Niger State	White
11.	DANKASCO Local	Jigawa State Nigeria	White
12.	E-8	NCRI, Badeji, Niger State	White
13.	S530	NCRI, Badeji, Niger State	Dirty white
14.	DANKASCO I	Jigawa State	Creamy white
15.	BATSARI Local	Katsina State	White
16.	ZABURAN Local	Jigawa State	White
17.	ALO	Ogbomoso, Oyo State	Black

Table 2: Statistical parameters for sesame floral characters

Traits	Range	Mean	S.E.
Length of flower	2.03 – 3.27	2.84	0.07
Length of ovary	0.30 – 0.45	0.37	0.01
Length of two upper anthers	0.30 – 0.40	0.35	0.01
Length of two lower anthers	0.25 – 0.40	0.33	0.01
Length of two upper filaments	1.00 – 1.17	1.02	0.01
Length of two lower filaments	0.90 – 0.99	0.92	0.01
Length of style	1.10 – 1.40	1.20	0.02
Length of capsule	2.33 – 2.98	2.58	0.05
Breadth of capsule	0.80 – 0.88	0.83	0.01
Number of seeds per capsule	38.67 – 57.67	50.69	1.40

Table 3: Correlation among analyzed sesame floral characters

Character	LOF	LOV	LUA	LLA	LUF	LLF	LOS	LOC	BOC	NSC
LOF	1.00									
LOV	0.70**	1.00								
LUA	-0.16	-0.17	1.00							
LLA	-0.17	-0.50*	0.49*	1.00						
LUF	0.37	0.56*	0.25	-0.24	1.00					
LLF	0.19	0.48*	0.41	-0.30	0.78**	1.00				
LOS	0.34	0.26	-0.16	-0.49*	0.26	0.23	1.00			
LOC	0.43*	0.20	-0.37	-0.45*	0.04	-0.14	0.77**	1.00		
BOC	0.28	0.02	-0.42*	-0.40	-0.34	-0.07	0.40	0.48*	1.00	
NSC	-0.33	-0.19	-0.47*	-0.24	-0.45*	-0.33	-0.17	-0.07	0.17	1.00

**P< 0.05; **P <0.01*

*LOF = Length of flower, LOV = Length of ovary, LUA = Length of two upper anthers, LLA = Length of two lower anthers, LUF = Length of two upper filaments, LLF = Length of two lower filaments, LOS = Length of stigma, LOC = Length of capsule, BOC = Breadth of capsule, NSC = Number of seeds per capsule.

Table 4: Eigenvectors and percentage explained variation by the first three principal components of the sesame floral characters

Characters	Eigenvectors		
	PC1	PC2	PC3
Length of flower	0.71	0.08	0.21
Length of ovary	0.75	0.25	-0.32
Length of two upper anther	-0.27	0.75	0.32
Length of two lower anther	-0.69	0.32	0.47
Length of two upper filament	0.56	0.70	-0.18
Length of two lower filament	0.48	0.66	-0.27
Length of style	0.74	-0.20	0.33
Length of capsule	0.66	-0.46	0.42
Breadth of capsule	0.39	-0.63	0.17
Number of seeds per capsule	-0.25	-0.61	-0.59
Eigenvalue	3.41	2.74	1.26
Individual percentage	34.13	27.46	12.62
Cumulative percentage	34.13	61.59	74.22

Seventeen accessions/landraces of sesame were broadly divided into two groups (Group A and B) using hierarchical clustering technique (Table 5). Group A had only one cluster (cluster I) containing five members with plants in this group recording the highest mean value for number of seeds per capsule (57.67) (Table 6). There were three clusters in Group B (cluster II, III, IV). In cluster II were seven accessions/landraces with plants recording highest mean value for length of the two lower anthers (0.40). Cluster III had three landraces from Jigawa and Katsina States. These three landraces are white seeded with the highest mean values for length (2.87) and breadth of capsules (0.86). Cluster IV had only two members with plants manifesting the highest mean values for most of the reproductive characters (length of flower, length of ovary, length of two upper anthers , length of two upper and lower filaments, and length of style). Dendrogram of relationship among the accessions and landraces (Fig. 1) revealed results that were consistent with the results of hierarchical clustering in Table 5. The same clustering pattern was observed in which all the accessions and landraces were recognized in the dendrogram with Euclidean dissimilarity coefficient mean of 12.5 delineating the main phenotypically related groups.

4 DISCUSSION

Medium variability were observed for floral characters such as length of ovary, length of flower, length of capsule and length of style of the accessions studied. This is promising from the view point of genetic improvement of flower size in sesame, which may subsequently translate into big capsule size and probably high seed yield. The importance of a trait is given by its discriminating power in the accessions and its stability of expression (Arriel et al., 2007). Floral characters are known to be expressively stable and in most cases less affected by the environment as suggested by their narrow to medium variability. An analysis of association between various plant characters helps in identifying the most important characters (Sarwar et al., 2005; Azeez and Morakinyo, 2011c). The strong positive correlations of length of ovary versus length of flower and length of capsule versus length of style can be exploited directly in the selection for long capsule with a view to increase the number of seed per capsule and subsequently bringing about improvement in sesame seed yield.

In this study, principal components analysis revealed that all characters took effect in the first two components. Characters that had higher values in the first ended up having lower values in the second and those characters that had lower values in the first ended up having higher values in the

second suggesting that all the characters considered are important in determining genetic variation. Length of flower, length of ovary, length of style and length of capsule were loaded with positive signs (0.71, 0.75, 0.74 and 0.66 respectively) and also exhibited high correlations. Traits with positive loading and high correlation might be influenced by the same gene or set of genes (Biabani and Pakniyat, 2008). Thus, selection based on these floral characters may be more efficient in screening for capsule size in sesame. This study has also shown the consistency between the dendrogram and the cluster grouping of the accessions, indicating that either of the tools is sufficient to illustrate diversity among the accession/landraces.

Table 5: Cluster composition of seventeen sesame accessions and landraces evaluated

Group	Cluster	Number of accessions	Accession code (No)
A	I	5	C-K2-1, BATSARI L, IBS, ALO, E-8
	II(b_1)	7	69B-882, EVA, PACH, GUMEL L, C-K2-2, S530, ZABURAN L
B	III($b_{2.1}$)	3	DAN, DANEKA I, DANKASCO L, DANKASCO I
	IV($b_{2.2}$)	2	AYK, 65-8B

Table 6: Cluster composition of seventeen sesame accessions and landraces

Characters	Clusters			
	I	II	III	IV
Length of flower	2.82	2.59	3.17	3.19
Length of ovary	0.36	0.30	0.40	0.45
Length of two upper anthers	0.33	0.37	0.33	0.38
Length of two lower anthers	0.25	0.40	0.33	0.30
Length of two upper filaments	1.04	1.00	1.00	1.17
Length of two lower filaments	0.94	0.90	0.90	0.99
Length of style	1.22	1.10	1.20	1.27
Length of capsule	2.77	2.33	2.87	2.53
Breadth of capsule	0.86	0.82	0.86	0.80
Number of seeds per capsule	57.67	52.33	44.33	38.67

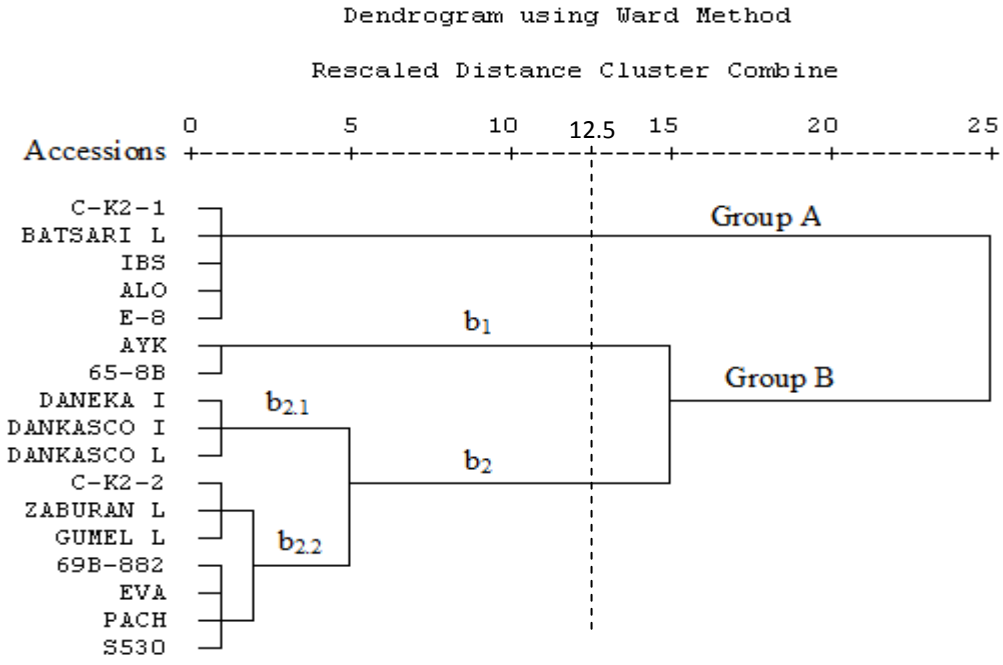

Figure 1: Dendrogram of seventeen accessions and landraces of sesame included in the study

The accessions were initially grouped into two groups consisting of four clusters. Accessions in cluster I showed variety of seed colors from white through light brown to black, while those in cluster II were all white seeded except 69B-882 with brown color. Accessions in cluster III were also white seeded whereas those in cluster IV consist of black (AYK) and brown (65-8B) seeded types. Clustering of accessions/landraces from different eco-geographical locations into one cluster were observed in this study which are attributable to the possibility of free exchange of breeding materials among widely separated locations (Banerjee and Kole, 2009). Some accessions/landraces of common geographical locations (Batsari L and Daneka I) were also observed to form different clusters, a situation that may be explained by their parental developmental traits, past history of selection and different outcrossing rates (Bhat et al., 1999). Accessions within each cluster seem to be more related genetically than members of other clusters based on one or two characteristics, indicating that members in the same cluster may represent one heterotic group. The use of accessions across distinct heterotic group as parents in crossbreeding results in the achievement of maximum variability for selection in the segregating population (Genet et al., 2005).

Comparatively, previous studies have shown that multivariate analyses of protein variation (Azeez et al., 2013), seed physical dimension (Azeez and Morakinyo, 2011a), and seed oil and fatty acid profile (Azeez and Morakinyo, 2011b) recorded 45.70 %, 88.96 % and 80.95 % for the first two principal components respectively. In some other investigations on sesame, multivariate analysis of morpho-physiological (Tabatabaei et al., 2011) and morpho-agronomic (Ercan et al., 2002) traits yielded 38.51 % and 45.30 % respectively for the first two principal components, while that of phytochemical characters recorded 91.86 %. (Laurentin et al., 2003). However, in the present study on floral characters, the first two principal components recorded 61.59 % suggesting medium variability for floral characters. Generally, biochemical (physio-chemical and fatty acid profile) characters and seed physical dimensions appear to be the most effective in distinguishing among the accessions/landraces, followed by the floral characters while protein, morpho-agronomic and physiological traits are probably the least effective. Nevertheless, the results of this investigation have underscored the usefulness of floral characters in delimiting taxa and subsequently distinguishing among the accessions/landraces of sesame.

5 CONCLUSION

Floral characters employed in this study have shown medium genetic variability. Correlation study revealed strong positive association for length of ovary versus length of flower and length of capsule versus length of style. The seventeen accessions and landraces of sesame evaluated using quantitative floral characters were divided into four clusters representing different heterotic groups. Multivariate analysis in this study has provided tools for bringing accessions that are genetically similar together and separating them from other members hence depicting genetic variation. This finding will enhance selection of good parent plant based on floral characters towards improvement of sesame yield in Nigeria.

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The effects of plant cover on population of pear psylla (*Cacopsylla pyricola*) and its predators

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ABSTRACT

Cacopsylla pyricola (Förster, 1848) (Hemiptera: Psyllidae) is a serious pest of pear in all pear growing areas. In the scope of an integrated pest management, a two consecutive years study was carried out to determine the effects of plant cover on pear psyllid population and its predators. Two treatments including plant cover and bare ground were applied in a randomized complete block design with three replicates. The sampling of the pest and its predators were done weekly by beating technique and leaf sampling. The data were subjected to analysis of variance (ANOVA). The results showed that plant cover had significant effect on the increase of predators on the trees ($P < 0.001$). The psyllid specialist predator, *Anthocoris nemoralis* (Fabricius, 1794), had the highest population among the pear psyllid predators (0.29 per sample). Plant cover had no significant effect on reducing the population of eggs, nymphs and adults of the pear psyllid. Despite the increase in the population of predators led by plant cover, lack of their effectiveness to reduce the pear psyllid population is discussed.

Key words: *Cacopsylla pyricola;* pear; predator; plant cover

IZVLEČEK

VPLIVI VAROVALNIH RASTLIN NA POPULACIJO MALE HRUŠEVE BOLŠICE (*Cacopsylla pyricola*) IN NJENE PLENILCE

Mala hruševa bolšica (*Cacopsylla pyricola* (Förster, 1848) (Hemiptera: Psyllidae) je pomemben škodljivec hrušk na vseh območjih njihove pridelave. V okviru integriranega zatiranja škodljivcev je bil v dveh zaporednih letih preučevan učinek poraslosti tal na populacijo navedene bolšice in njenih plenilcev. V popolnem naključnem bločnem poskusu s tremi ponovitvami sta bili preizkušani dve obravnavanji, in sicer vpliv golih in poraščenih tal. Vzorčenje škodljivcev in njihovih plenilcev je bilo opravljeno tedensko z metodama udarjanja vej in vzorčenja listov. Podatki so bili obdelani z analizo variance (ANOVA). Rezultati so pokazali, da je imel rastlinski pokrov značilni učinek na povečanje populacije plenilcev na drevesih ($P < 0.001$). Med vsemi plenilci male hruševe bolšice je bila vrsta *Anthocoris nemoralis* Fabricius, 1794, najbolj številčna (0,29 na vzorec). Poraščenost tal pa ni imela značilnega vpliva na zmanjšanje populacije jajčec, nimf in odraslih osebkov bolšice. V prispevku je analizirana neučinkovitost plenilcev na zmanjšanje populacije bolšic, kljub povečanju njihove populacije na zemljišču z zastrtimi tlemi z varovalnimi rastlinami.

Ključne besede: *Cacopsylla pyricola;* hruška; plenilec; poraslost tal

1 INTRODUCTION

The Pear psylla, *Cacopsylla pyricola* (Förster, 1848) (Hemiptera: Psyllidae), is a host specific pest of only pears and is present in all pear growing areas with considerable economic importance (Emami et al., 2014). The adults and nymphs suck the sap from the leaves and produce large, sticky drops of honeydew that can coat the tree and fruit. Psyllid feeding can cause the foliage to wilt and drop to the ground and fruit remains undersized. Prolonged infestations may kill the tree outright (Emami, 2014). Cover crops are widely used to reduce soil erosion by wind and water (Hargrove, 1991), produce organic matter, and reduce soil compaction and crusting and thus

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improve water infiltration and in some cases moisture retention (Letourneau et al., 2009). Cover crops also influence pest management of arthropods, as reviewed by Bugg (1992) and Bugg and Waddington (1994). Cover crops can be categorized to resident vegetation, planting alternate strip and commercial 'insectary mixes' of plants (Bugg, 1991). Understory weeds or 'resident vegetation' become assets when managed as cover crops (Zandstra and Motooka, 1978). Weeds sometimes play an important role in pest management systems and when specific weeds are not present, biological control of certain insects is often impossible (Zandstra and Motooka, 1978). Wilde (1960) showed that clean orchard cultivation tended to reduce predator numbers and favor high psyllid populations, probably because of higher orchard temperatures and lower relative humidity than those found in orchards with plant cover. Pear psyllids are favored by hot, dry conditions and damage caused by them is particularly devastating when there are prolonged periods of dry weather (Cross et al., 2010). Orchards with ground covers may have higher populations of certain natural enemies, largely due to increased habitat and alternate food sources for beneficial insects and mites; they also may have fewer problems with pests and mites (Flint, 1998). Orchard systems contain high plant diversity and perennial multi-strata designs that provide wealthy resources and habitats to living communities such as beneficial organisms (Simon et al., 2010). Research has shown an exceptionally strong relationship between higher natural enemy diversity and herbivore suppression in agricultural systems (Letourneau et al., 2009). Fye (1983) reported that cover crops in commercial pear orchards led to the build-up of generalist predators including *Nabis* sp., *Orius* sp., *Geocoris* sp., *Hippodamia convergens* Guérin-Méneville, 1842, *Coccinella transversoguttata richardsoni* Brown, 1962, *Chrysopa* spp., *Hemerobius* sp., and spiders. Despite the increase in the abundance of predatory and parasitoid insects led by the use of plant covers, it is still uncertain whether this will translate into reduced pest densities. In the present study, an investigation was performed on the effects of plant cover on pear psyllid and its predators in pear orchards, to determine the effectiveness of this strategy in regulating pest populations.

2 MATERIALS AND METHODS

2.1 Site and plants

Field studies were conducted in a 1-ha commercial pear orchard located at Isfahan, Iran, during the two consecutive years. The trees were 15-20 years old, *Pyrus communis* L. of the variety 'Shahmivea' which is the common pear variety in the study area. Plant cover was composed of a mix of resident weeds consisted of Lamb's quarters, *Chenopodium album* L. (~ 5 %), Liquorice, *Glycyrrhiza glabra* L. (~ 4 %), Purslane, *Portulaca oleraceae* L. (~ 3 %), Prickly lettuce, *Lactuca scariola* L. (~ 7 %), Sow thistle, *Sonchus asper* (L.) Hill. (~ 4 %), Dandelion, *Taraxacum officinale* Weber (~ 8 %), Wild carrot, *Daucus* sp. (~ 9 %), Plantain, *Plantago major* L. (~ 6 %), Couch grass, *Cynodon dactylon* (L.) Pers. (~ 4 %), Ground cherry, *Physalis* sp. (~ 4%), White Clover, *Trifolium reprens* L. (~ 19 %) and Alfalfa, *Medicago sativa* L. (~ 27 %). Change in the percentage of plants over the experimental area was about ± 1 %. Plant cover, represented by plant species that developed naturally for circa 10 years.

2.2 Experimental design

Two treatments consisted of plant cover and bare ground were applied in a randomized complete block design with three replicates. Each replicate was 1600 m^2 with 60 trees. Each treatment was randomly allocated in blocks. Between row of trees in bare ground treatment was kept free of vegetation by shallow tillage (10 cm deep). Tillage was repeated when the plant cover begin to emerge. In later treatment plant cover under the trees was removed by herbicide application. Management operations including fertilization, pruning and irrigation were applied similarly in treatments. A late-summer application of amitraz (Mitac) was made in both treatments to reduce densities of pear psylla and mites.

2.3 Sampling

Ten trees in each replicate were randomly selected at each sampling time. Pear psyllid adults and predators (larva/nymph and adult) of pear psyllid were sampled using the limb-jarring technique (Burts and Retan, 1973). A beat tray (45 × 45 cm) was covered with a white cloth, both to make the insect visible for counting and to act as substrate to which adult insect cling while they are being counted (Burts and Retan, 1973; McClure et al., 1982). Four limbs of a tree were randomly selected. The beat tray was held beneath the limb and it was rapped sharply three times with a section of stiff rubber hose (Horton et al., 2003). Dislodged adult psyllids and predators (adults and nymphs/larvae) which fell onto the tray were counted. Samples were taken in the morning when temperature was cool. Eggs and nymphs of psyllid were sampled by 20 randomly selected leaves per tree. The samples were separately placed into nylon labelled covers and taken to the laboratory in refrigerated containers. The upper and lower surface of the leaf was carefully examined using a stereomicroscope, where pear psyllid eggs and nymphs were counted and recorded.The sampling was initiated in early May and continued at weekly intervals until late September.

2.4 Data analyses

Data were square root ($x \pm 0.5$) transformed before analysis to standardize the variance. All data were subjected to a one-way analysis of variance (ANOVA) to compare the effect of treatments on pear psyllid and its predators. The comparison of means was performed using Duncan's multiple range test (DMRT) ($p < 0.05$). Data were analyzed by using SAS statistical software version 9.1. (SAS Institute Inc., 2004).

3 RESULTS

3.1 Population of the developmental stages of the pear psyllid

There was not a significant difference between treatments in the density of eggs, nymphs and adults of the pear psyllid (in the first year, egg: $P = 0.16$; nymph: $P = 0.06$; adult: $P = 0.1$; in the second year, egg: $P = 0.58$; nymph: $P = 0.09$; adult: $P = 0.1$,) (Fig. 1 and 2).

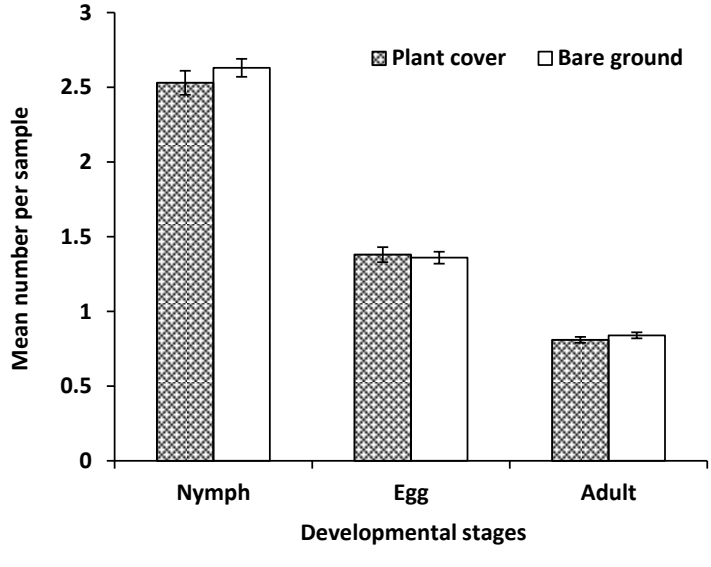

Figure 1: Mean number of developmental stages of *Cacopsylla pyricola* in the first year

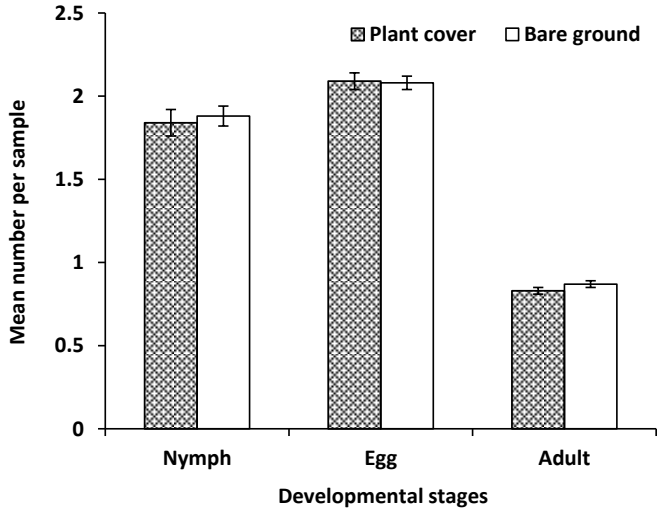

Figure 2: Mean number of developmental stages of *Cacopsylla pyricola* in the second year

3.2 The population of predators

The predators of pear psylla collected on beat tray over the duration of the study were eight species including *Anthocoris nemorum* Linnaeus, 1761. and *A. nemoralis* Fabricius, 1794 (Hemiptera: Anthocoridae), *Hippodamia variegata* Goeze, 1777, *Oenopia conglobata* Linnaeus, 1758, *Coccinell septempunctata* Linnaeus, 1758, *Scymnus syriacus* (Marseul, 1868) and *Adalia bipunctata* (Linnaeus, 1758) (Coleoptera: Coccinellidae) and *Chrysoperla carnea* (Stephens, 1836) (Neuroptera: Chrysopidae). There was a significant difference between treatments in the density of the predators of the pear psyllid (in the first year: $P < 0.001$; in the second year: $P < 0.001$). The psyllid specialist predatory bug, *A. nemoralis*, had the highest density among the pear psyllid predators (Figure 3 and 4), but there was not a significant difference between treatments in its population density (in the first year: $P = 0.06$; in the second year: $P = 0.09$). The other predators were generalist predators which were not closely associated with this pest and had lower population than specialist predator, *A. nemoralis* (Figure 3 and 4).

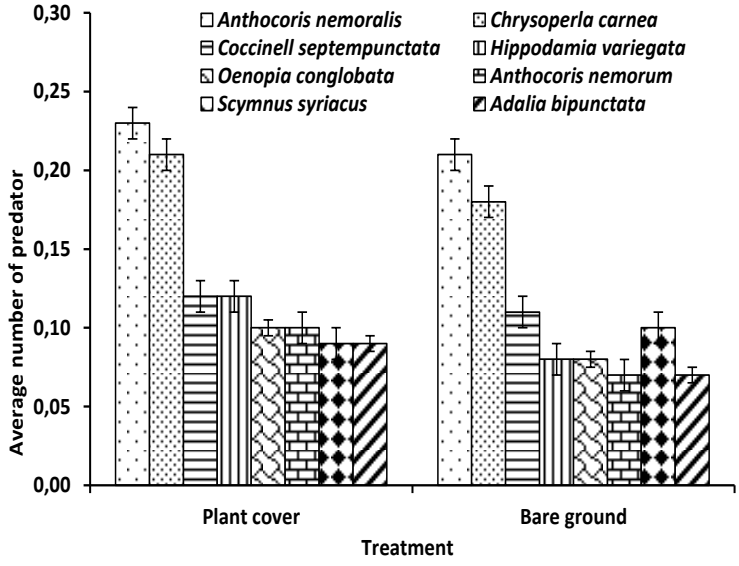

Figure 3: Average number of the pear psyllid predators per sample in the first year

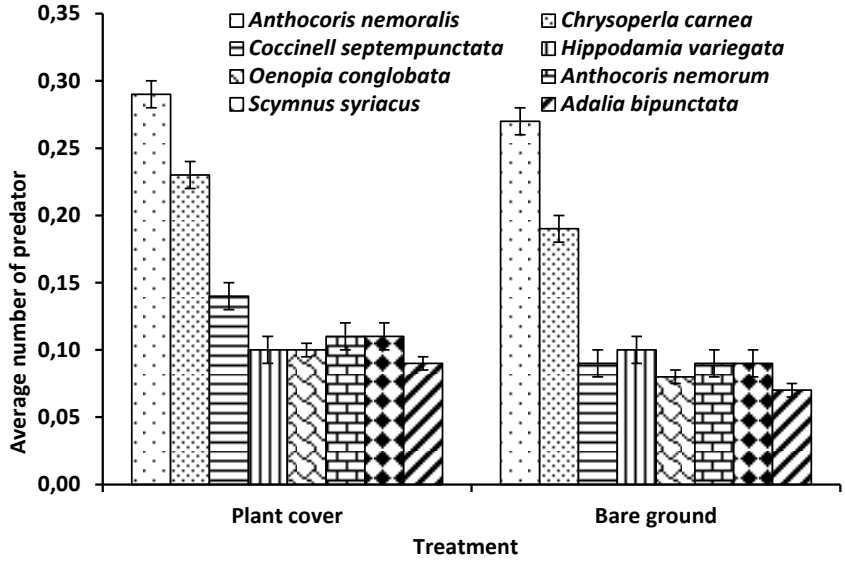

Figure 4: Average number of the pear psyllid predators per sample in the second year

4 DISCUSSION

The effect of ground cover on pest control is considered to be positive, null or negative when either the density of the pest arthropod of the fruit tree and fruit damage is lower, equal or higher, respectively, compared with control (Simon et al., 2010). Here, no significant difference was found between density of eggs, nymphs and adults of the pear psyllid in plant cover and bare ground treatments (Fig. 1 and 2). Thus, plant cover had nil effect on the pear psyllid control. Some studies have reported a decrease in herbivore density in the presence of ground cover vegetation (Aguilar-Fenollosa et al., 2011; Altieri and Schmidt, 1986; Beizhou et al., 2011; Irvin et al., 2006; Pfammatter and Vuignier, 1998; Rieux et al., 1999; Wyss, 1995; Wyss et al., 1995), whilst others have found no (Bone et al., 2009; Brown et al., 2008; Danne et al., 2010; Fitzgerald and Solomon, 2004; Horton et al., 2010; Jenser et al., 1999; Nyrop et al., 1994; Paredes et al., 2013; Paredes et al., 2015; Rodriguez et al., 2009) or negative effect (McClure et al., 1982; Meagher and Meyer, 1990a, 1990b; Spellman et al., 2006). All of these studies have shown that selection of the plant cover species in the orchard is important in preventing increased pest population. In pear orchards, both an increase in Anthocorid numbers and a decrease in pear psyllid prey are reported when a grassy ground cover is sown in the alleys compared with bare ground (Rieux et al., 1999). Non-crop vegetation can affect insect populations in a number of ways. They can provide a habitat for beneficial arthropods where they can find physical shelter, alternative hosts, pollen, nectar or water and perhaps a more favorable microclimate than is available within the cropped area, especially if it is a mono-crop (Dyer and Landis, 1997). Here, a significant presence of pear psyllid predators was displayed in plant cover treatment. Despite of the predators belonged to the families Anthocoridae, Coccinellidae and Chrysopidae; their presence had no effect on pear psyllid density. Wilde (1965) found green lacewings to be the most efficient predator of pear psylla followed by anthocorids and ladybird beetles. Rieux et al. (1999) reported that the main beneficial arthropods on pear tree were Empididae (Diptera) and Miridae (Hemiptera) in the natural ground cover area; and Forficulidae (Dermaptera) and Miridae in the bare ground area. Here, lack of pest suppression may be the result of disruption of biological control by alternative prey presence (Koss and Snyder, 2005), asynchrony between pest and their natural enemies (Fagan et al., 2002; Perdikis et al., 2011), lack of specificity and/or intra-guild predation (Paredes et al., 2013). Although plant cover enhanced predators, complex environments may provide pests with refuges and natural enemies may face

difficulties in locating their prey (Root, 1973; Barbosa, 1998; Finke and Denno, 2006; Hughes and Grabowski 2006). Horton et al. (2010) reported that despite the high densities of predators in the alfalfa cover crop, there was no statistical increase during 3 years of sampling in densities of predators in the canopy of trees having the alfalfa understory, and no effects on psylla densities. No significant correlation was displayed between predator abundance and pear psyllid control in a survey of 8 commercial pear orchards (Simon, 1999). Here, the psyllid specialist predatory bug, *A. nemoralis*, had the highest density among the pear psyllid predators (Figure 3 and 4). *A. nemoralis* is the most abundant predator in the pear orchards (Shaltiel and Coll, 2004; Emami et al., 2014) but often migrate into orchards too late and in too small numbers to effect timely and adequate natural regulation of pear psyllid populations (Cross et al., 2010). Most psylla natural enemies are arboreal (Booth, 1992) and relatively scarce in the cover crops (Fye, 1983), therefore, plant covers will not support them (Booth, 1992). Suitable hedgerows such as goat and grey willow, (*Salix caprea* L. and *S. cinerea* L.), hawthorn, (*Crataegus monogyna* Jacq.), stinging nettle, (*Urtica dioica* L.), common ash, (*Fraxinus excelsior* L.), and hazel, (*Corylus avellana* L.) (Cross et al., 2010) could act rather than plant covers as reservoirs for the development of pear psylla natural enemies (Booth, 1992). In conclusion, despite plant cover enhanced the population of some predators, this did not lead to reduce pear psyllid abundance, and so is not an optimal form of plant cover for inclusion in integrated pear psyllid management system in pear orchards.

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Effects of arbuscular mycorrhizal fungi and *Rhizobium* on ion content and root characteristics of green bean and maize under intercropping

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ABSTRACT

In order to evaluate arbuscular mycorrhizal fungi and rhizobium bacteria effects on leaf nitrogen (N) and phosphorus (P) concentration and root characteristics of green bean and maize under intercropping, experiment was carried out in the research field of College of Agriculture, Payame Noor University of Azna, Lorestan, Iran. In experiment, sandy loam soil with pH 7.3 and EC 0.49 dS m^{-1} was used.The treatments comprised three cropping systems (sole cropping of green bean and maize, and intercropping), and four inoculations (control, arbuscular mycorrhizal fungi, rhizobium and mix of arbuscular mycorrhizal fungi and rhizobium). The results showed that inoculation with rhizobium improved length, diameter, volume and area of green bean root.The highest of green bean N, P concentration and root dry mass were observed in sole culture of green bean inoculated with arbuscular mycorrhizal fungi. Moreover, root length, diameter, volume and area of maize increased by arbuscular mycorrhizal fungi, and total concentration of N and P enhanced with use of rhizobium in sole cropping. Although the usage of *Rhizobium* and AMF can be affected on increasing the root growth and nutrient uptake of crops, application of bacterium and fungi combination at the same time would not be suitable. Overall, intercropping of maize with green bean caused to increase of leaf N and P concentrations and root growth of maize.

Key words: inoculation; AMfungi; rhizobacteria; intercropping; root length; root area; root dry mass; phosphorus; nitrogen

IZVLEČEK

UČINKI ARBUSKULARNE MIKORIZE IN BAKTERIJ IZ RODU *Rhizobium* NA VSEBNOST N in P TER LASTNOSTI KORENIN V MEDSETVENEM POSEVKU KORUZE IN FIŽOLA

Z namenom ovrednotenja učinkov arbuskularnih mikoriznih gliv in bakterij iz rodu *Rhizobium* na listno vsebnost dušika (N) in fosforja (P) in na lastnosti korenin navadnega fižola in koruze v medsetvi je bil narejen poljski poskus na raziskovalnem polju College of Agriculture, Payame Noor University of Azna, Lorestan, Iran. Tla v poskusu so bila ilovnato-peščena s pH 7.3 in EC 0.49 dS m^{-1}. Obravnavanja so obsegala tri setvene sisteme (čista setev fižola in koruze in medsetve) in štiri inokulacije (kontrola, arbuskularne mikorizne glive, rizobium in mešanica arbuskularnih mikoriznih gliv in rizobium). Rezultati so pokazali, da je inokulacija z rizobijem izboljšala dolžino, premer, volumen in površino korenin fižola. Največja vsebnost N, P in največja suha masa korenin fižola sta bili izmerjeni v čistem posevku fižola inokuliranem z arbuskularnimi mikoriznimi glivami. Dolžina korenin, premer, volumen in površina korenin koruze so se povečali pri inokulaciji z arbuskularnimi mikoriznimi glivami, a vsebnost celukopnega N in P se je povečala le pri čistem posevku koruze in inokulaciji z rizobijem. Čeprav uporaba rizobijuma in arbuskularnih mikoriznih gliv lahko poveča rast korenin in privzem hranil poljščin pa njihova hkratna uporaba ni vedno primerna. Na osnovi raziskave lahko zaključimo, da medsetev fižola v koruzo povzroči povečanje listne vsebnosti N in P koruze in poveča rast njenih korenin.

Ključne besede: inokulacija; arbuskularne mikorizne glive; rizobakterije; medsetev; dolžina korenin; površina korenin; suha masa korenin; P; N

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1 INTRODUCTION

Fertilizer of nitrogen (N) and phosphorus (P) are crucial to the growth of all plants. However, uncontrolled use of chemical and pesticides can destroy environment. In addition, the prices of these chemicals are high. Accordingly, application of plant growth promoting rhizobacteria and symbiotic arbuscular mycorrhizal fungi (AMF) can perform the goals of sustainable agriculture.The AMF symbiosis is an association between the roots of higher plants and soil fungi that promotes plant development, especially under sub-optimal growth conditions (Koltai et al.,2010). Previous studies suggested that AMF can promote plant N uptake and improve plant N nutrition (Corkidi et al., 2002). The successful association between plants and AMF is a strategy to improve the nutritional status of both, which reduces the use of fertilizers especially P.These fungi increase the surface area of roots and help in absorbing some diffusion-limited nutrients such as Zn, Fe and Cu (Almagrabi and Abdelmoneim, 2012). During the formation of arbuscular mycorrhizae, fungal hyphae enter the rhizodermal, exodermal and cortical cell layers of the roots, reaching the inner cortex, where the functional units, the arbuscules, develop. The fungi also form hyphae outside of the plant, extending the root-soil interface to facilitate the uptake of nutrients such as phosphates and water (Kistner and Parniske, 2002). Moreover, the beneficial plant-microbe interactions in the rhizosphere are the primary determinants of plant health and soil fertility (Klyuchnikov and Kozherin, 1990). *Rhizobium* species can exist as free-living soil saprophytes or as N_2 fixing endo-symbionts of legume host plants that is within the root nodules of host legumes or in close association with the plant roots (Shamseldin et al., 2008). Rhizobium, root-colonizing bacteria are known to influence plant growth by various direct or indirect mechanisms. Plant growth promoting bacteria are reported to influence the growth, yield, and nutrient uptake by an array of mechanisms. Some bacterial strains directly regulate plant physiology by mimicking synthesis of plant hormones, whereas others increase mineral and nitrogen availability in the soil as a way to augment growth (Yasmin et al., 2007).

Many intercropping systems have proved to be better than sole crops in terms of yield (Zhang et al., 2007) because intercropping makes better use of one or more agricultural resources both in time and in space (Rodrigo et al., 2001). Intercropping maize and various legumes has been investigated with species such as cowpea (*Vigna unguiculata* L.) and various species of bean (Dahmardeh et al., 2009; Armstrong et al., 2008; Contreras-Govea et al., 2009). In intercropping, absorption of N, P and K is more than pure cultures (Kuo and Jellum, 2002). Plant nutrient uptake can be improved by intercropping (Li et al., 2003). Nitrogen (N) transfer from the N_2 fixing legume to the maize and other species has also been reported (He et al., 2009). According to the above, the objective of this paper was to investigate the interactiveeffects of AMF, *Rhizobium* inoculation and intercropping on the leaf N and P concentrations, and root characteristics of green bean and maize.

2 MATERIALS AND METHODS

Field experiment was conducted in spring of 2013 at the research farm of College of Agriculture, Payame Noor University of Azna (PNU), Lorestan, Iran (latitude 38°05′ N, longitude 46°17′ E, and 1360 m above sea level). The climate of area is cold-dry and the average annual rainfall is 160 mm. Soil of experiment was a sandy loam-type; with pH and EC were 7.3 and 0.49 dS m^{-1}, respectively. The mean values of soil total N, available P and C contentswere 0.70 %, 15 mg kg^{-1} and 7.8 g kg-1, respectively. The study was laid out a factorial based on randomized complete block with three cropping systems (sole cropping of green bean and maize, and intercropping), and four inoculations (control, AMF, *Rhizobium* and mix of AMF and *Rhizobium*). Seeds of maize (*Zea mays* 'S.C. 704') and green bean (*Phaseolus vulgaris*‘Derakhshan’) were obtained from the Khoram Abad Agricultural Jihad Institute. The pattern of intercropping was a replacement series.The experimental plots consisted of 7 rows with 50 cm distance between rows and 20 cm between plants in the row. Fertilizers providing 15 kg of N (KNO_3) per hectare was applied at

sowing time. The mycorrhizal inoculum contained soil, plant roots and fragments of *Glomus mosseae* (T.H. Nicolson & Gerd.) Gerd. & Trappe (obtained from Turan Biotech Co), and symbiovar *trifolii* of mesorhizobium bacteria (*Rhizobium leguminosarum* (Frank 1879) Frank 1889) was obtained from the Mehr Asia Biotechnology Company (MABCo.). Before inoculation for more adhesion of inoculums, the seeds surface was mixed with 15 % sugar completely for 2 hours (Shariati et al., 2015). Seeds were washed with distilled water then inoculation was performed by a suspension of *Rhizobium* at the dose of 500 g per 100 kg^{-1} seed in the darkness at 20 - 26 °C. Finally, seeds were dried in the shade for 2 h. For AMF treatment, the amount of 15 g of mycorrhizal inoculum was placed 3 cm below of each seed at 2ndApril 2013. Irrigation was carried out as required to keep the soil water content near field capacity and weeds were controlled by hand. Plants were harvested and measured at flowering stage. To measure root characteristics, including root length, root diameter, root volume, root area and root dry mass, sampling was done at flowering stage. Root sampling was based on root profile observations and soil sample analysis. For each plant sample, a block of soil (66 cm length × 17 cm width × 20 cm depth) was extracted from the center region surrounding the plant. Each 20 cm layer of soil was placed into nylon netting bags, and sampling was done to 200 cm. Roots were washed and collected after the soil was passed through a 0.5 mm sieve using a hose and nozzle attachment. Root length (cm) and root area were obtained according to Newman (1966) and Bohn (1979) methods, respectively. Root volume was calculated by difference of initial water volume with second water volume after putting it in.

At flowering stage, five plants of each treatment were collected randomly. All samples were heat-treated at 105 °C for 30 min, dried at 70 °C and then ground into fine powder (passed through a 2 mm mesh screen). Nitrogen was analyzed by a micro-Kjeldahl procedure after digestion with H_2SO_4-H_2O_2 (Nelson and Sommers, 1973).Total P of leaves was estimated after digestion in di-acid mixture 9:1 ratio (HNO_3:$HClO_4$) using the standard methods described by AOAC (1970). Experimental factors were determined from analysis of variance (ANOVA) using the generalized linear model (GLM) procedure in SAS (SAS Institute, Cary, NC, USA). The mean values were compared by Duncan's test at 0.05 level of probability.

3 RESULTS AND DISCUSSION

3.1 Green bean traits

3.1.1 Nitrogen content

Analysis of variance indicated that the effects of cropping system, *Rhizobium* and interaction of cropping system × *Rhizobium* and AMF × *Rhizobium* were considerable on nitrogen content (Table 1).The results showed that application of rhizobium and AMF under different cropping systems increased nitrogen content of green bean. The maximum nitrogen content was recorded in sole culture of bean with AMF by 86.59 % and the minimum was observed in intercropping without inoculation by 49.4 % (Table 1). Nitrogen is an essential constituent of proteins, nucleic acids, some carbohydrates, lipids, and many metabolic intermediates involved in synthesis and transfer of energy molecules (Davis, 1980). Optimum growth of leguminous plants is usually dependent on symbiotic relationships with AMF and N_2-fixing bacteria (Xavier and Germida, 2003). AMF infection of plant roots usually stimulates plant growth through effects on nutrient uptake, nodulation, nitrogen fixation, and water supply (Redecker et al.,1997).

3.1.2 Phosphorus content

Leaf phosphorus content was significantly ($P \leq 0.01$) affected by cropping system, *Rhizobium* and interaction of cropping system × AMF and AMF × *Rhizobium* (Table 1).The highest phosphorus content was achieved in sole culture of bean with AMF; in contrast, the lowest was obtained in sole culture without inoculation (Table 1). Phosphorus plays a fundamental role in the very large number of enzymic reactions that depend on phosphorylation. Phosphorus is essential for cell division and development of meristem tissue (Vessey, 2003). AMF has strong mycelia, which expand the area of roots available

for absorption of nutrients, especially phosphorus (Ortas, 2012). Fungi showed high solubilization of P with reduction in the pH of the medium. The reduction in the soil pH, increase in available P and organic carbon was greater in inoculated by fungi as compared to non-inoculated which may be attributed to ability of such microorganisms to excrete organic acids, thereby decrease the pH and increase the concentration of phosphorus in soil by mechanisms involving chelation and exchange reactions (Reyes et al., 2006).There are many reports stating that P absorption and availability would be increased in mycorrhizally inoculated plants (Martin et al., 2012; Grace et al., 2009). AMF colonization can significantly promote plant P uptake from the soil, so that other functions are often inextricably linked with the improvement of P nutrition status (Cozzolino et al., 2010).

3.1.3 Root characteristics

Presented results in Table 1 clearly show that root dry mass, length, diameter, volume, area of green bean was noticeably influenced by cropping system, AMF and interaction of cropping system × *Rhizobium* and AMF × *Rhizobium* ($P \leq 0.01$).The effect of rhizobium was remarkable on root length, diameter and area. Moreover, the interaction of cropping system × AMF had considerable effect only on root volume and area (Table 1).

The maximum root dry mass of green bean was obtained in sole culture of bean with AMF treatment, while the minimum was produced under sole culture and none-inoculation (Table 1). AMF increased the surface area of roots and thus helped in absorbing some diffusion-limited nutrients. Based on the mean comparison result the highest root length, diameter, volume and area were recorded in mono-cropping inoculated with rhizobium; however, the lowest were observed in intercropping with use of rhizobium and AMF combination (Table 1). *Rhizobium* increases plant growth by various ways such as production of plant growth hormones, vitamins, siderophores, by solubilisation of insoluble phosphates, induction of systemic disease resistance and enhancement in stress resistance (Hussain et al., 2009). Zahir et al.,(2004) found that the inoculation by rhizobium increased root elongation and root dry mass in wheat. Induction of longer roots with increased number of root hairs and root laterals is a growth response attributed to IAA production by other bacteria. Reductionof root length, diameter, volume and area by use of dual rhizobium and AMF may be due to antagonistic activity of rhizobial inoculation (Gachande and Khansole, 2011).

Table 1: Effects of cropping system, *Rhizobium* and AMF on nitrogen and phosphorus content, and root characteristics of green bean. Means are average values of three replicates ± standard errors. Within the column, different letters indicate statistically significant difference between treatments.

Cropping system	Inoculation	Nitrogen content (%)	phosphorus content (ppm)	Root dry mass (g)	Root length (cm)	Root diameter (mm)	Root volume (mm^3)	Root area (cm^2)
Mono cropping	Control	59.9±1.02de	16.3±0.84d	3.25±0.77e	10±1.38c	6.01±0.37b	16.6±1.1b	53.4±1.28b
	AMF	86.5±3.05a	31.6±1.03a	8.61±0.96a	11.2±1.48b	5.67±0.23e	12±1.03d	38.7±1.58d
	Rhizobium	77.5±1.8ab	30.5±1.49ab	6.73±1.03b	15.8±1.03a	7.13±0.52a	19.1±1.06a	61.7±1.25a
	AMF+*Rhizobium*	62±1.93cd	27.6±1.25ab	5.83±0.68bc	11.9±1.93b	6.18±0.65b	14.1±1.126c	46.0±1.43c
Intercropping	Control	49.4±1.13e	22.1±0.87c	5.22±0.93c	9.04±1.37d	5.38±0.58d	11.6±1.26d	36.3±1.79e
	AMF	54.7±2.9de	16.5±0.63d	4.78±0.88cd	8.67±1.71d	5.2±0.44d	11.1±0.93d	35.6±1.07e
	Rhizobium	73.2±1.16bc	26.8±1.59b	5.1±0.63cd	9.5±1.67cd	5.52±0.58cd	11±0.96d	36.7±1.28de
	AMF+*Rhizobium*	74.4±1.29b	18.1±0.78cd	3.85±0.72de	5.94±1.44e	4.36±0.52e	6.67±0.61e	22.2±1.56f
Means within columns followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range tests.								
Analysis of variance								
Cropping system (C)		**	**	**	**	**	**	**
R (*Rhizobium*)		**	**	ns	**	**	ns	**
AMF (Arbuscular Mycorrhizal)		ns	ns	**	**	**	**	**
C×R		**	ns	**	**	**	**	**
C×AMF		ns	**	ns	ns	ns	**	**
R×AMF		**	**	**	**	**	**	**
C×R×AMF		**	**	**	*	ns	**	**

ns= non-significant, * significant in 5 %, ** significant in 1 %

3.2 Maize traits

3.2.1 Nitrogen content

The statistical analysis of the data revealed that the effects of cropping system and interactive effects of AMF × *Rhizobium* were remarkable on nitrogen content of maize plants (Table 2). The maximum nitrogen content was recorded in sole culture of maize with rhizobium and sole culture with AMF by almost 80 %. In contrast, the lowest belonged to mono-cropping of maize with combination of AMF and rhizobium by 65 % (Table 2). Mirzai et al., (2010) reported that the effect of the inoculation of a mixture of several free-living rhizobacteria have enhanced nitrogen accumulation of plants. Das and Saha (2005) indicated that the effect of non symbiotic N_2 fixing bacteria (*Azotobacter* and *Azospirillum*) were found significantly improved inorganic and organic nitrogen content in rhizosphere.

As an average, intercropping enhanced nitrogen content of maize in comparison with sole culture. Nitrogen transfer from the N_2-fixing legume to the maize and other species has also been reported (He et al., 2009), reducing the need for N fertilizer. Li et al., (2003) showed that plant nutrient uptake can be improved by intercropping. Growing plant species with differing root architecture in the same field also can increase nutrient use efficiency. Therefore, intercropping may be an important strategy to use N efficiently and to reduce the risks of N leaching.

3.2.2 Phosphorus content

Presented results in Table 2 clearly show that phosphorus content of maize was considerably influenced by cropping system, *Rhizobium*, AMF and interaction of cropping system × *Rhizobium* and AMF × *Rhizobium*. Inoculated maize plants with rhizobium under sole culture and also inoculated with AMF under intercropping had the highest of phosphorus content among other treatments. The lowest of phosphorus content was found under sole culture by 9.28 ppm (Table 2). Plant growth promoting rhizobacteria stimulate plant growth directly either by synthesizing hormones such as indole-3-acetic acid or by promoting nutrition, for example, by phosphate solubilization or more generally by accelerating mineralization processes (Kannahi and Kowsalya, 2013; Baniaghil et al., 2013).

Abdel-Fattah and Mohamedin (2000) demonstrated that phosphorus and nitrogen contents in shoots and roots of AMF sorghum plants were significantly greater than those of non-inoculated with AMF plants. Over 90 % of plants will engage in AMF symbiosis, which mainly improves the nutrient uptake of phosphorus, and several other nutrients (Bonfante, 2003). As a result of the increased uptake of P, plants inoculated with mycorrhizae frequently produce higher yields than do those without AMF (Martin et al., 2012). Maize is more competitive than green bean for phosphorus, so phosphorus supply for green bean in intercropping was less than its sole crop.

3.2.3 Root characteristics

Root dry mass was significantly ($P \leq 0.01$) affected by cropping system, *Rhizobium* and interaction of cropping system × AMF, cropping system × *Rhizobium* and AMF × *Rhizobium*. Effects of all treatments were noticeable on root length, diameter, volume and area (Table 2). The greatest root dry mass was recorded in intercropping without inoculation by 39.47 g. The treatment of intercropping with AMF had the maximum root length, diameter, volume and area. However, the least of root dry mass, length, diameter, volume and area were observed in sole culture of maize (Table 2).

Li et al., (2006) showed that roots of maize had not only penetrated deeper than those of the faba bean but had also spread under the faba bean strip in a maize and faba bean intercropping system. Adikuet al. (2001) indicated that although the roots of maize and cowpea had extended into the rhizospheres of each other, the encroachment on part of maize was much greater. In the maize and green bean intercropping that we studied, interspecific interactions resulted in higher N and P uptake in maize but lower in green bean. Such interactions were defined as asymmetric interspecific facilitation between the intercropped species by Li et al. (2006), who suggested that asymmetric interspecific facilitation results from greater lateral deployment of roots and increased root length of one crop, and that compatible spatial root distribution of the intercropped species contributes to the symmetric interspecific facilitation observed in faba bean and maize intercropping. Results of our experiment demonstrated that intercropping favored lateral spread of maize roots possibly the main reason for the superiority of maize over green bean in terms of root growth, N and P uptake.

Table 2: Effects of cropping system, *Rhizobium* and AMF on nitrogen and phosphorus content, and root characteristics of maize. Means are average values of three replicates ± standard errors. Within the column, different letters indicate statistically significant difference between treatments.

Cropping system	Inoculation	Nitrogen content (%)	phosphorus content (ppm)	Root dry mass (g)	Root length (cm)	Root diameter (mm)	Root volume (mm^3)	Root area (cm^2)
Mono cropping	Control	69±2.3c	9.28±1.3c	11.8±1.49e	58.8±2.35e	13.7±0.25e	20.1±2.16h	121±2.45e
	AMF	80.6±3.33a	14±1.67c	29.9±2.11b	121±3.59b	19.3±0.47b	97.5±3.21d	385±4.98b
	Rhizobium	82.2±2.37a	16.3±1.35a	25.8±2.03c	72.6±2.9d	15.2±0.19d	68±2.93e	249±3.59c
	AMF+*Rhizobium*	65±3.48c	15±0.94ab	22±1.01d	77.4±2.51d	15.7±0.58d	25±1.02g	155±3.59d
Intercropping	Control	79±3.36a	15.6±1.36ab	39.4±2.57a	104±3.55c	18.2±0.29c	49.3±3.283f	253±4.68c
	AMF	75.1±2.36c	16±1.13ab	31.3±2.18b	161±2.93a	22.7±0.76a	152±3.42a	554±5.22a
	Rhizobium	77.3±2.55bc	15.2±1.25ab	24.8±3.51cd	105±1.94c	19.3±0.87b	111±4.08c	358±4.59b
	AMF+*Rhizobium*	78±2.56b	14.9±1.03ab	25.4±2.42c	88.8±2.31c	16.9±0.42c	132±3.97b	383±4.32b
Means within columns followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range tests.								
Analysis of variance								
Cropping system (C)		*	**	**	**	**	**	**
R (*Rhizobium*)		ns	**	**	**	**	**	**
AMF (Arbuscular Mycorrhizal)		ns	*	ns	**	**	**	**
C×R		ns	**	**	**	**	**	**
C×AMF		ns	ns	**	**	**	**	**
R×AMF		**	**	**	**	**	**	**
C×R×AMF		**	*	**	**	ns	**	**

ns= non-significant, * significant in 5 %, ** significant in 1 %

4 CONCLUSION

Plants grown from seeds dressed with rhizobium showed high root length, diameter, volume and area for green bean, and N and P content for maize over the control. However, N and P content of green bean and root characteristics of maize in soil inoculated with AMF improved compared to none-inoculated. Although the usage of rhizobium and AMF can be affected on increasing the root growthand nutrient uptake of crops, application of bacterium and fungi combination at the same time would not be suitable. This study showed that maize and green bean intercropping could increase root growth of maize. As an average, compared with mono-cropped, the N and P content of green bean were decreased by intercropping; however, for maize were intensified. In fact, green bean as a legume can help nutrient uptake such as N and P of maize slightly. Similar trend was observed in root characteristics of green bean and maize. Therefore, intercropping of maize and green bean caused to increase of leaf N and P concentrations and root growth of maize.

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Inducing salt tolerance in sweet corn by magnetic priming

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ABSTRACT

This study evaluates seed germination and growth of sweet corn under NaCl stress (0, 50, and 100 mM), after exposing the seeds to weak (15 mT) or strong (150 mT) magnetic fields (MF) for different durations (0, 6, 12, and 24 hours). Salinity reduced seed germination and plant growth. MF treatments enhanced rate and percentage of germination and improved plant growth, regardless of salinity. Higher germination rate was obtained by the stronger MF, however, the seedling were more vigorous after priming with 15 mT MF. Proline accumulation was observed in parallel with the loss of plant water content under 100 mM NaCl stress. MF prevented proline accumulation by improving water absorption. Positive correlation between H_2O_2 accumulation and membrane thermostability (MTI) was found after MF treatments, which revealed that MF primed the plant for salinity by H_2O_2 signaling. However, over-accumulation of H_2O_2 after prolonged MF exposure adversely affected MTI under severe salt stress. In conclusion, magnetic priming for 6 hours was suggested for enhancing germination and growth of sweet corn under salt stress.

Key words: hydrogen peroxide; maize; malondialdehyde; plant water content; proline; seed germination; seedling growth

Abbreviations: DM: dry mass, FM: fresh mass, MDA: malondialdehyde, MF: magnetic field, MGT: mean germination time, MTI: membrane thermostability index, PWC: plant water content, ROS: reactive oxygen species.

IZVLEČEK

INDUKCIJA TOLERANCE NA SOL SLADKE KORUZE S PREDHODNIM OBRAVNAVANJEM SEMEN Z MAGNETNIM POLJEM

V raziskavi sta bili ovrednoteni kalitev in rast sladke koruze v razmerah solnega stresa (NaCl; 0, 50, in 100 mM), po izpostavitvi semen šibkemu (15 mT) in močnemu (150 mT) magnetnemu polju (MF) v trajanju 0, 6, 12, in 24 ur. Slanost je zmanjšala kalitev semen in rast rastlin. Obravnavanje z magnetnim poljem je povečalo hitrost in odstotek kalitve ter izboljšalo rast rastlin, ne glede na slanost. Kalitev je bila značilno večja pri obravnavanju z močnejšim magnetnim poljem, kalice so bile bolj vitalne pri obravnavanju z 15 mT MF. Akumulacija prolina je bila opažena sočasno z izgubo vsebnosti vode v razmerah močnega NaCl stresa. Obravnavanje z magnetnim poljem je izboljšalo absorbcijo vode in preprečilo akumulacijo prolina. Pozitivna korelacija med akumulacijo H_2O_2 in termostabilnostjo membran (MTI), ki je bila opažena po obravnavanju z MF je pokazala, da so bile tako obravnavane rastline odpornejše na slanost preko H_2O_2 signalizacije. Kljub temu je prekomerna akumulacija H_2O_2, kot posledica podaljšane izpostavitve MF, negativno vplivala na MTI v razmerah solnega stresa. Zaključimo lahko, da predobravnavanje semen z magnetnim poljem za 6 ur pospeši nihovo kalitev in rast rastlin v razmerah solnega stresa.

Ključne besede: vodikov peroksid; koruza; malondialdehid; vsebnost vode; prolin; kalitev; rast kalic

Abbreviations: DM: dry mass, FM: fresh mass, MDA: malondialdehyde, MF: magnetic field, MGT: mean germination time, MTI: membrane thermostability index, PWC: plant water content, ROS: reactive oxygen species.

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1 INTRODUCTION

Salinity was always known to be a constraint to agricultural production. In the 21st century, however, it is predicted that enhanced soil salinization due to climate change and warming of the planet will be the major challenge in agriculture. The deleterious effects of salt stress on plant are associated with low osmotic potential of soil solution (osmotic stress), nutritional imbalance, specific ion effect (salt stress), and interactions among these factors (Ashraf, 2004). Most of the plant species exhibit over-sensitive responses to salt stress at seed germination and early growth stages of seedling (Cuartero et al., 2006). Seed germination may be delayed or prevented by salt stress (Lin et al., 2011). Several strategies can be employed to overcome the adverse effects of salinity. In addition to the use of the classic breeding and plant genetic transformation of crops, employing seed pre-sowing treatments as a valuable strategy may facilitate seed germination and seedling growth under abiotic stress such as salinity (Iqbal and Ashraf, 2010).

Magnetic field (MF) treatments have shown to enhance seed germination, plant vigor and productivity (Vashisth and Nagarajan, 2010; Radhakrishnan and Kumari, 2012), and delay the senescence process in plant organs (Piacentini et al., 2001). Such effects may be due to promotion of gene expression, protein biosynthesis, enzymes activity, cell reproduction and overall metabolism of plant (Stange et al., 2002; Atak et al., 2007; Vashisth and Nagarajan, 2010). Rapid germination and vigor of seedlings is linked with enhancement of activities of α-amylase, dehydrogenase and protease in seeds following MF exposure (Vashisth and Nagarajan, 2010; Radhakrishnan and Kumari, 2012). Seeds that have been treated by MF for a short time generate more extensive root system as well as more vigorous shoot compared to untreated seeds (Florez et al., 2007; Vashisth and Nagarajan, 2010). Therefore, MF may improve germination parameters and initial growth of non-standard seeds (Aladjadjiyan, 2002).

In spite of the positive effects of MF on plant, there are limited numbers of studies that examine the effects of MF treatments on plant responses to environmental stresses. It has shown that magnetic and electromagnetic treatments enhance the saline-alkali tolerance (Xi et al., 1994), enhance seed germination and seedling growth under heat stress (Ružič and Jerman, 2002; Cakmak et al., 2010), and improve plant osmotic tolerance (Karimi et al., 2012). In contrast, Yao et al. (2005) showed that MF treatments enhanced sensitivity of cucumber seedlings to UV irradiation. Therefore, the current study was conducted to investigate whether the benefits of pre-sawing MF exposure would improve sweet corn seed germination and vigor under salt stress, and to determine the effects of MF intensity and exposure duration on plant responses to abiotic stress.

2 MATERIAL AND METHODS

Uniform seeds of sweet corn (*Zea mays* var. *saccharata* 'SC 404') were selected and disinfected with 0.3 % fungicide Benomyl (GYAH Corp., Iran) for 15 min and thoroughly washed with sterilized distilled water. The seeds were soaked for 3 hours (h) in distilled water and then air dried prior to starting the experiment. Twenty-five seeds were placed between two Whatman No. 1 filter papers laid in the Petri dishes and incubated at 30/25 °C day/night temperature, 12 h photoperiod with light intensity of 30 W m^{-2}. Salt treatments were applied by adding NaCl solutions to the Petri dishes (0 - control, 50 and 100 mM). The Petri dishes were irrigated with 5 ml salt solution of the respective treatment every 24 hours. The filter beds were changed every 48 hours to avoid salt accumulation.

The MF treatments were induced using two cubic magnets for each Petri dish. The geometric characteristics of the magnets were 100 mm length, 30 mm width and 20 mm thickness (Figure 1). They were arranged at 0, and 5.5 cm apart from bottom and top of the Petri dishes to generate 150 and 15 mT magnetic fields, respectively. The intensity of magnetic fields was provided by the

Department of Materials Science and Engineering, Engineering School of Shiraz University. The seeds were exposed to the strong and weak magnetic fields for different durations (0, 6, 12, and 24 h). The Petri dishes were kept at least 100 cm apart, to avoid the influence of the magnets on each other.

Figure 1: The geometric characteristics of the magnets used in the experiments (100×30×20 mm).

Seed germination was evaluated according to the guidelines issued by the International Seed Testing Association (ISTA, 2004). The number of germinated seeds was recorded every 6 hours for a period of 5 days. A seed was considered germinated when its seminal root had emerged ≥ 2.0 mm. Total germination was recorded as overall percentage of germinated seeds. The germination rate was determined by measuring the length of time required for maximum germination percentage, and by calculating mean germination time (MGT) using the following formula (Eq. 1):

$$MGT = \sum_{i=1}^{n} N_i Ti \; / \sum_{i=1}^{n} N_i \qquad (1)$$

In this formula: *Ti* is the initial time and *Ni* is the number of germinated seeds between *Ti*-1 and *Ti*.

To evaluate the early growth of the plant in response to the MF and the NaCl stress, another experiment was conducted under the same conditions as described above. Ten uniform and sterilized seeds were subjected to 0, 50 and 100 mM NaCl, after exposing to the magnetic treatments as describe in the first experiment. After 7 days, the seedlings were harvested and length, fresh and dry mass of root and shoot were measured. Dry mass was measured after drying at 70 °C for 72 hours. Plant water content was calculated according to fresh and dry mass of the seedlings.

Proline was measured in 200 mg of dried shoot tissue using the method described by Bates et al. (1973). The plant material was homogenized in 3 % sulfosalicylic acid and the extract was reacted with glacial acetic acid and ninhydrin in boiling water. The reaction mixture was extracted with toluene. The absorbance was measured at 520 nm by spectrophotometry (Shimadzu model 160 A). L-proline was used as standard.

The membrane thermostability index (MTI) was assessed by measuring electrolyte leakage derived from the shoot tissue by adopting an electrical conductivity meter (Ohm 419). MTI was applied to find out changes in cell membrane permeability according to the technique of Arora et al (1998) with slight modifications. Concentration of hydrogen peroxide (H_2O_2) was determined in plant tissues according to the method described by Velikova et al. (2000). Shoot and root tissue was homogenized in trichloroacetic acid (TCA) and centrifuging, the supernatant was added to potassium phosphate buffer and KI. The absorbance was measured spectrophotometrically at 390 nm (Shimadzu model 160 A). Lipid peroxidation was determined by calculating the quantity of malondialdehyde (MDA) using the thiobarbituric acid reactive substances method explained by Heath and Parker (1968). Shoots and roots of the plants were homogenized in TCA and after centrifuging, the supernatant was mixed with thiobarbituric acid (TBA) in 20 % TCA. The mixture was then heated up at 95 °C for 30 minutes. After cooling and centrifugation at 10,000 × g for 10 minutes, the supernatant absorbance was verified at 532 and 600 nm. MDA content (nmol g^{-1} FM) was calculated according to extinction coefficient of 155 mM cm^{-1}, after withdrawing the non-specific absorbance at 600 nm.

The treatments were arranged as a factorial experiment based on a completely randomized design comprising 3 factors (NaCl levels, MF intensities and MF exposure durations) with 4 replications. The data were subjected to an analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) at $P \leq 0.05$ was used for comparing the means. Spearman bivariate correlation test was used to investigate the correlation between the measured parameters. The statistical analyses were performed by SPSS (v 21.0).

3 RESULTS

Salinity treatments did not affect germination percentage during the first 24-h after incubation (Table 1). At this stage, the highest germination percentage was observed in 150 mT (28.6 %), and the control treatment had the lowest value (4.7 %). The highest germination percentage was found in the 6-h MF exposure treatment (25.3 %). Salt stress induced by 100 mM NaCl significantly reduced germination percentage at 48-h stage. Magnetic exposure enhanced seed germination, however, no significant difference was observed between 15 and 150 mT intensities and the highest seed germination was observed in the 6-h and 12-h MF exposure durations. After 72-h hours, the adverse effects of 50 mM NaCl stress became evident on seed germination. Effects of magnetic exposure on enhancing seed germination was significant at this stage, however, no significant difference was observed between 15 and 150 mT intensities (79.0 and 82.5 %, respectively). Seeds exposed to MF for either 6 or 12 hours showed the highest germination at this stage (86.0 and 81.5 %, respectively). Final germination percentage was significantly reduced under salt stress and no significant difference was found between 50 and 100 mM NaCl treatments. The highest percentage of germinated seeds was obtained in the 6-h magnetic treatment (89.5 %).

The time required for maximum germination percentage was significantly increased by 50 and 100 mM NaCl treatments (78.6 and 77.2 h, respectively) when compared to the control (68.6 h). MF exposure significantly reduced the time for max germination. A significant interaction effect of NaCl stress and MF exposure duration on the time required for max germination was detected (Figure 2). The interaction effect showed that by increasing NaCl concentration in medium, time for maximum germination of non-magnetic treated seeds significantly increased, however, the time was reduced to the control level in the 6-h and 12-h magnetic treated seeds. MGT, indicating overall seed germination rate, was significantly reduced by application of 100 mM NaCl (72.7 h). MGT of the non-MF treated seeds was 75 h. The time was significantly reduced to 72.6 and 69.1 h by 15 and 150 mT MF intensities, respectively. The lowest MGT was found in 6-h and 12-h MF exposure durations (69.7 and 70.9 h, respectively).

Table 1: Effects of salinity stress and magnetic treatments on seed germination of *Zea mays* var. *saccharata*

	Germination [%] – 24h	Germination [%] – 48h	Germination [%] – 72h	Max germination [%]	Time to max germination [h]	MGT [h]
NaCl [mM]						
0	18.0	65.8^{a†}	86.5^{a}	89.7^{a}	68.6^{b}	70.9^{b}
50	18.7	62.6^{a}	78.9^{b}	84.2^{b}	78.6^{a}	71.0^{b}
100	14.3	50.3^{b}	73.4^{b}	80.7^{b}	77.2^{a}	72.7^{a}
MF Intensity [mT]						
0	4.7^{c}	49.3^{b}	72.0^{b}	82.7	84.5^{a}	75.0^{a}
15	10.7^{b}	60.6^{a}	79.0^{a}	83.8	73.8^{b}	72.6^{b}
150	28.6^{a}	63.7^{a}	82.5^{a}	87.4	70.3^{b}	69.1^{c}
MF Exposure Duration [h]						
0	4.7^{d}	49.3^{b}	72.0^{b}	82.7^{b}	84.5^{a}	75.0^{a}
6	25.3^{a}	67.0^{a}	86.0^{a}	89.5^{a}	71.7^{b}	69.7^{c}
12	19.0^{b}	64.5^{a}	81.5^{a}	86.5^{ab}	70.8^{b}	70.9^{c}
24	11.8^{c}	53.2^{b}	72.8^{b}	80.8^{b}	73.8^{b}	72.4^{b}
ANOVA						
NaCl	ns	**	**	**	**	**
Intensity	**	*	*	ns	**	**
Duration	**	**	**	**	*	**
NaCl×Int.	ns	ns	ns	ns	ns	ns
NaCl×Dur.	ns	ns	ns	ns	*	ns
Int.× Dur.	ns	ns	ns	ns	ns	ns
NaCl×Int.×Dur.	ns	ns	ns	ns	ns	ns

** and *: Significant at 0.01 and 0.05 levels, respectively; ns: Non-significant.

†. Means separation by DMRT ($P \leq 0.05$).

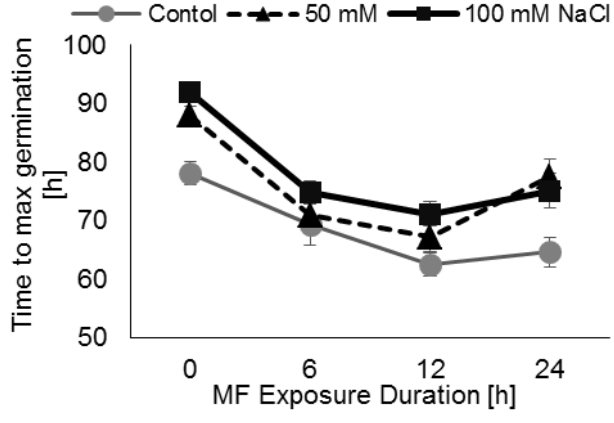

Figure 2: The interaction effect of magnetic field exposure duration and NaCl concentration in medium on time required for maximum seed germination percentage of *Zea mays* var. *saccharata* (Data are means ± SEM of four independent samples).

Table 2 represents the effects of MF treatments and NaCl stress on growth parameters of sweet corn seedlings. Salt stress significantly reduced the fresh mass (FM) of sweet corn shoot. The highest shoot FM was obtained in the control treatment (248.8 mg) and the lowest FM was observed in 100 mM NaCl treatment (216.9 mg). Magnetic exposure significantly increased the shoot FM; however, no significant differences were found between the weak and the strong MF or between the different magnetic exposure durations. Shoot dry mass (DM) was significantly affected by MF intensity and was significantly higher in 15 mT MF.

A significant reduction in root FM was observed under 100 mM NaCl stress. Exposing to 15 mT MF significantly increased root FM (160.1 mg)

and the highest root FM was found in the 12-h and 24-h MF treatments (143.5 and 144.7 mg, respectively). Root DM was significantly reduced under 50 mM (9.3 mg) and 100 mM NaCl stress (6.6 mg) when compared to control treatment (11.9 mg). MF treatments significantly increased root DM, however, no significant differences were found between 15 and 150 mT intensities or between the different MF exposure durations.

Table 2: Effects of salinity stress and magnetic treatments on fresh mass (FM) and dry mass (DM) of *Zea mays* var. *saccharata* seedling

	Shoot FM [mg]	Shoot DM [mg]	Root FM [mg]	Root DM [mg]
NaCl [mM]				
0	248.8^{a†}	21.2	143.6^{a}	11.9^{a}
50	227.1^{ab}	19.1	125.4^{a}	9.3^{b}
100	216.9^{b}	18.0	100.2^{b}	6.6^{c}
MF Intensity [mT]				
0	198.9^{b}	16.9^{b}	94.7^{b}	6.6^{b}
15	242.9^{a}	23.4^{a}	160.1^{a}	11.1^{a}
150	245.9^{a}	17.3^{b}	117.3^{b}	9.2^{a}
MF Exposure [h]				
0	198.9^{b}	16.9^{b}	94.7^{b}	6.6^{b}
6	243.3^{a}	21.7^{a}	119.6^{ab}	10.7^{a}
12	247.7^{a}	19.4^{ab}	143.5^{a}	9.3^{a}
24	242.6^{a}	19.5^{ab}	144.7^{a}	10.4^{a}
ANOVA				
NaCl	**	ns	**	**
Intensity	*	**	**	*
Duration	*	*	*	*
NaCl×Int.	ns	ns	ns	ns
NaCl×Dur.	ns	ns	ns	ns
Int.× Dur.	ns	ns	ns	ns
NaCl×Int.×Dur.	ns	ns	ns	ns

** and *: Significant at 0.01 and 0.05 levels, respectively; ns: Non-significant.
†. Means separation by DMRT ($P \leq 0.05$).

Plant biomass was significantly reduced under both 50 mM (28.4 mg) and 100 mM NaCl stress (24.6 mg) when compared to control treatment (33.1 mg). The highest plant biomass was found in 15 mT MF treatments. However, no significant differences were observed between 6-h, 12-h and 24-h MF exposure durations (Table 3). Shoot:root DM was significantly increased by both 50 mM and 100 mM NaCl stress, respectively. MF treatments significantly reduced shoot to root ratio, however, no significant differences were found between 15 and 150 mT MF intensities. Shoot to root ratio of the 12-h and 24-h MF treatments was significantly lower when compared to the other treatments.

Salinity significantly reduced shoot length and the lowest plant height (38.4 mm) was found under 100 mM NaCl stress (Table 3). Shoot length of sweet corn seedlings significantly increased by MF treatments; however, no significant differences were observed between 15 and 150 mT intensities and between the different MF exposure durations. Root length was significantly reduced under salt stress treatments. The highest root length (84.8 mm) was observed in 15 mT MF treatments and the lowest value was found in non-magnetic treated plants (50.1 mm). Although MF exposure significantly increased root length of sweet corn, no significant differences were found between 6-h, 12-h and 24-h MF exposure durations.

Table 3: Effects of salinity stress and magnetic treatments on fresh mass (FM) and dry mass (DM) of *Zea mays* var. *saccharata* seedling

	Biomass [mg]	Shoot:Root [Dry mass]	Shoot Length [mm]	Root Length [mm]
NaCl [mM]				
0	33.1^{a†}	2.0^{b}	44.5^{a}	90.2^{a}
50	28.4^{b}	2.2^{b}	41.2^{ab}	54.6^{b}
100	24.6^{c}	4.3^{a}	38.4^{b}	55.7^{b}
MF Intensity [mT]				
0	23.5^{c}	4.0^{a}	35.9^{b}	50.1^{c}
15	34.5^{a}	2.3^{b}	43.1^{a}	84.8^{a}
150	26.5^{b}	2.3^{b}	44.4^{a}	69.9^{b}
MF Exposure [h]				
0	23.5^{b}	4.0^{a}	35.9^{b}	50.1^{b}
6	32.4^{a}	2.8^{ab}	43.4^{a}	75.4^{a}
12	28.7^{a}	2.4^{b}	45.1^{a}	77.7^{a}
24	29.9^{a}	2.0^{b}	42.6^{a}	75.5^{a}
ANOVA				
NaCl	**	**	**	**
Intensity	**	*	*	*
Duration	*	*	*	*
NaCl×Int.	ns	ns	ns	ns
NaCl×Dur.	ns	ns	ns	ns
Int.× Dur.	ns	ns	ns	ns
NaCl×Int.×Dur.	ns	ns	ns	ns

** and *: Significant at 0.01 and 0.05 levels, respectively; ns: Non-significant.
†. Means separation by DMRT ($P \leq 0.05$).

Plant water content (PWC) was significantly reduced by 100 mM NaCl stress. MF exposure significantly increased PWC, however, no significant differences were observed between the MF intensities or between the different MF exposure durations (Table 4). Significant proline accumulation was observed in shoot under 100 mM NaCl. MF exposure significantly prevented proline accumulation and the lowest proline concentration was found in 6-h magnetic exposure (Table 4).

Table 4: Effects of salinity stress and magnetic treatments on plant water content (PWC), membrane stability index (MSI) and concentration of proline, malondialdehyde (MDA) and H_2O_2 in shoot of *Zea mays* L. var. *saccharata*

	PWC [mg plant^{-1}]	Proline [μmol g^{-1} DM]	MTI [%]	H_2O_2 [μmol g FM^{-1}]	MDA
NaCl (mM)					
0	359.3^{a†}	186.3^{b}	81.7^{a}	2.99^{b}	0.100^{b}
50	324.1^{ab}	186.6^{b}	77.2^{a}	3.11^{b}	0.118^{b}
100	292.5^{b}	239.8^{a}	65.9^{b}	3.48^{a}	0.173^{a}
MF Intensity (mT)					
0	270.1^{b}	282.2^{a}	72.1^{b}	3.23	0.151
15	368.5^{a}	177.8^{b}	78.7^{a}	3.21	0.120
150	336.7^{a}	184.0^{b}	80.5^{a}	3.44	0.123
MF Exposure (h)					
0	270.1^{b}	282.2^{a}	71.9^{c}	3.23	0.141^{a}
6	330.5^{a}	157.3^{c}	78.1^{b}	3.12	0.102^{b}
12	362.5^{a}	203.4^{b}	80.1^{a}	3.13	0.118^{b}
24	327.4^{a}	182.1^{bc}	70.9^{c}	3.30	0.158^{a}

Continued overleaf / nadaljevanje na naslednji stran

ANOVA					
NaCl	**	**	**	**	**
Intensity	*	*	*	ns	ns
Duration	*	*	*	ns	*
NaCl×Int.	ns	ns	ns	ns	ns
NaCl×Dur.	ns	**	*	*	ns
Int.× Dur.	ns	ns	ns	ns	ns
NaCl×Int.×Dur.	ns	ns	ns	ns	ns

** and *: Significant at 0.01 and 0.05 levels, respectively; ns: Non-significant.
†. Means separation by DMRT ($P \leq 0.05$).

The interaction of MF exposure duration and NaCl concentration significantly affected proline concentration in shoot (Figure 3). The interaction effect indicated that magnetic exposure for 6 or 12 hours reduced proline concentration in the plant shoot to the control level, however, a significant increase in proline concentration was observed in the seedling exposed to 24-h MF treatment in combination with salt stress treatments. A significant positive correlation was observed between PWC and proline concentration in the leaves (Figure 4).

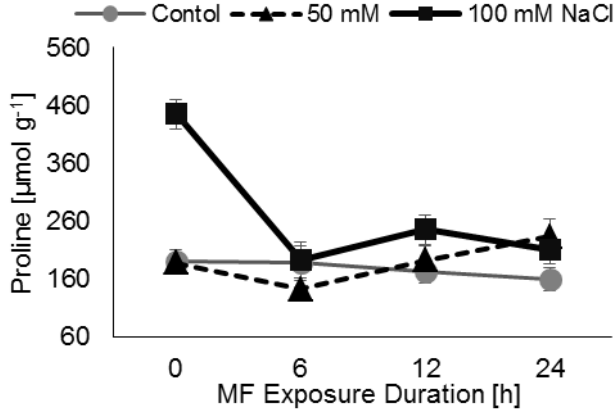

Figure 3: The interaction effect of magnetic field exposure duration and NaCl concentration in medium on concentration of proline in shoot of *Zea mays* var. *saccharata* (Data are means ± SEM of four independent samples)

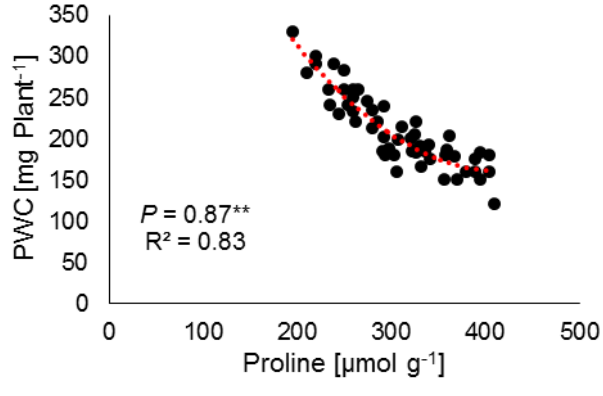

Figure 4: Correlation between plant water content (PWC) and proline concentration in the shoot of *Zea mays* var. *saccharata*

Application of 100 mM NaCl significantly reduced MTI in shoot by 15.8 % (Table 4). MF pretreatments significantly improved MTI as the highest MTI was obtained in 12-h MF exposure (80 %). MTI was significantly affected by the interaction effect of salt stress and magnetic exposure duration (Figure 5). The interaction effect indicated that although MF pretreatment of seed enhanced MTI in the seedlings under salt stress, by increasing MF exposure up to 24 hours a significant reduction in MTI was observed under 100 mM NaCl stress. In this order, the 6-h treatment enhanced plasma membrane thermostability of salt affected plants up to the control level. A significant accumulation of H_2O_2 in plant tissues was observed in 100 mM NaCl treatment (Table 4). A significant interaction effect of NaCl level and MF exposure duration on H_2O_2 was found, which indicated that magnetic exposure induced H_2O_2 accumulation in plant of non-saline treatment. However, exposing the seeds for 6 or 12 hours to MF reduced H_2O_2 accumulation in the plants under salt stress, although the trend was not statistically significant. On the other hand, MF treatment for 24 hours did not reduce H_2O_2 content under 100 mM NaCl stress (Figure 5). A positive correlation was found between H_2O_2 content and plasma membrane thermostability index (Figure 6).

Accumulation of MDA was observed in the plants under salt stress (Table 4), which in parallel MTI significantly reduced (Figure 6). The highest MDA concentration was detected in plants under 100 mM NaCl treatment. Magnetic exposure of seeds for 6 and 12 hours significantly reduced MDA content in plant and no significant difference was detected between the week and the strong magnetic fields.

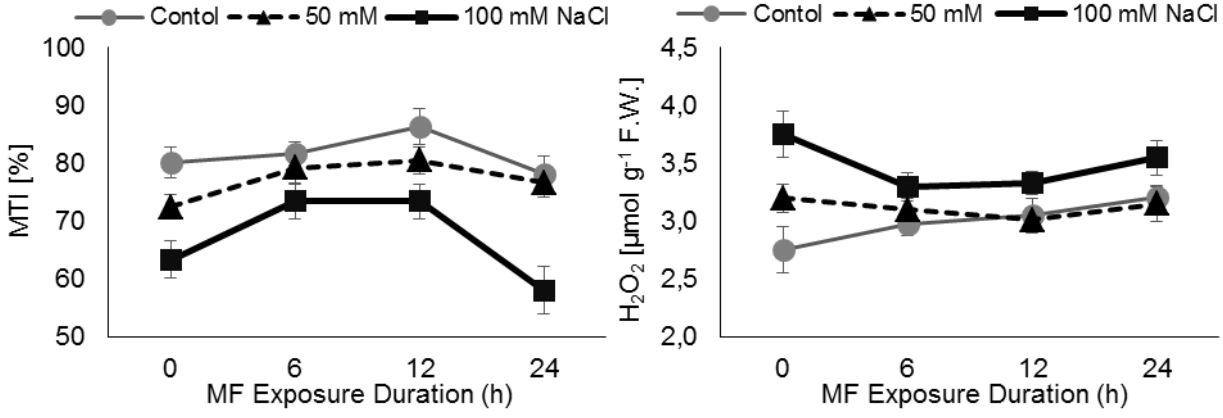

Figure 5: The interaction effect of magnetic field exposure duration and NaCl concentration in medium on plasma membrane thermostability index (MTI) in shoot of *Zea mays* var. *saccharata* (Data are means ± SEM of four independent samples)

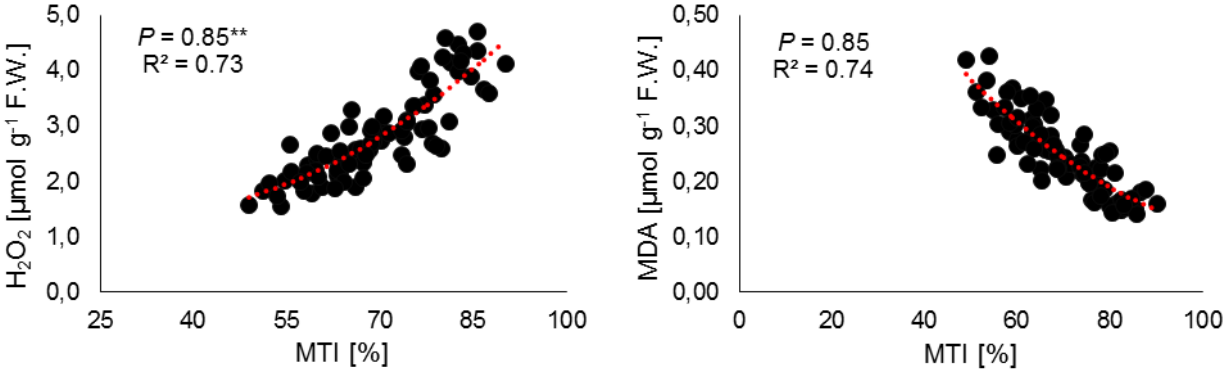

Figure 6: Correlation between membrane stability index (MSI) and concentration of malondialdehyde (MDA) and H_2O_2 in the shoot of *Zea mays* var. *saccharata*

4 DISCUSSION

The model plant which was used for this experiment, *Z. mays* var. *saccharata*, is a salt sensitive species (Chinnusamy et al., 2005; Cha-Um and Kirdmanee, 2011). In recent years, improving salt tolerance of this species has attained more attention in breeding programs (Bänziger et al., 2006; Cha-Um and Kirdmanee, 2011). In the current study adverse effects of salt stress were observed on the plant during seed germination and early growth stages. Ashraf and Rauf (2001) also reported the adverse effects of NaCl stress on sweet corn during seed germination and seedling growth stages. It has not been clarified which component of salt stress mainly affects plant during seed germination. Our results indicated that salt stress did not affect sweet corn seed germination during early 24 hours after salt treatment, however, the adverse effects of 100 mM NaCl stress became evident after 48 h. The detrimental effects of salinity were increased by time, as the final seed germination was equal in 50 and 100 mM NaCl treatments. Similar results were found in the case of germination rate indices. The results suggest that during the early stages, seed germination rate is inhibited through reducing imbibition due to osmotic impact of salt stress. However, with increasing the contact between salts and the seeds over time, the germination percentage is limited due to ion toxicity. A recent report by Lin et al. (2011) confirms this conclusion.

Ramoliya et al. (2006) stated that salinity suppresses shoot growth more than root growth. Similar results are evident in Ashraf and Rauf (2001) research on sweet corn. However, in the current study, salt stress did not affect biomass accumulation in plant shoot. Accordingly, the reductions in shoot FM and elongation were due to water deficit under salt stress. Cha-Um and Kirdmanee (2011) stated that limitation of water availability in growing medium due to reduced osmotic potential is detrimental to sweet corn growth under salt stress. In the current study, the reduced PWC which was observed under salt stress confirms this finding. Root showed more susceptibility to salt stress as biomass accumulation in root was reduced to less than 25 % when compared to shoots under 100 mM NaCl stress. Therefore, the reduced biomass of the seedlings under salt stress was mainly due to the inhibition of root growth. The reduction in biomass accumulation in plant organs is a result of slower growth and development due to osmotic effect of salinity (Shani and Ben-Gal, 2005) and a decline in net photosynthesis (Arfan et al., 2007). Moreover, enhanced respiration of cell under this situation (Jacoby et al., 2011) and injuries to cells in growing points result in reduction in overall sink capacity and plant growth (Moradi and Ismail, 2007). The detrimental effects of NaCl stress on sweet corn growth in terms of dry matter production and water absorption was previously shown by other authors (Shenker et al., 2003).

Different types of pre-sawing treatments can be used for improving seed germination and seedling establishment under abiotic stress conditions (Ashraf and Rauf, 2001; Iqbal and Ashraf, 2010). Several reports have showed the positive effects of magnetic treatments on seed germination and plant growth (Aladjadjiyan, 2002; Podlesni et al., 2004; Yao et al., 2005; Fl´orez et al., 2007). However, reports on evaluating the performance of MF-primed plants under abiotic stress are scarce. The results of the current study indicated that magnetic priming treatments enhance seed germination and growth of sweet corn seedling under non-saline and salt stress conditions. Yao et al. (2005) and Fl´orez et al. (2007) stated that increasing MF intensity enhances seed germination and plant growth. In the current study, although the stronger MF significantly reduced MGT and time required for obtaining the final germination percentage, increasing MF intensity did not affect the final germination percentage. These effects were mainly due to increasing germination percentage till 24 h after exposure and such effects were gradually reduced afterward. Hence, it can be concluded that increasing MF intensity mainly improves rate of germination. This positive effect appears not to be durable and is vanished over time.

The results revealed that enhancement of water absorption after MF exposure was a major factor in the mechanism of improvement of seed germination and seedling growth under salt stress. Other studies also showed that seeds or plants exposed to MF absorb more moisture (Garcia and Arza, 2001; Karimi et al., 2012). The mechanism

of MF treatment on promoting seed imbibition is not completely known, but it may be a result of the changes in intracellular levels of Ca^{++} and other ionic current density across cellular membrane which increase osmotic pressure and cellular membrane capacity to absorb more water (Dhawi and Al-Khayri, 2011). In a previous study, we found that MF exposure improves osmotic stress tolerance of plant by enhancing osmotic adjustment capability and improving water availability to plant (Karimi et al., 2012). Moreover, an increase in activity of enzymes after MF exposure can be involved in higher seed germination and vigor under salt stress (Vashisth and Nagarajan, 2010). Atak et al. (2007) reported that the peroxidase activity increases as plants passes through MF. Moreover, Sahebjamei et al. (2009) showed that activity of superoxide dismutase also enhances in cell after MF exposure. Enhancement of ROS scavenger enzymes is critical for salt tolerance of plant.

In the current study, a significant increase in concentration of proline in the root of non-primed plants was observed under salt stress. The accumulation of proline in sweet corn in response to salt stress was reported in previous studies (Cha-Um and Kirdmanee, 2011). Proline accumulation is a widespread response of plants to environmental stresses (Anjum, 2008), which is shown to be involved in defense of plants against salinity and osmotic stress (Asraf, 2004; Karimi et al., 2012). Accumulation of ions in tissues and/or tissue dehydration under salt stress may trigger proline accumulation. In this study, proline accumulation in shoot was in parallel with reduction of PWC. The results revealed that water deficit is the primarily responsible for proline accumulation in plant under salt stress. The effects of MF treatments on promoting PWC and preventing proline accumulation confirm this hypothesis. Another possible conclusion is that the accumulation of proline may be regarded as a good index of salt stress pressure on this species. These conclusions are in accordance with Cha-Um and Kirdmanee (2011) that showed proline accumulation in salt sensitive maize cultivar in response to salt stress is significantly higher than the salt tolerant genotype. Similar results also have been reported in other crops such as rice and sorghum, grown under salt stress (de Lacerda, et al., 2005; Demiral and Türkan, 2005).

Although MF exposure enhanced sweet corn germination capacity, some trends in reducing seed germination and seed germination rate were observed by increasing MF exposure duration up to 24 h. Generally, seed germination and seedling growth after 24-h exposure to MF were less than the other MF treatments. On the other hand, MF intensity 15 mT was also more effective than 150 mT in improving seedling growth parameters. Carbonel et al. (2000) and Vashisth and Nagarajan (2010) also reported that strong magnetic fields may adversely affect seed germination and plant growth. The mechanism of such detrimental effects of exposing to strong magnetic fields or prolonged MF treatments are unknown; however, MF may induce mutation and DNA damage in plant (Ager and Radul, 1992) and other organisms (Zmyslony et al., 2000). Robison et al. (2002) also showed that electromagnetic treatments decrease DNA repair rate. On the other hand, lower seeds germination and seedlings growth can be related to overproduction of hydrogen peroxide in plant and increased lipid oxidation after exposing seeds to stronger MF or prolonged MF treatments (Podleoeny et al., 2005). Our results confirmed the accumulation of H_2O_2 in plant shoot after magnetic exposure. However, the increase in H_2O_2 concentration in plant was positively correlated with enhancement of MTI. Recent studies have shown that reactive oxygen species (ROS) such as H_2O_2 at low doses act as major signals and secondary messengers in regulating plant acclimation responses to environmental stresses (Foyer and Noctor, 2005). A molecular signal system by sensing small changes in H_2O_2 levels controls gene expression to activate cell responses to environmental stresses (Vanderauwera et al., 2005). Thus plant by sensing redox changes in cell becomes prepared to the particular needs of abiotic stress situations (Gechev et al., 2002). In this order, Vanderauwera et al. (2005) identified 20 genes, including the transcription factor DREB2A, which were induced in response to H_2O_2 and abiotic stress situations. DREB2A is a key regulator of cell responses to dehydration (Yoshida et al., 2014). Our results revealed that magnetic exposure activates plant defense system by inducing H_2O_2 accumulation in cell, which results in faster and stronger response to abiotic stress. However, overproduction of H_2O_2 was observed in the 24-h exposure treatment that explains the adverse

effects of prolonged magnetic exposure on seed germination and plant growth.

According to Ashraf and Rauf (2001), accumulation of high levels of Na^+ and Cl^- in sweet corn probably trigger the injuries to the plasma membrane by affecting ion homeostasis in cell under salt stress. Physiological resonance causes ROS overproduction and induces lipid peroxidation in cell under this condition, which eventually leads to plasma membrane injuries and malfunction (Foyer and Shigeoka, 2011). In the current study, plasma membrane thermostability significantly reduced under 100 mM NaCl stress and in seedlings of 24-h MF treatment. Accumulation of MDA, the by-product of non-enzymatic lipid peroxidation, in parallel with overproduction of H_2O_2 and loss of MTI confirmed peroxidation of membrane lipids under salt stress. These observations suggest that although MF treatments may reduce oxidative pressure on plant under salt stress, accumulation of high doses of H_2O_2 after prolonged MF exposure may adversely affect plant performance under severe abiotic stress.

In conclusion, this study showed that the positive effects of MF on germination and early growth of plant depend on the intensity and the duration of MF exposure. The results revealed that stronger MF exposure enhances rate of seed germination, however, using stronger MF or increasing MF exposure duration do not necessarily exert positive effects on final seed germination percentage and plant growth. MF exposure was found to improve plant water absorption capability and induce H_2O_2 signaling in cells, priming plant to deal with salt stress. Therefore, magnetic pretreatment for a short duration was suggested as an applicable approach to improve seed germination and seedling performance under salt stress. Such treatments are simple, cheap, and environmental friendly; however, the results suggested that this technique should be optimized for each species.

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Fungal pathogens associated with crown and collar rot of apple trees in southern Syria

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ABSTRACT

Crown and collar rot of apple trees is a destructive and widespread disease in most areas of the world. Surveys have been done to describe disease symptoms, estimate the disease incidence, and identify the pathogens associated with this disease in southern Syria. Disease incidence was 0.08 – 10 % in most studied sites, only in Alroom location it was up to 14.7 % in 2014 and 17.8 % in 2015, with average of 11.8 %. Symptoms included small pale green leaves, sparse foliage, and a reddish-brown discoloration of inner bark of the infected area at the base of infected tree. The isolated fungi belonged to genera and form genera *Phytophthora*, *Rosellinia*, *Rhizoctonia*, *Phialophora*, *Acremonium*, *Pestalotiopsis*, *Cylindrocarpon* and *Verticillium*. *Phytophthora* was isolated from all infected trees, and was the most frequent pathogen (53.7 % of total isolates). *Phytophthora* isolates recovered from crown cankers of apple were identified as *P. cactorum* (91.5 %) and *P. cambivora* (8.5 %). The results of this study are the first report of crown and collar root rot of apple in Syria.

Key words: crown and collar rot; apple tree; associated fungi;, *Phytophthora;* Syria

IZVLEČEK

GLIVNI PATOGENI, POVEZANI Z ODMIRANJEM KROŠNJE IN GNILOBO KORENINSKEGA VRATU JABLANE V JUŽNI SIRIJI

Odmiranje krošnje in gniloba koreninskega vratu jablane je uničujoča in široko razširjena bolezen na večini njenih pridelovalnih območij. Na območju južne Sirije so bile opravljene raziskave za opis bolezenskih znakov, pogostosti pojavljanja bolezni in prepoznavanja njenih povzročiteljev. Na večini raziskanih mest se je bolezen pojavljala z 0,08 do 10 %, samo na lokaciji Alroom pa do 14,7 % v letu 2014 in do 17,8 % v letu 2015, povprečno 11,8 %. Bolezenski znaki so bili majhni bledo zeleni listi, redka olistanost in rdeče-rjavo obarvano ličje okuženih delov na bazi debel dreves. Izolati gliv so pripadali naslednjim rodovom gliv: *Phytophthora*, *Rosellinia*, *Rhizoctonia*, *Phialophora*, *Acremonium*, *Pestalotiopsis*, *Cylindrocarpon* in *Verticillium*. Glive iz rodu *Phytophthora* so bile najpogostejši pathogen in izolirane iz vseh okuženih dreves, 53,7 % vseh izolatov. Izolati vrst iz rodu *Phytophthora*, ki so se razvili iz rakov v krošnji jablan, so bili določeni kot vrsti *P. cactorum* (91,5 %) in *P. cambivora* (8,5 %). Izsledki te raziskave so prvi o pojavljanju bolezni odmiranja krošenj in gnilobe koreninskega vratu jablane v Siriji.

Ključne besede: odmiranje krošnje; gniloba koreninskega vratu; jablana; z boleznijo povezane glive; *Phytophthora;* Syria

1 INTRODUCTION

Crown rot is an important soil-borne disease of apple *Malus domestica* Borkh. and pear trees *Pyrus communis* L. in most production regions of the world (Jeffers and Wilcox, 1990; Thomidis et al., 2002). Symptoms generally include: reduced tree vigor and growth, yellowing or chlorosis of leaves and eventual collapse or death of the tree (Nakova, 2010). Crown rots and trees declining and dying are frequently misdiagnosed as suffering from root asphyxiation, and sometimes confused with those suffering from winter injury (Ellis, 2008). Crown rot advances rapidly and trees collapse after the

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first warm weather in spring (Teviotdale and Gubler, 1999).

Several *Phytophthora* spp. are known to cause crown, collar and root rots of apple trees in most areas of the world where this crop is grown (Erwin and Ribeiro, 1996; Jeffers and Wilcox, 1990; Judelson and Blanco, 2005; Brasier, 2008). *Ph. cactorum* (Lebert & Cohn) J. Schröt. has been the species most frequently associated with crown and root rot of apple throughout much of the world, but other species of *Phytophthora* have been associated with crown rot of apple or pears trees such as *Ph. cambivora* (Petri) Buisman., *P. citricola* Sawada, *P. cryptogea* Pethybr. & Laff., *P. drechsleri* Tucker, *P. megasperma* Drechsler,, *P. parasitica* Dastur, *Pseudomonas syringae* Van Hall, 1904, and several unidentified *Phytophthora* spp. (Jeffers et al., 1981; Jeffers and Aldwinckle, 1986, 1988; Browne et al., 1995; Thomidis et al., 2002). Crown rot incidence was highly variable, depending on rootstock, pathogen, and flooding duration (Wilcox, 1993). Ellis (2008) states that wet soils that remain saturated for extended periods are required for disease development. Periods of 24 h or more of saturated soil favor *Phytophthora* infections. Conversely, good soil drainage and more frequent but shorter irrigation reduce the risk of root and crown rot (Teviotdale and Gubler, 1999).

In Syria, no previous studies have been conducted on decline and death of apple trees. The purpose of this study was to discover disease symptoms, to evaluate the incidence of this disease, and to identify the fungi associated with crown rot of apple trees in southern Syria where apples and pears are grown.

2 MATERIALS AND METHODS

2.1 Surveying and sampling

The survey was conducted from January 2014 to May 2015 in apple orchards distributed in eight different locations in Daher Aljabal region where apples are grown in the province of Swaida in southern Syria. Percentage of infected orchards at each site, and the percentage of infected trees in each orchard was estimated, then the disease incidence in each site was calculated.

A hundred and forty nine samples of infected plant materials (stem base) were collected from 35 different orchards of approximately 408 acres**.**

2.2 Fungal Isolation

Necrotic crown tissues were removed from diseased apple trees, thoroughly washed with running water, surface sterilized with 70 % ethanol for 30 seconds, washed with sterile distilled water, dipped in 6 % sodium hypochlorite for 5 minutes, washed twice with sterile distilled water, dried on a sterilized filter paper, and cut into small pieces (2 x 5 mm). They were then plated into Petri dishes with two media: potato dextrose agar PDA (39 g/l) and cornmeal agar CMA (30 g/l cornmeal and 20 g/l agar in 1l of sterile water) both supplemented with antibiotic (Amoxicillin 100 ppm). Petri dishes were incubated in darkness at 21ºC ± 2 for 7 days. Pure cultures were made by additional transfers (several sub-culture). Fungal isolates were identified based on their morphology according to International Mycological Institute (I.M.I.) Descriptions of Pathogenic Fungi and Bacteria (Minter and Cannon 2015).

3 RESULTS AND DISCUSSION

3.1 Incidence and distribution of apple crown rot

Incidence of the disease varied significantly depending on the location and on the orchards within the same site. The percentage of orchards where the disease was observed ranged from 0 % to 100 %. The disease incidence was 0.08 - 17.8 %, and the average of the disease incidence was 11.8 % in Daher Aljabal region (Table 1).

Table 1: Incidence of crown and collar rot disease of apple trees in southern Syria

Site	Date	Number of Orchards	Area of studied orchards (hectares)	Percentage (%) of infected orchards	Disease incidence (%)
Ayon Alsoufer	30/01/2014	5	6.3	80	2.5
Albassaa	09/02/2014	7	9.6	71.42	9.6
Alshoaf Algharbi	05/06/2014	5	11.5	20	0.08
Almazlaghat	11/08/2014	10	6.8	10	0.1
Almouaker	11/08/2014	2	2.0	100	10
Alroom	11/08/2014	4	3.0	100	14.7
	03/5/2015	4	3.0	100	17.8
Aljeemah	08/09/2014	1	1.2	100	0.8
Kanawat	09/09/2014	1	0.4	0	0
	2014	**35**	**43.8**	**47.52**	**11.8**

Disease incidence ranged from 0.08 % to 10 % in most studied sites, only in Alroom location it was 14.7 % in 2014 and 17.8 % in 2015. This high percentage of infected trees was more often observed in lowland areas of the orchards, especially when soils are clay, heavy and poorly drained. In fact, similar results have been reported in some previous studies (Wilcox, 1998; Hickey and Yoder, 2001). Nakova (2010) showed that crown rot spread was 2-3 % in most gardens, and only in an apple orchard in Bjaga (Plovdiv region) it was up to 8-10 % and also pointed out that disease spread is favored by wet, heavy and poorly-drained soils, and heavy rains also provoke disease symptoms.

3.2 Symptoms of apple crown rot

The trees infected by crown and collar rots showed symptoms including bud break delay in early spring, the presence of small pale green leaves, sparse foliage and the absence of vigorous growth of terminal shoots. In late spring, leaves of infected trees show reddish discoloration and drop down. Twigs and branches dieback. Infected trees may decline slowly, or they may die suddenly in the latter part of the growing season.

A reddish-brown discoloration of inner bark can be seen by removing the outer bark layer of the infected area at the base of infected tree several centimeters above the ground surface (Fig.1). These symptoms were reported in many previous studies of *Phytophthora* root and crown rot (Ellis, 2008; Nakova, 2003, 2010). We also noted that trees showing symptoms of crown rot disease were more sensitive to be infected by bacterial canker and papery bark disease caused by *Pseudomonas syringae* pv. *syringae*. Approximately 30 % of the trees infected by crown rot disease were also infected later by bacterial canker.

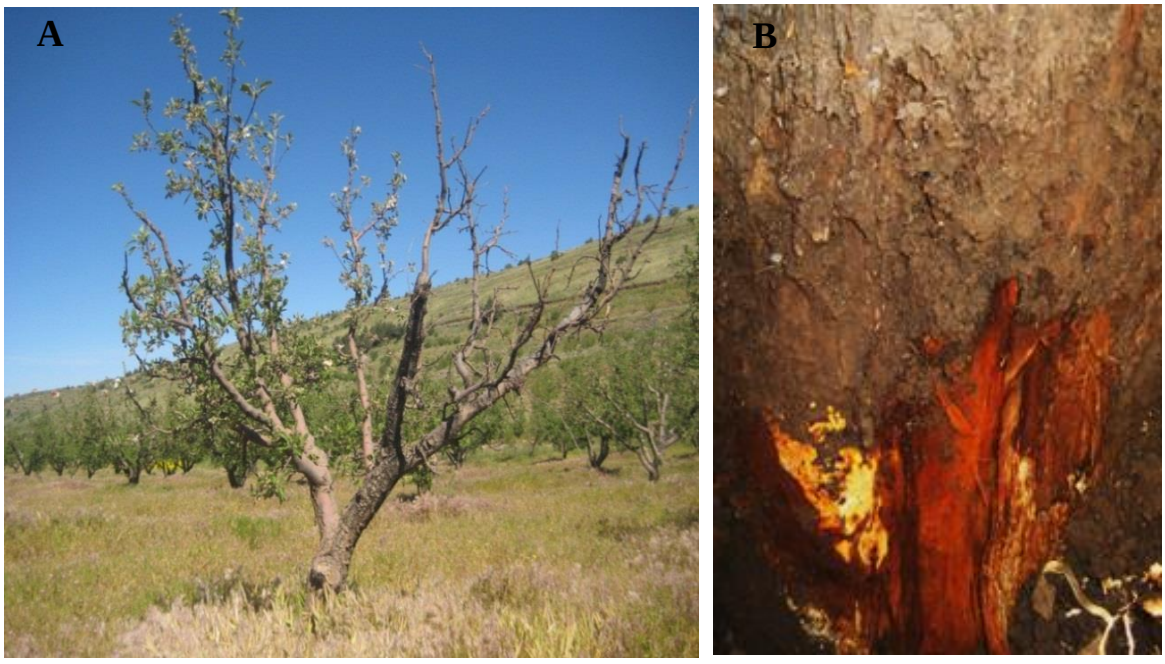

Figure 1: (A) Infected tree showing sparse small chlorotic leaves, die back of branches. The tree was later infected by bacterial canker. (B) A reddish-brown discoloration of inner bark of the infected area at the base of infected tree.

3.3 Fungi associated with crown rot of apple trees

Two hundred sixty isolates were obtained from crown cankers of apple trees. A hundred seventy five were identified according to their morphology. The isolated fungi belonged to genera and form genera such as *Phytophthora*, *Rosellinia*, *Rhizoctonia*, *Phialophora*, *Acremonium*, *Pestalotiopsis*, *Cylindrocarpon* and *Verticillium*. However, the relative dominance of individual species in the fungal community isolated from apple varied among the orchards, and some fungi of this complex were absent in specific orchards. *Phytophthora* was isolated from all infected trees, and dominated the fungal population (53.7 % of total isolates) recovered from crown cankers of apple. According to their morphology, 91.5 % of *Phytophthora* isolates were identified as *P. cactorum*, and 8.5 % as *P. cambivora*.

Colonies of *P. cactorum* were whitish, fluffy or smooth on PDA. Zoosporangia were oval to elongated (lemon shape) with papillae. Terminal or intercalary chlamydospores were found. Antheridia and oogonia, as well oospores, were formed in large numbers (Fig. 2). Colonies of *P. cambivora* were whitish, fluffy with dense aerial hypha. Zoosporangia were nonpapillate, ellipsoid or ovoid. Chlamydospores were absent.

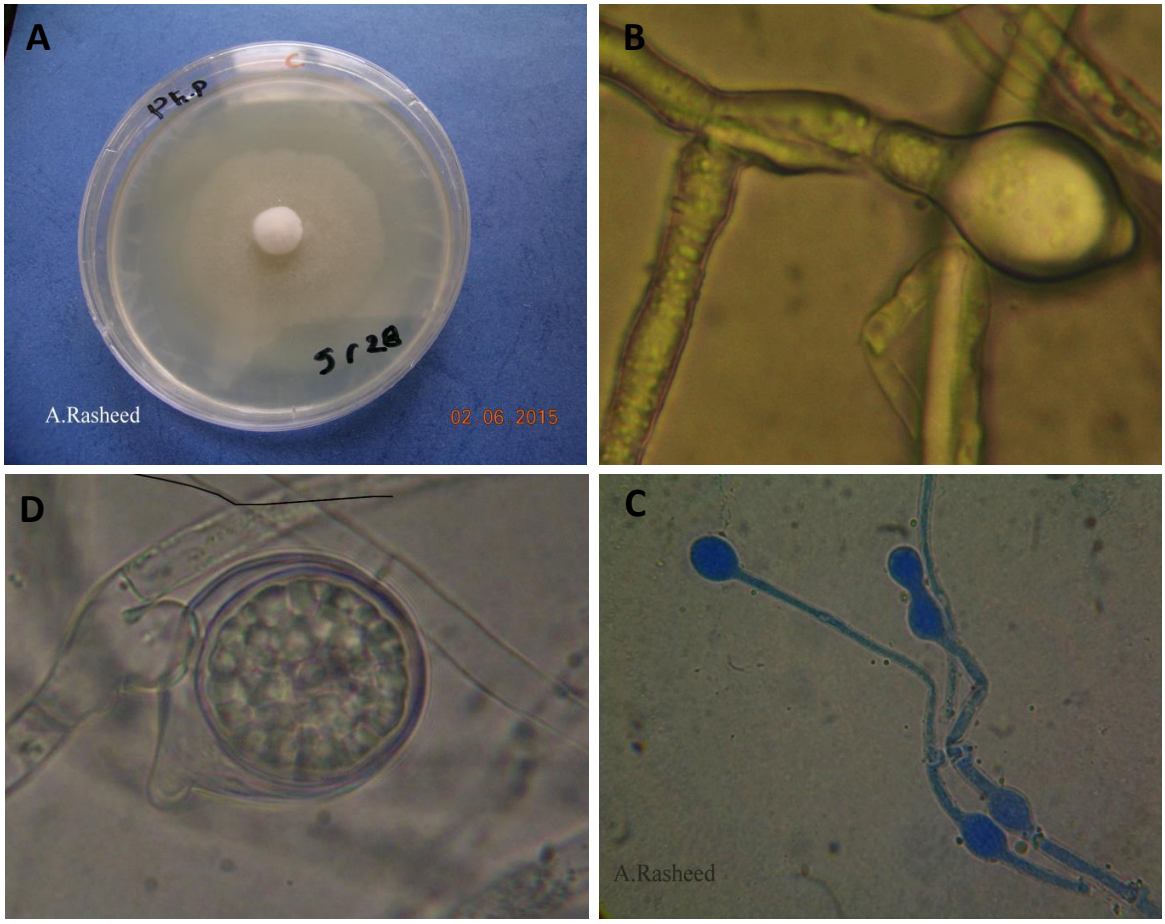

Figure 2: (A) colony of *P. cactorum* on PDA. (B) Limon shape sporangium with papillae. (C) Terminal and intercalary chlamydospores. (D) Sexual organs (antheridium and oogonium).

The morphology of sporangia, and general morphology of isolates of the two identified *Phytophthora* species were similar to those described in many studies (Waterhouse, 1963, 1970; Nakova, 2010; Welsh, 2011). Matheron et al. (1988) showed that *P. cactorum* and *P. cambivora* were highly virulent and caused rapid decline and death of apple seedlings, and these pathogens also were the most frequently isolated *Phytophthora* spp. in commercial apple orchards in Arizona.

The frequency of other fungi associated with crown and collar rot of apple trees varied from 1.7 % for *Verticillium* sp. to 18.3 for *Rhizoctonia solani* J.G. Kühn (Table 2).

This study showed that *P. cactorum* was the most important fungal species causing crown and collar rot of apple in Swaida where the apple trees are grown in southern Syria. In fact, *P. cactorum* has been the species most frequently associated with crown and root rot of apple throughout much of the world (Jeffers et al. 1981; Jeffers and Aldwinckle, 1986, 1988; Browne et al. 1995; Thomidis et al., 2002). Isolation of *P. cactorum* has been possible only from the margins of active lesions. There is evidence that the activity of *P. cactorum* is inhibited in rotted tissues by the antagonistic effect of one or more secondary organisms. These results are in according to those of Welsh (2011).

Table 2: Percentage of fungi associated with crown and collar rot of apple

Fungi	Ro	Rh	Ph	Phi	Ac	P	C	V
Number of isolates	11	32	94	13	10	5	7	3
Percentage %	6.3	18.3	53.7	7.4	5.7	2.9	4	1.7

Ro: *Rosellinia,* Rh: *Rhizoctonia* sp., Ph: *Phytophthora* sp., Phi: *Phialophora* sp., Ac: *Acremonium* sp., P: *Pestalotiopsis* sp., C: *Cylindrocarpon* sp., V: *Verticillium* sp.

In addition to *Phytophthora* spp., a fungal complex consisting of *Rosellinia* sp. (6.3 %), *Rhizoctonia solani* (18.3 %), *Phialophora* sp. (7.4 %), *Acremonium* sp. (5.7 %), *Pesalotiopsis* sp. (2.9 %), *Cylindrocarpon* sp. (4 %) and *Verticillium* sp. (1.7 %), was associated with crown and collar rot of apple. Many studies conducted in Washington State indicated that a fungal pathogen complex, consisting of ***Nectria radicicola*** Gerlach & L. Nilsson (*Cylindrocarpon destructans* (Zinssm.) Scholten), *Phytophthora cactorum*, *Pythium* spp., and *Rhizoctonia solani* was the predominant cause of replant disease of apple trees, and were consistently isolated from symptomatic trees in all orchards, and these fungi were pathogenic to apple in greenhouse tests (Mazzola, 1998; Mazzola et al., 2001; Braun, 1995). *Pestalotiopsis* spp. have been reported to cause root and crown rot of strawberry in Spain (Chamorro et al., 2016), canker and twig dieback of blueberry in Chile (Espinoza et al., 2008), black foot of grapevine in Portugal (Oliveira et al., 1998). In fact, the soilborne fungus *Rosellinia necatrix* Berl. ex Prill., is the causal agent of white root rot disease on numerous plant species, including apple (Pasini et al., 2016).

4 CONCLUSIONS

Crown rot of apple in Swaida in the south of Syria is associated with many fungi, but the main cause of this disease, and the most frequently isolated pathogen is *P. cactorum*. The result of this study is the first report of crown rot of apple trees in Syria.

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Vroča točka v mestu: povezava ekosistemskih storitev in biotske pestrosti mestnih zelenih površin

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IZVLEČEK

Zelene površine v mestih ter ekosistemske storitve (ES), ki se na teh površinah oblikujejo, nudijo prebivalcem različne posredne in neposredne koristi. Nabor teh storitev je odvisen tudi od biotske pestrosti (BP) določene zelene površine. Namen raziskave je bil ugotoviti povezavo med BP in naborom ES v določeni kategoriji zelenih površin. V izbranih devetih kategorijah v Mestni občini Ljubljana, smo opravili 108 vegetacijskih popisov v jesenskem in spomladanskem času. Vsaki kategoriji smo nato ocenili nabor zaznanih ES na terenu ter jih primerjali z zaznanimi ES takih površin iz literature. V rezultatih je tako po številu vrst kot po številu ES izstopala kategorija gozdov. Ostale kategorije so bile v sestavi vegetacije med seboj podobne, največja podobnost je bila v kategorijah s travišč no vegetacijo. Ugotovljena je bila pozitivna zveza med številom rastlinskih vrst in številom zaznanih ES v posamezni kategoriji zelenih površin.

Ključne besede: urbani ekosistemi; zelena infrastruktura; rastlinska vrstna pestrost; kategorije zelenih površin; Ljubljana

ABSTRACT

CITY HOTSPOT: LINKAGES BETWEEN ECOSYSTEM SERVICES AND BIODIVERSITY OF URBAN GREEN AREAS

Green areas in cities and their ecosystem services (ES) offer residents various benefits. The range of services depends on biodiversity of a green space. The aim was to determine the relationship between biodiversity in different categories of green areas in the city and the ecosystem services, which appear in it. We made 108 relevés in the autumn and spring time, within nine categories of green areas in the Municipality of Ljubljana. In each category the range of ES was assessed based on field analysis and compared with literature assessed ecosystem services. Results showed that the category of forests differ from others. Other categories were similar to each other, in particular grassland categories. Also, a positive relationship linkage was found - more plant species mean more assessed ecosystem services in a specific green infrastructure category.

Key words: biodiversity; ecosystem services; green infrastructure; green spaces; green infrastructure categories; Ljubljana

1 UVOD

Povezavo urbanih okolij ter narave v mestu najlažje prepoznamo in opazujemo v različnih oblikah mestnih zelenih površin. Že leta 1996 je Evropska komisija objavila, da so zelene površine v mestih enako pomembne kot ostala mestna infrastruktura (European Commission, 1996). Zelene površine, tudi zelena infrastruktura, so lahko gozdovi, parki, mestni vrtički, športna igrišča, pokopališča, vrtovi in dvorišča (Gómez-Baggethun and Barton, 2012). V mestu predstavljajo javno ali zasebno površino, prekrito z vegetacijo, ki je posredno ali neposredno povezana s svojimi uporabniki (Baycan-Levent in sod., 2009). Te površine vplivajo na kakovost življenja v

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This article is based on the Master Thesis of Špela Železnikar. Supervisor: Assist. Prof. Ph. D. Klemen Eler, co-supervisor: Prof. Ph. D. Marina Pintar

mestu, na klimo v mestu, zagotavljajo svež zrak ter zmanjšujejo vplive onesnaževanja. Prav tako omogočajo medsebojno interakcijo prebivalcev, v njih lahko pridobivamo različne surovine ter delujejo kot vidna in zvočna pregrada. Te koristi za človeka imenujemo ekosistemske storitve (ES), ki se v urbanih območjih v različnih kategorijah zelenih površin različno izražajo (Cilliers in sod., 2011). Koncept ES, je bil oblikovan v poročilu »Millennium Ecosystem Assessment« (2005), ki poudarja, da mora biti splošna klasifikacija ES prilagojena vrstam ekosistemov, v katerih opredeljujemo storitve.

Z naraščanjem priljubljenosti koncepta ES se vse bolj izpostavlja vprašanje, kako so ES in BP povezani med seboj ter ali je BP že sama po sebi ekosistemska storitev (Jax in Heink, 2015). Še danes, čeprav obstajajo številne raziskave, ki nakazujejo pozitiven odnos med BP in naborom ES (Egoh in sod., 2009, Cardinale, 2011, Isbell in sod., 2011, Mace in sod., 2012, Harrison in sod., 2014), ni veliko jasnega o njuni povezavi ter mehanizmu te povezave (Loreau in sod., 2001, Harrison in sod., 2014). Zanimanje za ES mestnih ekosistemov (Bolund in Hunhammar, 1999) se pojavi kmalu po začetkih vrednotenja (glej Costanza in sod., 1997). Raziskovalci in načrtovalci mest iz različnih disciplin poudarjajo vlogo mestne BP pri zagotavljanju ES za povečanje blaginje ljudi v hitro urbanizirajočem se svetu (Botzat in sod., 2016).

Na zelene površine ter njihovo BP v mestu vpliva tudi urbanizacija. Tako nastajajo spremenjeni ekosistemi, oziroma nove, unikatne oblike mestnih zelenih površin (McKinney, 2006; Schneider in sod., 2012; Bolund in Hunhammar, 1999). Díaz in sod. (2005) ter Balvanera in sod. (2006) navajajo, da je dokazan pozitiven vpliv večje BP na količino in kakovost ES. Te so pogosto odvisne od funkcionalnih lastnostih osnovnih rastlinskih združb, ki te ekosisteme sestavljajo (De Bello in sod., 2010). Med njimi je vegetacija dober pokazatelj BP, saj oblikuje strukturo organizmov na višjih trofičnih ravneh (Smith in sod., 2006). Vegetacija mestnih zelenih površin deluje predvsem kot bio-indikator ekološke funkcije za mesto (Borysiaket in sod., 2014).

Zelene površine v mestih ter ES, ki se na teh površinah oblikujejo, nudijo prebivalcem različne posredne in neposredne koristi. Namen naše raziskave je bil ugotoviti povezavo med BP in naborom ES v različnih kategorijah zelenih površin. Z raziskavo preverjamo hipotezo, da se z večanjem števila rastlinskih vrst v posamezni kategoriji zelenih površin povečuje nabor zaznanih ES.

2 MATERIALI IN METODE

2.1 Območja raziskave

Na območju Mestne občine Ljubljana smo izbrali devet kategorij zelenih površin, ki smo jih razdelili na štiri lokacije popisov (Preglednica 1). Te kategorije so bile kategorizirane v projektu Green Surge, (7. okvirni program EU za raziskave, FP7-ENV.2013.6.2-5-603567) (GS, 2016).

Preglednica 1: Izbrane kategorije zelenih površin, razdeljene po območjih popisov v Mestni občini Ljubljana
Table 2: Areas of relevés within urban green infrastructure categories in Municipality of Ljubljana

Kategorije zelenih površin	Območja popisov
Gozd	Rožnik, Golovec, Pržan, Tomačevski prod
Park	Tivoli, Zvezda, Arturo Toscanini, Navje
Drevoredi	Cesta v Rožno dolino, Vojkova cesta ob ARSO, Celovška cesta, Žale
Igrišča	Mostec, Golf Ljubljana, Rugby Gunclje, Kodeljevo
Zelene površine ob blokih	Šišenska cesta (Šiška), Glinškova ploščad, Jamova cesta, Nusdorferjeva ulica
Vrtovi ob hišah	Vič (Idrijska ulica), Šentvid (Kozlarjeva pot), Bežigrad (Dunajska cesta), Štepanjsko naselje (Ob potoku)
Vrtički	Livada, Litostroj, draveljski vrtovi, krakovski vrtovi
Njive	Kleče, Polje, Barje, Podutik
Ruderalna območja	trgovska cona Rudnik, stadion Stožice, gramoznica Stanežiče, parkirišče za železniško postajo Ljubljana

2.2 Popisi rastlinske pestrosti

Na vsaki lokaciji smo izvedli vegetacijski popis na naključno razporejenih treh kvadratnih ploskvah v velikosti 9 m^2. Skupaj smo tako opravili 216 vegetacijskih popisov, 108 (9 x 4 x 3) v pomladnem ter 108 v jesenskem času (9 x 4 x 3). Posamezne popisne ploskve so bile iste za jesenske in pomladanske popise.

Vegetacijski popis smo izvedli po Braun-Blanquetovi metodi (povzeto po Dierschke, 1994), s pomočjo katere smo ocenjevali zastopanost posameznih vrst višjih rastlin po kombinirani lestvici, ki združuje številčnost in pokrovnost posamezne vrste. Vzorčili smo samo pojavljanje spontane vegetacije in ne sajenih rastlin. Popisi so potekali v jesenskem (med 30. 8. 2013 in 18. 10. 2013) in spomladanskem času (med 3. 5. 2014 in 26. 6. 2014). Poleg popisov na 9 m^2 ploskvah smo popisali tudi rastlinske vrste (spontano pojavljanje) v okolici ploskev. Pri popisih v gozdovih smo upoštevali vertikalno strukturiranost združbe z ugotavljanjem zastopanosti vrst po treh plasteh: drevesni (lesnate vrste nad 5 m višine), grmovni (lesnate vrste od 0,5 do 5 m višine) in zeliščni. Za vsak posamezni popis smo ugotovili število rastlinskih vrst na popis in izračunali vrednost Shannon-Wiennerjevega indeksa pestrosti. Ocene po Braun-Blanquetovi kombinirani lestvici smo za izračun indeksa pretvorili v deleže z upoštevanjem sredin razredov pokrovnosti (5 = 87,5 %, 4 = 62,5 %, 3 = 37,5 %, 2 = 17,5 %, 1 = 5 %, + = 0,5 %). Iz njih smo nato izračunali skupno ugotovljeno pokrovnost vegetacije oziroma korigirane deleže. Na lokacijah v kategoriji gozdov smo za vrste, ki so se pojavljale v več plasteh, upoštevali deleže ločeno po teh plasteh. Shannon-Wiennerjev indeks pestrosti za posamezen popis smo izračunali kot vsoto produktov korigiranih deležev (p_i) in logaritmov korigiranih deležev vseh vrst v danem popisu:

$$H = -SUM[(p_i) \times \ln(p_i)] \quad (1)$$

Razlike v številu vrst in vrednosti Shannon-Wiennerjevega indeksa med lokacijami in kategorijami zelenih površin smo ugotavljali z analizo variance (ANOVA) z lokacijami popisov kot bloki ter s Tukeyevim *post-hoc* preizkusom, pri katerem smo upoštevali Bonfferonijevo korekcijo za število primerjav. Različnost/ podobnost celotne vrstne sestave vegetacije po lokacijah in kategorijah zelenih površin smo ugotavljali s korespondenčno analizo z odstranjenim trendom; vhodno matriko so predstavljale standardizirane vrednosti pokrovnosti vrst v deležih. Opravili smo več korespondenčnih analiz – posebej za spomladanske in posebej za jesenske popise ter z in brez upoštevanja gozdnih ploskev.

2.3 Ekosistemske storitve

S pomočjo iskalnega portala ISI Web of Knowledge smo opravili pregled literature na temo zaznanih ES v mestnih zelenih površinah.

Uporabili smo naslednje iskalne kriterije (v angleških ustreznicah): ekosistemske storitve - parki, ekosistemske storitve - gozd (mestni), ekosistemske storitve - obcestna vegetacija, ekosistemske storitve - degradirana območja, ekosistemske storitve - vrtički, ekosistemske storitve - njive, ekosistemske storitve - zasebni vrtovi, ekosistemske storitve - igrišča/športne površine ter ekosistemske storitve - zelene površine ob blokih. Rezultati iskanja z zaznanimi storitvami znotraj posamezne kategorije zelenih površin v mestu so predstavljeni v Preglednici 2. Na podlagi podatkov, ki smo jih pridobili iz literature ter terenskih popisov lokacij, smo nato v izbranih kategorijah zelenih površin v Ljubljani z ekspertno oceno ocenili, katere od storitev, ki so bile za posamezno kategorijo zelenih površin omenjene v literaturi, so v teh kategorijah v Ljubljani dejansko prisotne. Ekspertne ocene smo opravili trije avtorji prispevka individualno, skupno oceno pa smo sestavili na podlagi večinskega mnenja za vsako kombinacijo kategorije zelene površine in v literaturi zaznane ES. V analizi smo tudi primerjali, koliko ES za posamezno kategorijo zelene površine smo našli z literaturnim pregledom in koliko ES smo na podlagi te ekspertne ocene pripisali našim proučevanim zelenim površinam v Ljubljani.

Preglednica 2: Seznam ekosisiemskih storitev znotraj posameznik kategorij mestnih zelenih površin iz pregleda literature (znak plus predstavlja zaznano ES v literaturi, navedeni na dnu preglednice)

Table 2: List of assessed ecosystem services in different urban green areas, based on literature review (symbol + represents an assessed ecosystem service appearing in the literature, provided at the bottom of the table)

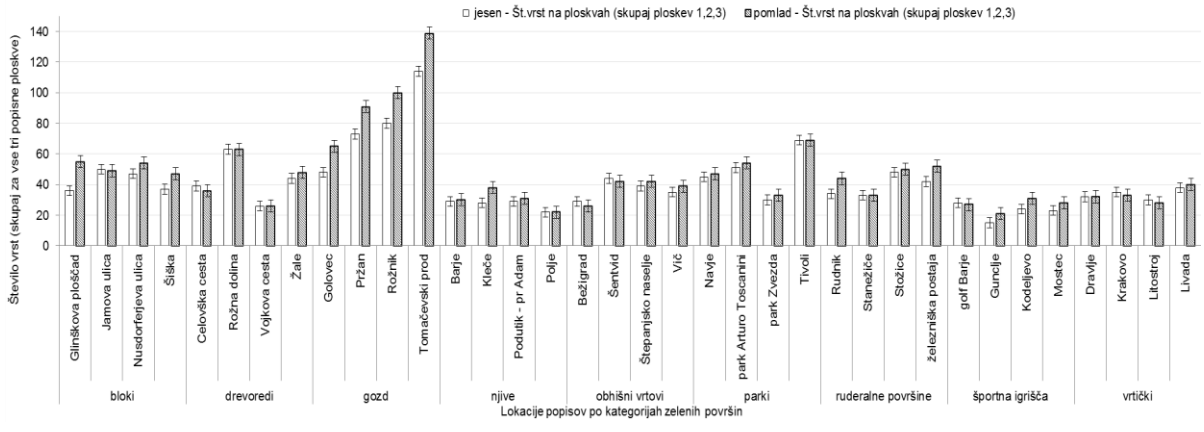
Ekosistemske storitve / kategorije zelenih površin v mestu	PARKI	GOZD (mestni)	DREVOREDI	DEGRADIRANA OBMOČJA	VRTIČKI	NJIVE	ZASEBNI VRTOVI	IGRIŠČA	ZELENE POVRŠINE OB BLOKIH
Regulacija vode	+	+	+	+	+	+	+	+	+
Življenjski prostor rastlin, živali	+	+	+		+		+		
Genetski vir	+		+				+	+	
Skladiščenje ogljika	+		+		+		+	+	
Fiksacija CO_2		+				+			
Biološki nadzor škodljivcev	+	+		+	+	+			
Kakovost zraka	+	+	+	+	+	+	+		+
Vpliv na klimo mesta	+	+	+		+	+	+		+
Zmanjšanje onesnaženosti z hrupom	+		+	+					
Regulacija T	+	+	+	+	+		+		
Proizvajanje O_2		+				+			
Ustvarjanje sence			+						+
Opraševanje	+	+			+	+			
Varnost pred erozijo		+							
Ohranjanje narave/biodiverzitete		+		+	+	+	+		
Produkcija biomase/Recikliranje odpadkov				+					
Surovine (hrana, les, drugi materiali)	+	+			+	+	+		
Turizem	+	+				+		+	
Estetska vrednost	+	+	+		+	+			+
Vpliv na fizično dobro počutje	+	+	+	+	+		+	+	+
Psihološki pozitivni vplivi	+	+	+		+			+	
Rekreacija	+		+		+	+	+	+	+
Kulturna dediščina			+		+	+			
Izobraževalna vrednost		+	+		+	+			+
Omogočanje socialnih interakcij	+			+	+				
Vir literature	Vir 1 ; Vir 2	Vir 3; Vir 4; Vir 5	Vir 6; Vir 7	Vir 8; Vir 9	Vir 10; Vir 11; Vir 12	Vir 13; Vir 14; Vir 15;	Vir 16	Vir 17	Vir 18; Vir 19

Opomba: Vir 1 – Kovacs, 2012; Vir 2 – Buchel in Frantzeskaki, 2015; Vir 3 – Gorriz-Mifsud in sod., 2016; Vir 4 – Mislimshoeva in sod., 2016; Vir 5 – Platon in sod., 2015; Vir 6 – Weber in sod., 2014; Vir 7 – Saumel in sod., 2016; Vir 8 – Bardos in sod., 2016; Vir 9 – Haase in sod., 2014; Vir 10 – Barthel in sod., 2010; Vir 11 – Camps-Calvet in sod., 2016; Vir 12 – Middle in sod., 2014; Vir 13 – Ma in sod., 2015; Vir 14 – Soy-Massoni in sod., 2016; Vir 15 – Firbank in sod., 2013; Vir 16 – Cameron in sod., 2012; Vir 17 – Dai in sod., 2016; Vir 18 – Krellenberg in sod., 2014; Vir 19 – Norouzian-Maleki in sod., 2015

3 REZULTATI IN DISKUSIJA

3.1 Rastlinska pestrost

Skupno smo popisali 288 različnih rastlinskih vrst. Po vrstni sestavi sta najbolj izstopali kategoriji gozdov in parkov, kjer smo popisali največjo in najbolj raznoliko vrstno sestavo vegetacije. Na gozdni lokaciji Tomačevski prod je bilo tako v jesenskem kot v spomladanskem času popisanih največ vrst. V jesenskem času je bilo najmanj vrst popisanih na ploskvi njive na Polju, v spomladanskem času na ploskvi igrišča Kodeljevo. Na lokaciji parka Tivoli je bila največja razlika v popisu rastlinskih vrst znotraj in izven ploskve, enako število vrst znotraj in izven ploskve smo popisali na gozdni ploskvi na Golovcu (Slika 1).

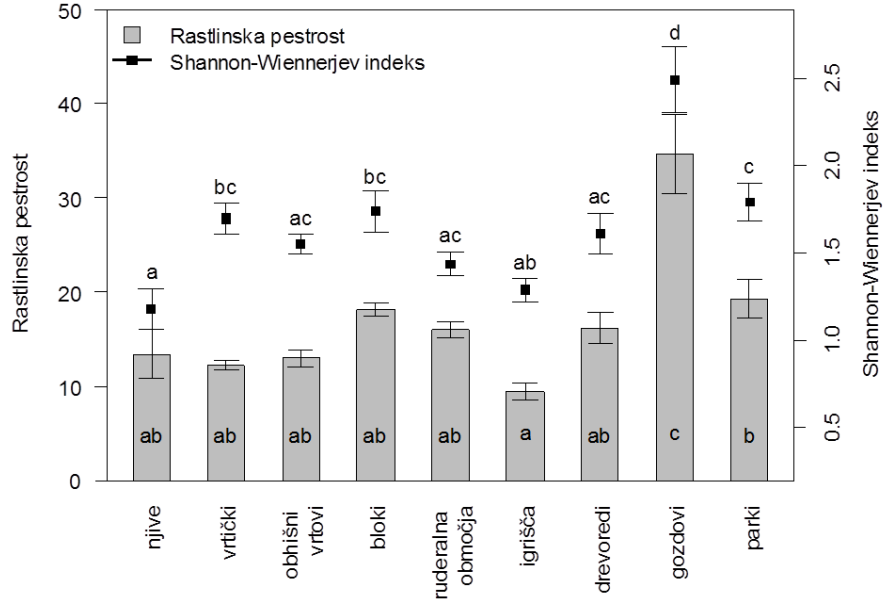


Slika 1: Število vrst na popisnih ploskvah in v neposredni okolici teh ploskev po posameznih popisnih lokacijah, za jesenske in spomladanske popise v Mestni občini Ljubljana.

Figure 1: Number of species on the plots and around plots for individual locations for the autumn and spring relevés in the Municipality of Ljubljana.

Največ rastlinskih vrst je bilo popisanih v kategoriji gozdov, sledi kategorija njive, površine ob blokih in drevoredi ter ruderalne površine. Kategoriji z najmanj popisanimi vrstami sta bili igrišča in obhišni vrtovi. Izračunana povprečja števila vrst znotraj posameznih kategorij zelenih površin prikazujejo naslednje rezultate. Največ vrst smo popisali v kategoriji gozdov (68 vrst), sledita ji kategoriji ruderalnih površin (28 vrst) in bloki (26 vrst). Sledijo kategorije drevoredi in njive (obe 25 vrst) ter parki (24 vrst) in vrtički (22 vrst). Najmanj vrst smo v povprečju popisali v kategoriji športnih igrišč (14 vrst). Najmanjša variabilnost med lokacijami popisov je bila opažena med lokacijami vrtičkov, največja med lokacijami v kategoriji obhišnih vrtov in gozdov. Shannon-Wiennerjev indeks je bil največji v kategoriji gozdov, sledijo ji vrtički, parki in bloki. Najmanjši je bil v kategoriji njiv. Največja razlika med jesenskimi in spomladanskimi vrednostmi indeksa je bila izračunana v kategoriji igrišč (0,30), najmanjša v kategoriji vrtičkov (0,07).

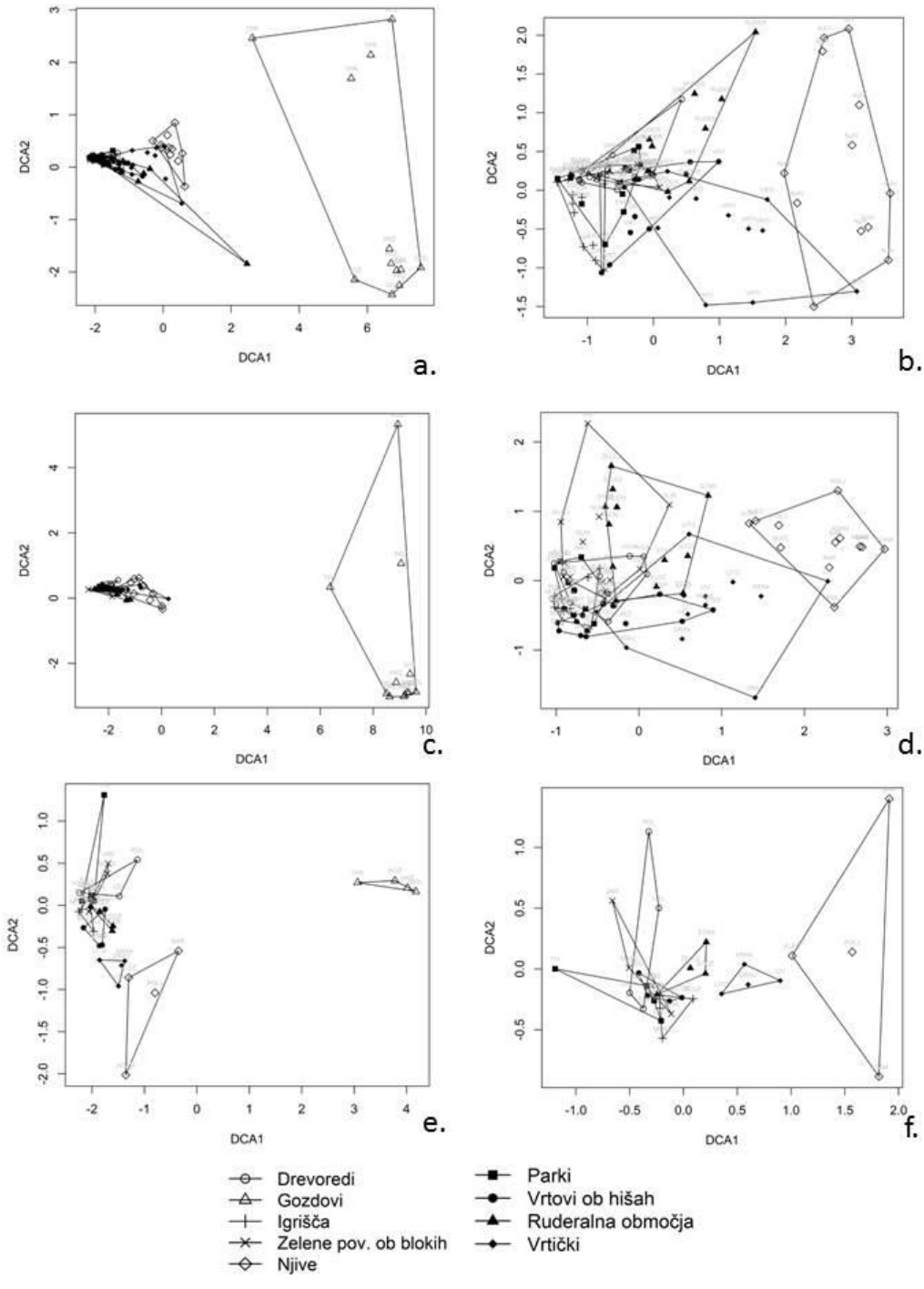
Primerjava celotne ugotovljene rastlinske pestrosti ter povprečij Shannon-Wiennerjevega indeksa med posameznimi kategorijami zelenih površin v Ljubljani kaže, da se statistično značilno od ostalih kategorij po številu rastlinskih vrst razlikuje le kategorija gozdov (Slika 2). Med kategorijami vrtički, obhišni vrtovi, njive, ruderalna območja, drevoredi in zelenice ob blokih ni statistično značilnih razlik v številu rastlinskih vrst. Rezultati rastlinske pestrosti v kategoriji igrišč in parkov se prekrivajo s kategorijami vrtički, obhišni vrtovi, njive, drevoredi, zelenice ob blokih in ruderalna območja. V vrednostih Shannon-Wiennerjevega indeksa je statistično značilna razlika opazna med kategorijo gozdov in vsemi ostalimi kategorijami. Med drugimi kategorijami proučevanih zelenih površin ni statistično značilnih razlik. Statistično značilnih razlik nismo ugotovili niti med kategorijami zelene površine ob blokih, obhišni vrtovi, vrtički, parki, drevoredi in ruderalna območja.



Slika 2: Podatki rastlinske pestrosti in povprečij Shannon- Wiennerjevega indeksa po posameznih kategorijah zelenih površin v Mestni občini Ljubljana

Figure 2: Number of species and average values of Shannon - Wienner index by categories of green infrastructure in the Municipality of Ljubljana

Korespondenčno analizo podobnosti popisov v celotni sestavi rastlinskih združb smo opravili za jesenski in spomladanski del popisov posebej. Sestava ter različnost sestave (variabilnosti med lokacijami in ploskvami) kažeta na to, da so gozdne ploskve precej drugačne od preostalih tako v jesenskem kot v spomladanskem obdobju popisa (Slika 3 – jesenski popisi). Zaradi tega smo korespondenčno analizo ponovili z upoštevanjem samo negozdnih lokacij (Slika 3e in 3f). Zaradi večjega deleža enoletnih vrst so nekoliko ločene njivske površine in delno vrtički. Podobnost je posebej opazna na površinah s travišču vegetacijo – zelene površine ob blokih, drevoredi, igrišča, vrtovi ob hišah in parki.

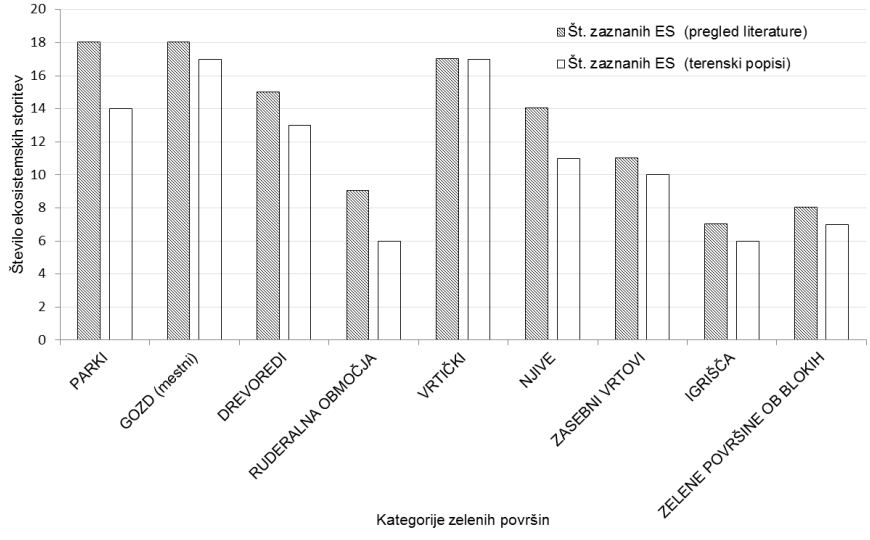


Slika 3: Rezultati (ordinacijski diagrami za prve dve osi) korespondenčne analize za sestavo rastlinskih združb na zelenih površinah v Ljubljani; a) ordinacija jesenskih popisov upoštevajoč pokrovnost vrst; b) enako kot a) le brez gozdnih ploskev; c) ordinacija spomladanskih popisov zelenih površin v Ljubljani na prvih dveh oseh korespondenčne analize, upoštevajoč pokrovnost vrst; d) enako kot c) le brez gozdnih ploskev; e) ordinacija popisanih lokacij, upoštevajoč le prisotnost vrst (združeni jesenski in spomladanski popisi treh ploskev na lokacijo); f) enako kot e) le brez gozdnih ploskev.

Figure 3: Correspondence analysis results (ordination diagrams for the first two axes): a) autumn relevés of land cover in green spaces in Ljubljana; b) same as a. except forest relevés; c) spring relevés of land cover in green spaces of Ljubljana; d) same as c. except forest relevés; e) autumn and spring relevés (three plots per location), showing only the results of the presence of the species; f) same as e. except forest relevés.

3.2 Ekosistemske storitve

Največ storitev (izračunano je bilo povprečje iz vseh lokacij znotraj posamezne kategorije) je bilo na podlagi ekspertne ocene na terenu zaznano v kategoriji gozd ter vrtički, sledita kategoriji parki in drevoredi. Povprečno najmanj storitev smo na terenu zaznali v kategoriji igrišč in ruderalnih območij. Med popisnimi lokacijami sta pri terenskih popisih izstopali gozdni lokaciji Rožnik in Golovec z največjim številom zaznanih ES, najmanj pa smo jih ugotovili na lokaciji gramoznice Stanežiče v kategoriji ruderalnih območij. Na proučevanih kategorijah zelenih površin smo največkrat določili naslednje ES: vpliv na kakovost zraka v mestu, vpliv na fizično dobro počutje ter rekreacija. V najmanj kategorijah sta bili ugotovljeni varstvo pred erozijo ter produkcija biomase. Največja razlika v številu ES najdenimi v literaturi in številu ES za naše lokacije po ekspertni oceni je prisotna v kategoriji parki (Slika 4). Sledita ji kategoriji njive in ruderalna območja, kjer razlika znaša 3 storitve.

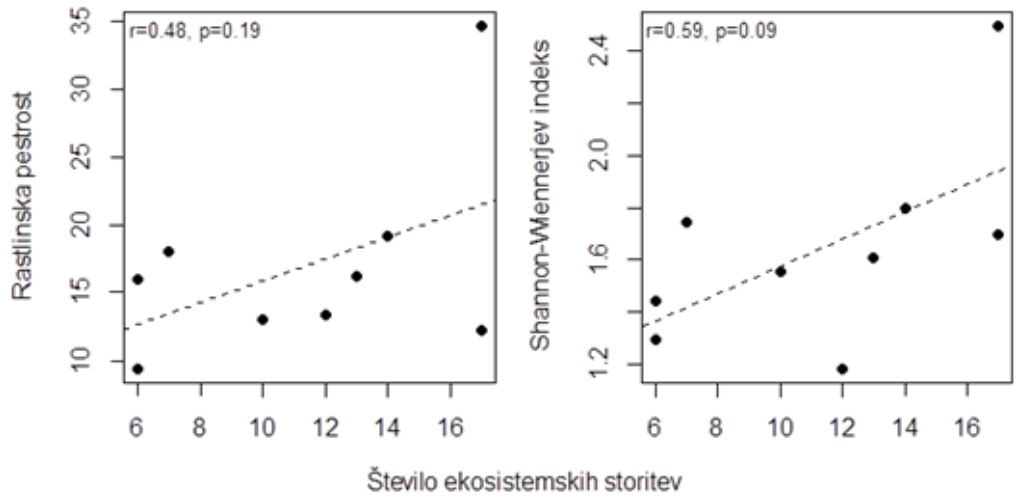


Slika 4: Povprečno število ES na podlagi pregleda literature ter število storitev po ekspertni oceni v izbranih kategorijah zelenih površin v Mestni občini Ljubljana

Figure 4: Average number of ecosystem services reported in the literature and the number of assessed services according to the expert evaluation for the selected categories of green infrastructure in the Municipality of Ljubljana

3.3 Povezanost rastlinske pestrosti in ekosistemskih storitev

Glede na podatke, prikazane na Sliki 5, lahko povzamemo, da je bilo največ vrst popisanih v kategoriji gozdov, ta kategorija je imela tudi največ ES. V nadaljnji analizi smo izračunali Pearsonov koeficient korelacije (statistično značilen pri $p < 0,01$) med spremenljivkama število vrst na ploskvi in povprečnim število ES v posamezni kategoriji ter vrednostjo Shannon-Wiennerjev indeks in povprečnim številom ES. Koeficient je bil v prvem primeru 0,48, pri primerjavi pestrosti Shannon-Wiennerjevega indeksa pa 0,59. Iz tega lahko sklepamo, da je med spremenljivkama majhna pozitivna povezanost oz. pri večjem številu vrst pričakujemo tudi več ES.



Slika 5: Primerjava skupnega števila popisanih rastlinskih vrst ter povprečnega števila ES na posamezni lokaciji zelenih površin v Mestni občini Ljubljana (levo); primerjava Shannon-Wiennerjevega indeksa pestrosti ter povprečnega števila ES v posamezni kategoriji zelenih površin v Mestni občini Ljubljana (desno)

Figure 5: Number of inventoried plant species and average number of ES at each location of green infrastructure categories in the Municipality of Ljubljana (left); Shannon-Wienner inedx compared with the average number of ES of each category of green infrastructure categories in the Municipality of Ljubljana (right)

3.4 Diskusija

Mesta so s strani človeka tako izrazito vplivana okolja z uničeno ali znatno spremenjeno vegetacijo, da je v njih nabor in pomen ES prvotnih naravnih ekosistemov v največji možni meri predrugačen. Kljub temu morajo mesta tudi kot takšni intenzivno spremenjeni ekosistemi mestnim prebivalcem nuditi pomembne storitve, kot so vzdrževanje življenjskih razmer (Odum, 1989), varnost (Costanza in sod., 2006), dobro počutje (TEEB, 2011), zdravje (Maas in sod., 2006) ter socialne interakcije (EEA, 2011). Kot smo omenili že v uvodu, se nakazuje, da je nabor ES in njihova kvaliteta v povezavi tudi z biotsko pestrostjo mestnih območij, posebej zelenih površin. To povezavo smo na različnih kategorijah zelenih površin preverili tudi za mesto Ljubljana.

Med proučevanimi zelenimi površinami je pričakovano izstopala kategorija gozdov, saj gre za ekosisteme najbližje prvotnim oz. naravnim, katerih vrstna sestava, različnost in strukturiranost življenjskih oblik se zelo razlikuje od drugih ZP. Večina vrst, ki jih najdemo v gozdovih, težko uspeva v kaki drugi kategoriji zelenih površin v Ljubljani. Druge kategorije namreč sodijo v skupino t. i. motenih habitatov, kjer se po bolj ali manj celotni površini izvaja teptanje s hojo ljudi ali vozili, košnja idr., kar onemogoča ali otežuje rast lesnatih vrst, razen če so te izrecno vzdrževane. Prav lesnate vrste oz. gozdni ekosistemi naj bi najbolj zmanjševali odtok površinske vode (Villarreal in Bengtsson, 2005), je pa bila ta ES v naši raziskavi ugotovljena tudi za večino negozdnih kategorij. Povsod, kjer tla namreč niso nepropustna (asfaltirana, betonirana) in so porasla z vegetacijo, je ta storitev ugotovljena.

Le nekoliko manj ES kot za gozdove smo jih ugotovili za parke. Ljubljana med evropskimi mesti ni izrazito bogata z deležem parkovnih površin (Braquinho in sod., 2015), kar je dokaj značilno za nekoliko manjša mesta z dobro dostopnostjo zelenih površin izven mesta. Poleg tega se skoraj v center Ljubljane zajedata dve gozdni območji (Rožnik in Golovec), ki se v evidencah ne vodita kot parkovni površini, imata pa podobni funkciji. Rezultati kažejo, da se največji park v Ljubljani (Tivoli) razlikuje od ostalih izbranih parkovnih površin tako po številu ES kot tudi po številu rastlinskih vrst. Park Tivoli je del krajinskega parka, v njem velja posebna ureditev, prav tako je po svoji površini tudi največji in po svojih funkcijah za mestno prebivalstvo bolj raznolik.. Pomembna vloga parkovnih površin ugotovljenih na primeru Ljubljane ter zelenih površin ob blokih, ki jih lahko pojmujemo kot pomanjšane parke, ni presenetljiva. Hardin in Jensen (2007) sta npr.

ugotovila, da te površine značilno zmanjšujejo temperaturo v mestu, saj vegetacija teh večjih površin s pomočjo evapotranspiracije zmanjšuje temperaturo. Prav tako drevesa v mestu, ki so del tako mestnih gozdov kot parkovnih površin in drevoredov, vplivajo na temperaturo v mestu z ustvarjanjem sence (Bolund in Hunhammar, 1999).

Kategorijam vrtičkov, skupnostnih vrtov in vrtov ob hišah smo ugotovili veliko storitev, povezanih s socialno funkcijo. Med glavne socialne koristi, ki jih take površine omogočajo uvrščamo: izobraževanje o naravi in pridelavi hrane, socialna kohezija, ki združuje ljudi iz različnih okolij, ki imajo skupni interes za vrtnarjenje in zdravstvene koristi z zmerno telesno aktivnostjo, predvsem za starejše ljudi (Speak in sod., 2015). To lahko potrdimo tudi z našimi rezultati, saj so bile te storitve ugotovljene v vseh lokacijah popisov v kategoriji vrtičkov.

Mestno kmetijstvo je v Ljubljani in tudi drugih mestih različnih oblik (vrtički, skupnostni vrtovi, zasebni vrtovi, zelene strehe), vendar imajo vse te oblike skupen namen. McGranahan in sod. (2005) navajajo, da mnogim mestnim prebivalcem te površine predstavljajo pomemben vir hrane ter vir dodatne oskrbe in zaslužka. To velja tudi v Ljubljani, saj kategoriji zasebnih vrtov in mestnih vrtičkov glede na povpraševanje mestnih prebivalcev pridobivata na pomenu.

Pri vseh zelenih površinah v mestu smo ugotovili njihov pomen za kakovost zraka ter vpliv na mestno mikroklimo. Glavni onesnaževalci v mestu, kot so industrija, transport in ogrevanje pripomorejo k slabšanju kakovosti življenja v mestu. Nowak (1996) ugotavlja, da različne oblike vegetacije v mestu vplivajo na kakovost zraka z odstranjevanjem onesnažil, predvsem ozona, dušikovih oksidov in žveplovega dioksida. To poteka z absorpcijo in adsorpcijo na liste in druge dele mestnih dreves in ostale vegetacije. Rezultati kažejo, da se je to kot pomembno izkazalo predvsem v kategoriji gozd, parki, zelene površine ob blokih, vrtički in drevoredi, kjer je več drevesne vegetacije, v ostalih kategorijah te ES niso bile ugotovljene kot pomembne.

V kategoriji ruderalnih površin je število pripisanih ES zelo majhno, čeprav smo na teh lokacijah popisali razmeroma veliko rastlinskih vrst, vendar pa je funkcionalna pestrost popisane vegetacije manjša (pretežno gre za steblikaste zelnate vrste zgodnjih in srednjih faz sukcesije). Manjkajo večja drevesa in grmi in s tem tudi številne storitve, ki jih srečamo v tistih kategorijah, kjer so ti rastlinski tipi dominantni. Pozitivni prispevek teh lokacij je lepši videz mesta, saj namesto zapuščenih, sivih površin ta območja prerašča zelena in občasno tudi cvetoča (sub)spontana vegetacija. Na tem mestu ne moremo mimo problematike invazivnih vrst, ki se pojavljajo na teh površinah in se hitro širijo. Čeprav te vrste omogočajo ozelenitev zapuščenih površin v mestu in tako v očeh prebivalcev prispevajo k ozelenitvi mesta, so lahko pomemben vir propagulov teh invazivnih vrst, ki tako lahko vdirajo na druge površine, kjer niso zaželene in tam povzročajo gospodarsko škodo, težave pri zdravju ljudi ali pa izrinjajo avtohtono vegetacijo.

V primerjavi povezanosti med številom ES in velikostjo biotske pestrosti smo ugotovili pozitivno zvezo med številom vrst v združbi in številom ugotovljenih ES – več vrst smo ugotovili tam, kjer je tudi več ES. Pri tem je treba opozoriti, da s to raziskavo ne moremo dokazati vzročno posledične zveze, torej da večja biotska pestrost omogoča večje število ES, saj sta tako število vrst kot število ES lahko hkrati posledici drugih dejavnikov- npr. režima motenj oz. režima upravljanja z ekosistemom. Nasploh je potrebno opozoriti, da je proučevanje ekologije mest izredno težavno, saj se v mestih izrazito prepletajo in medsebojno učinkujejo naravni, ekonomski, kulturnozgodovinski in sociološki dejavniki. Raba mestnih zelenih površin in s tem povezane ES posamezne površine se lahko za isto kategorijo izrazito razlikujejo in niso v celoti pojasnljive. Določene vzorce in uporabnost izsledkov pri načrtovanju mestnih zelenih površin bo zato možno pridobiti le ob sintezi večjega števila podobnih analiz.

4 SKLEPI

Zelene površine zagotavljajo široko paleto ES v urbanih območjih. Storitve, povezane z pridelavo hrane, biotsko raznovrstnostjo, opraševanjem in rekreacijo so najbolj značilne in prepoznane v teh kategorijah. Te storitve imajo velik pomen v mestih, predvsem zaradi velike gostote prebivalcev, prometa in pozidanih površin v mestu. V prvi vrsti velja poudariti, da tudi v Ljubljani najvidnejšo vlogo igrajo zgoraj omenjene ES. To se kaže predvsem v vplivu, ki ga imajo zelene površine in pripadajoče ES na kakovost zraka v mestu, uravnavanje onesnaženosti okolja, omogočanje rekreacije in socialnih interakcij ter človekovo dobro počutje.

Zato se je v prihodnje potrebno osredotočiti na načine načrtovanja in upravljanja mest, ki bodo ohranjala ključne komponente ekosistemov, v prvi vrsti naravno biotsko raznovrstnost, ki zagotavlja največ ES. Izguba ES lahko v mestih privede do mnogih negativnih vplivov. Predvsem bodo to vplivi na gospodarstvo, socialo, zdravje ljudi ter kulturo mesta S pravim načinom upravljanja se izboljša dolgoročno zdravje ekosistemov in posledično zagotavljanja obstoj ekosistemskih storitev, ki so ključnega pomena za preživetje vseh živih bitij v mestu.

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Dovzetnost bub plodove vinske mušice (*Drosophila suzukii* (Matsumura, 1931)) za okužbo z entomopatogenimi glivami

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IZVLEČEK

Zatiranje plodove vinske mušice (PVM) (*Drosophila suzukii* (Matsumura, 1931), Diptera, Drosophilidae) je težavno, ker ima vrsta izjemen razmnoževalni potencial, je polifagna in ima ostro nazobčano leglico, s katero lahko predre povrhnjico zdravih plodov, v katere nato izleže jajčeca. Poleg tega se odrasle žerke lahko zabubijo v tleh, kjer so zavarovane pred insekticidi. Naša hipoteza je bila, da bodo talne glive, ki so patogene za žuželke, znatno zmanjšale izleganje mušic iz okuženih bub PVM. Bube PVM smo okuževali z več entomopatogenimi in talnimi glivami: a) v substratu, okuženim s konidiji gliv, b) z neposrednim nanosom suspenzije gliv na bube ter c) z namakanjem bub v suspenzijo gliv. Gliva *Metarhizium brunneum* Petch izolat H.J.S. 1154 je značilno zmanjšala izleganje PVM v okuženem substratu, bioinsekticid Naturalis (na podlagi entomopatogene glive *Beauveria bassiana* (Bals.-Criv.) Vuill.) pa v poskusih neposredne izpostavitve. Poskus namakanja bub v suspenzijo gliv, s katerim smo želeli določiti IC_{50} izleganja bub, je bil neuspešen. Sklepamo, da je razvojni stadij bube PVM prekratek, da bi glive izrazito vplivale na izleganje odraslih osebkov PVM. Skladno z našimi rezultati in objavljeno literaturo, bi bilo smiselno preučiti potencial entomopatogenih gliv v biotičnem varstvu neposredno na odraslih osebkih PVM.

Ključne besede: biotično varstvo rastlin; entomopatogene glive; jagodičevje; škodljivci; talne glive; virulenca; žuželke

ABSTRACT

SUSCEPTIBILITY OF SPOTTED WING DROSOPHILA (*Drosophila suzukii* (Matsumura, 1931)) PUPAE TO ENTOMOPATHOGENIC FUNGI

Spotted wing drosophila (*Drosophila suzukii* (Matsumura, 1931), Diptera, Drosophilidae) management is difficult mainly because of its short generation time, polyphagy and serrated ovipositor, but also because its larvae can pupate in the orchard soil and are thus protected from insecticide applications. We hypothesized that insect-pathogenic soil fungi could successfully infect *Drosophila suzukii* pupae in soil environment. We tested several entomopathogenic or soil fungi against pupae in a) conidia-spiked soil, b) via direct applications of conidia, and c) by dipping pupae into conidial suspensions. *Metarhizium brunneum* Petch strain H.J.S. 1154 significantly reduced fly emergence in conidia spiked soil and bioinsecticide Naturalis (based on entomopathogenic fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. in direct exposure tests. Our attempt to determine IC_{50} of pupal hatching rate by dipping *D. suzukii* pupae into conidial suspensions was unsuccessful. We conclude that the pupal stage is probably too brief to allow entomopathogens to cause a significant reduction of fly emergence. According to our results and published articles, the fungal biocontrol potential would probably best be evaluated in spray applications against adult flies.

Key words: biological control; entomopathogenic fungi; insect-pathogenic soil fungi, insect; organic; pest; soft fruit; virulence

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1 UVOD

Plodova vinska mušica (PVM) (*Drosophila suzukii* (Matsumura, 1931), Diptera, Drosophilidae) izvira iz Azije in je od l. 2008 zastopana v Severni Ameriki in Evropi, kjer povzroča škodo na jagodičevju (Cini in sod., 2014). Od ostalih sorodnih vrst vinskih mušic, ki se prehranjujejo na gnijočih in poškodovanih plodovih, se samice PVM razlikujejo po ostro nazobčani leglici, s katero lahko samice odlagajo jajčeca v nepoškodovane zoreče plodove in povzročijo večje izgube pridelka (Lee in sod., 2011). V domači strokovni literaturi sta o škodljivcu in možnih načinih njegovega zatiranja že pisala Bohinčeva in Trdan (2014).

PVM se razmnožuje hitro in ima skozi celo rastno dobo na razpolago številne gostiteljske rastline, zato je njeno zatiranje oteženo. Poleg tega lahko dorasle žerke zapustijo plod in se zabubijo v tleh sadovnjaka, kjer so varne pred insekticidi (Cuthbertson in sod., 2014a). Proučevanih je bilo že več strategij varstva v nasadih ameriških borovnic, jagodnjaka, malinjaka (e.g. Bruck in sod., 2011; Van Timmeren and Isaacs, 2013) in drugih sadnih vrst, toda pri ekološki pridelavi sadja je raba insekticidov močno omejena, poleg tega njihova raba negativno vpliva na koristne organizme. Več raziskav v svetu je bilo usmerjenih v proučevanje parazitoidov in plenilcev (Chabert in sod. 2012; Gabarra in sod. 2015; Rossi Stacconi in sod. 2015; Woltz in sod. 2015), entomopatogenih ogorčic (Cuthbertson in sod., 2014b; Gargani in sod., 2013; Woltz in sod., 2015) in entomopatogenih gliv (Naranjo-Lázaro in sod., 2014) z namenom učinkovitega biotičnega zatiranja PVM. V nobeni od omenjenih raziskav pa niso neposredno preizkušali vpliv izolatov entomopatogenih gliv (EPF) na razvojni stadij bube PVM.

Naša hipoteza je bila, da lahko entomopatogene glive uspešno okužijo bube PVM v testnem substratu. Znano pa je, da so lahko, poleg entomopatogenih gliv, tudi talne glive patogene za žuželke, kar smo že dokazali na zgledu kapusove muhe *Delia radicum* (Linnaeus, 1758) (Razinger in sod., 2014a, b). Cilji raziskave so bili določiti zmanjšanje izleganja mušic a) zaradi posredne, substratne, izpostavitve glivam in b) neposredne izpostavitve glivam, ter c) določiti inhibicijo izleganja (IC_{50}) bub prek namakanja bub v suspenziji konidijev različnih talnih in entomopatogenih gliv.

2 MATERIALI IN METODE

2.1 Nanos gliv in bioinsekticidov

Preskušali smo glive *Metarhizium brunneum* Petch (izolata H.J.S. 1154 in 1868), *Trichoderma atroviride* Bissett (izolat 1873), *Clonostachys rosea* (Link) Schroers (izolat 1884), in *Beauveria bassiana* (Bals.-Criv.) Vuill. (izolata 2121 and 2122). V poskusih smo konidije dodajali testnemu substratu v vodni suspenziji. Glive smo gojili ter preverili viabilnost konidijev, kot je opisano v Razinger in sod. (2014b). Bioinsekticid Laser 240 SC (a.s. spinosad, 22,75 % w/w, Dow Agrosciences, Dunaj, Avstrija) smo uporabili kot pozitivno kontrolo. Bioinsekticid Naturalis (a.s. *B. bassiana*, 2,3 x 10^7 CFU ml^{-1}, Andermatt biocontrol AG, Grossdietwil, Švica) smo uporabili kot referenčni biotični pripravek. Neionski detergent Tween 80 (0,1 %) smo uporabili kot negativno kontrolo.

2.2 Gojenje plodove vinske mušice

PVM smo gojili v 30×30×30 cm plastičnih insektarijih v komori v nadzorovanih razmerah: dan:noč 14:10 h pri 21 °C in 77 ± 3 % RH. Mušice so imele na razpolago vodovodno vodo ter umetno hrano (20 g agarja, 20 g sladkorja, 10 g pšenične moke, 50 g suh pekovski kvas, 500 ml vodovodne vode, 400 g naribanih ekoloških jabolk, 500 ml ekološkega jabolčnega soka, 50 ml jabolčnega kisa in 4 g nipagina (methyl 4-hydroxybenzoate, Sigma-Aldrich)), kamor so legle jajčeca in v kateri so se razvijale ličinke.

2.3 Zasnova poskusov

2.3.1 Substratna izpostavitev bub plodove vinske mušice

Štiristo gramom na zraku posušenega šotnega komercialnega substrata za presajanje

(Tonsubstrat, Klasmann-Deilmann GmbH, Nemčija) smo dodali 40 ml suspenzije konidijev in 40 ml sterilne demineralizirane vode. Substrat s konidiji smo temeljito premešali s sterilno lopatico v plastični posodi, da smo dobili končno koncentracijo 4×10^6 živih konidijev g^{-1} zračno-suhega substrata. 40-gramske alikvote substrata s konidiji smo dodali v posamezne 250 ml poskusne posodice. V vsako posodico smo dodali pet 1-3 dni starih bub PVM. Pokrove testnih posod smo preluknjali z iglo. Poskus smo izvajali v enakih razmerah kot smo gojili mušice (opisano zgoraj). Vsak dan do osem dni po okužbi smo beležili število izleglih mušic. Neizlegle bube smo prestavili na vodni agar (1 %) in po sedmih dneh določili stopnjo okužbe z glivami.

2.3.2 Neposredna izpostavitev bub plodove vinske mušice

Pet 1-3 dni starih bub PVM smo dali v posamezno jamico na multi-plošči s 6 jamicami. 50 µl suspenzije konidijev s koncentracijo 1×10^8 živih konidijev ml^{-1} smo odpipetirali neposredno na bube. Vsak dan do osem dni po okužbi smo beležili število izleglih muh. Neizlegle bube smo prestavili na vodni agar (1 %) in po sedmih dneh določili stopnjo okužbe z glivami.

2.3.3 Določanje 50 % inhibicije izleganja bub (IC_{50})

Deset 1-3 dni starih bub PVM smo namočili v suspenzije konidijev, 0,1 % Naturalis, ali Tween 80 (0,1 %) kot negativno kontrolo, za 30 s, ob sočasnem rahlem mešanju. Preskušali smo naslednje koncentracije suspenzij konidijev: 10^8, 10^6, 10^5, 10^4, 10^2 in 0 živih konidijev ml^{-1}. Po namakanju smo neadsorbirane suspenzije konidijev ali ostale testne tekočine odstranili tako, da smo izpostavljene bube prestavili na sterilne papirne brisače. Po 10 izpostavljenih bub smo prestavili v posamezno jamico na multi-plošči s šestimi jamicami. V vsako jamico smo dodali 1 g umetne hrane. Umetno hrano smo zamenjali po 7 dneh, da smo preprečili razvoj naslednjega rodu PVM. Negativna kontrola je bila izvedena v šestih ponovitvah, ostali postopki pa v treh. Eno ponovitev je predstavljalo 10 bub v posamezni jamici. Poskus smo opazovali 0, 2, 3, 4, 7, 10 in 16 dni po izpostavitvi (PI) in beležili število izleglih mušic.

2.4 Analiza podatkov

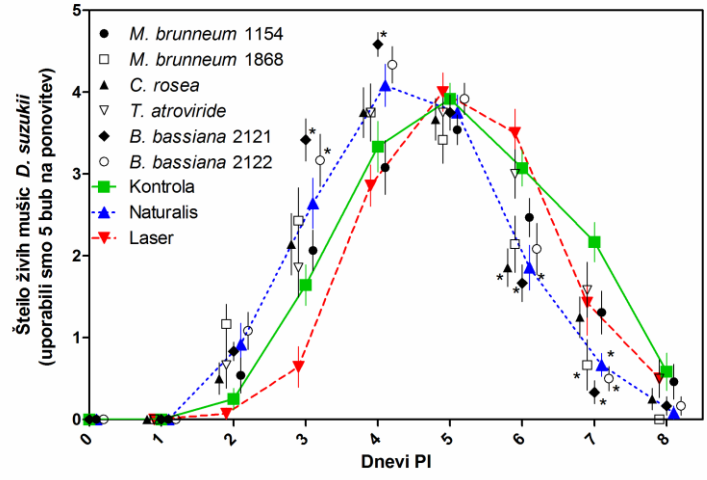
Podatke iz poskusov substratne in neposredne izpostavitve bub PVM smo analizirali za normalnost razporeditve z D'Agostino-Pearson omnibus K2 testom. Iz teh podatkov smo izračunali parameter 'dolgoživost mušic', kot povprečje vsote živih mušic opaženih v vseh ponovitvah v vseh dneh opazovanj poskusov. Ta parameter nam je služil kot ocena posrednega vpliva obravnavanj na mušice (odrasle osebke). Statistično značilnost razlik med obravnavanji in negativno kontrolo smo izračunali z dvosmerno analizo variance s faktorjema 'čas po izpostavitvi' in ' obravnavanje' ter Bonferronijevim post-testom. Kumulativno stopnjo izleganja smo obdelali z enosmerno analizo variance in Dunnettovim post-testom. Podatke iz poskusa Določanje IC_{50} smo analizirali z dvosmerno analizo variance z dejavnikoma 'koncentracija konidijev' in 'čas po izpostavitvi' ter Bonferronijevimi post-testi (Gaddum, 1948; Motulsky, 1995). Razlika med obravnavanji in negativno kontrolo je bila značilna pri pogoju $P < 0,05$, in je označena v grafih oz. v tabeli z zvezdico (*). Število neodvisnih ponovitev poskusov in ponovitve v posameznem poskusu so navedene v naslovih grafov oz. tabele. Statistične analize smo opravili s programom GraphPad Prism 5.00 (GraphPad Software, Inc., La Jolla, CA, ZDA).

3 REZULTATI

3.1 Substratna izpostavitev bub plodove vinske mušice

Na število živih mušic v poskusu substratne izpostavitve bub je značilno vplival čas izpostavitve ($F_{8,\ 942} = 389$; $P < 0.0001$) in interakcija dejavnikov 'čas izpostavitve' in 'obravnavanje' ($F_{64,\ 942} = 4,79$; $P < 0.0001$), medtem ko samo 'obravnavanje' ni imelo značilnega vpliva ($F_{8,\ 942} = 1,6$; $P = 0,1210$). Bonferronijev post-test je pokazal značilno večje število živih mušic v kontrolnem obravnavanju v primerjavi z obravnavanjem *B. bassiana* (seva 2121 in 2122) na tretji dan in pri sevu 2122 tudi četrti dan po izpostavitvi (PI). Značilno manj živih mušic v

primerjavi s kontrolo je bilo v skupinah okuženih z glivama *C. rosea* in *B. bassiana* (2121) ter pripravkom Naturalis šesti dan PI, ter glivama *M. brunneum* (1868) in *B. bassiana* (2121 in 2122) ter pripravkom Naturalis sedmi dan PI (Slika 1). Najbolj patogen izolat glive (*M. brunneum* 1154) je povzročil 15 % (značilno), bioinsekticid Naturalis pa 5 % zmanjšanje (neznačilno) celokupnega izleganja mušic v primerjavi s kontrolno skupino. Obravnavanja niso značilno vplivala na parameter 'dolgoživost mušic' (Tabela 1). Največ neizleglih in okuženih bub smo opazili v skupinah okuženih z glivama *M. brunneum* (1154 in 1868) in *B. bassiana* (2121 in 2122).

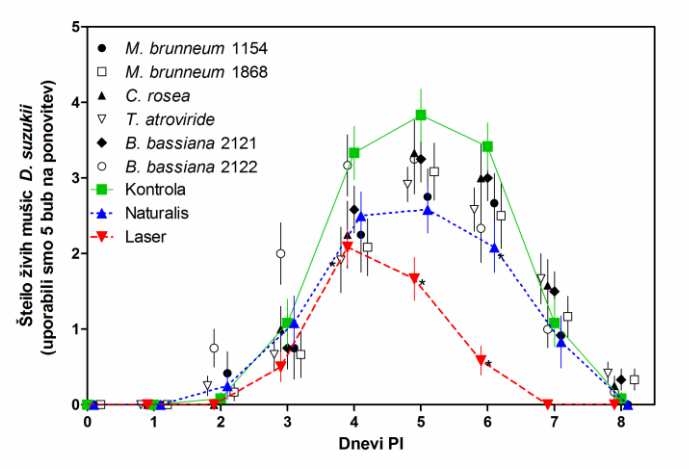


Slika 1: Število živih mušic vrste *D. suzukii*, ki so se izlegle iz bub, vstavljenih v substrat, ki je bil okužen z različnimi glivami, ali obdelan z bioinsekticidom, v odvisnosti od časa po izpostavitvi. Zvezdica (*) označuje značilno razliko od kontrolnih vzorcev (P < 0,05). Predstavljena so povprečja ± standardna napaka. Uporabili smo pet bub na ponovitev. Število ponovitev (N), združenih iz dveh poskusov, je bilo 14. Podatkovne točke na grafu smo zamaknili do ± 0,2 enote za preprečevanje prekrivanja. Naturalis - bioinsekticid na podlagi glive *Beauveria bassiana* izolat ATCC 74040. Laser 240 SC - bioinsekticid na podlagi aktivne snovi spinosad; PI - po izpostavitvi oz. okužbi.

Figure 1: The number of living *D. suzukii* flies emerged from pupae placed into soil inoculated with different fungi or treated with bioinsecticide. Asterisk (*) denotes a significant difference from control samples (P<0.05). Data presented are means ± standard error. Five pupae per replicate were used. Number of replicates (N) pooled from two experiments was 14. Data points on the graph were nudged up to ± 0.2 units to prevent overlapping. Naturalis – bioinsecticide based on *Beauveria bassiana* isolate ATCC 74040. Laser 240 SC– insecticide based on spinosad; Dnevi PI – days post infection.

3.2 Neposredna izpostavitev bub plodove vinske mušice

Na število živih mušic v poskusu neposredne izpostavitve sta značilno vplivala dejavnika 'obravnavanje' ($F_{8, 891}$ = 9,40; p < 0,0001) in 'čas po izpostavitvi' ($F_{8, 891}$ = 184; p <0,0001), hkrati pa tudi njuna interakcija ($F_{64, 891}$ = 2,12; p < 0,0001). Bonferronijev post-test je pokazal značilno manj preživelih mušic, v primerjavi s kontrolo, v skupinah okuženih z glivo *T. atroviride* četrti dan PI, z bioinsekticidom Laser 240 SC peti in šesti dan PI in pripravkom Naturalis šesti dan PI (Slika 2). Obravnavanja so značilno vplivala na parameter 'dolgoživost mušic' pri testu neposredne izpostavljenosti ($F_{8, 99}$ = 12,2; p < 0,0001). Najbolj patogen izolat (*M. brunneum* 1154) je povzročil 8 % (neznačilno), Naturalis pa 21 % (značilno) zmanjšanje celokupnega izleganja mušic v primerjavi s kontrolno skupino. Skupine žuželk, ki so bile okužene z glivo *M. brunneum* (1154 in 1868), ter pripravki Naturalis in Laser 240 SC, so imele značilno manjši parameter 'dolgoživost mušic', v primerjavi s kontrolo (Tabela 1). Največ neizleglih okuženih bub smo opazili v skupinah, okuženih z glivami *M. brunneum* (1154 in 1868), *C. rosea*, *B. bassiana* (2121) in pripravkom Naturalis.



Slika 2: Število živih mušic vrste *D. suzukii*, ki so se izlegle iz bub neposredno okuženih z različnimi glivami ali obdelanih z bioinsekticidom, v odvisnosti od časa po izpostavitvi. Zvezdica (*) označuje značilno razliko od kontrolnih vzorcev (P < 0,05). Predstavljena so povprečja ± standardna napaka. Uporabili smo pet bub na ponovitev. Število ponovitev (N), združenih iz dveh poskusov, je bilo 12. Podatkovne točke na grafu smo zamaknili do ± 0,2 enote za preprečevanje prekrivanja. *Naturalis* - bioinsekticid na osnovi glive *Beauveria bassiana* izolat ATCC 74040. Laser 240 SC- bioinsekticid, ki temelji na aktivni učinkovini spinosad; PI - po izpostavitvi oz. okužbi.

Figure 2: The number of living *D. suzukii* flies emerged from pupae directly infected with different fungi or treated with bioinsecticide. Asterisk (*) denotes a significant difference from control samples (P<0.05). Data presented are means ± standard error. Five pupae per replicate were used. Number of replicates (N) pooled from two experiments was 12. Data points on the graph were nudged up to ± 0.2 units to prevent overlapping. Naturalis – bioinsecticide based on *Beauveria bassiana* isolate ATCC 74040. Laser 240 SC – insecticide based on spinosad; Dnevi PI – days post infection.

Tabela 1: Parameter 'dolgoživost mušic', izračunan kot povprečje vsote živih mušic v vseh ponovitvah v vseh dneh opazovanj poskusov. Zvezdica (*) označuje značilno razlikovanje od negativne kontrole (P < 0,05). Predstavljena so povprečja ± standardna napaka. Uporabili smo pet bub na ponovitev. Število ponovitev (N), združenih iz dveh poskusov, je bilo 14 v poskusu substratne izpostavitve in 12 v poskusu neposredne izpostavitve.

Table 1: Parameter Fly longevity, calculated as the replicate average of the sum of living flies observed at all observation days. Asterisk (*) denotes a significant difference from control samples (P<0.05). Data presented are means ± standard error. Five pupae per replicate were used. Number of replicates (N) pooled from two experiments was 14 in soil test and 12 in direct exposure test.

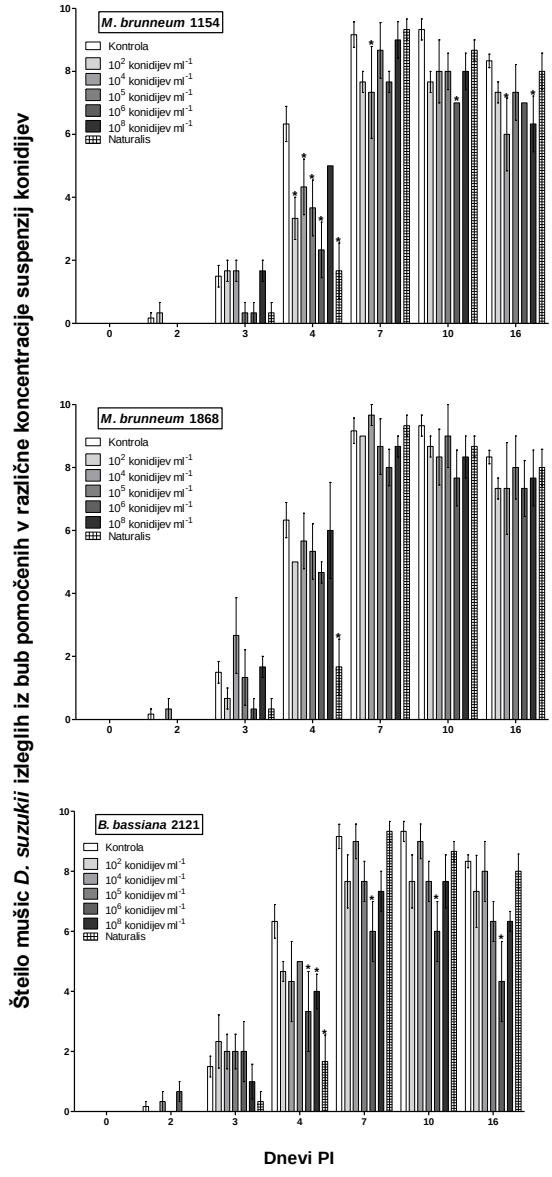
Obravnavanje	**Substratna izpostavitev**	**Neposredna izpostavitev**
	Dolgoživost mušic (a.u.)	Dolgoživost mušic (a.u.)
M. brunneum (1154)	13,5 ± 0,68	9,8 ± 0,83*
M. brunneum (1868)	13,8 ± 0,81	10,0 ± 0,59*
C. rosea (1884)	13,9 ± 0,81	11,4 ± 0,71
T. atroviride (1873)	15,3 ± 0,72	10,4 ± 0,73
B. bassiana (2121)	14,8 ± 0,45	11,5 ± 0,90
B. bassiana (2122)	15,3 ± 0,62	12,7 ± 0,75
Kontrola	14,9 ± 0,70	12,9 ± 0,48
Naturalis[a]	14,1 ± 0,83	9,3 ± 0,67*
Laser 240 SC[b]	13,0 ± 0,77	4,8 ± 0,39*

[a] Bioinsekticid na podlagi glive *Beauveria bassiana* izolat ATCC 74040, uporabljen v priporočeni koncentraciji 0,1 % (v / v).

[b] Bioinsekticid na podlagi spinosada (22,75 % m/m aktivne učinkovine), uporabljen v priporočeni koncentraciji 0,1 % (v / v).

3.3 Določanje 50 % inhibicije izleganja bub (IC_{50})

Na število mušic, izleglih iz bub okuženih z glivo *M. brunneum* 1868 v poskusu določanja IC_{50}, je značilno vplivala 'koncentracija konidijev' ($F_{6, 119} = 7,94$; $p < 0,0001$) in 'čas po izpostavitvi' ($F_{6, 119} = 380$, $p < 0,0001$) ter njuna interakcija ($F_{36, 119} = 2,41$; $P = 0,0002$). Bonferronijevi post testi so pokazali značilno manj izleglih mušic v primerjavi s kontrolo v skupinah okuženih z 10^2 ml^{-1} viabilnih konidijev glive *M. brunneum* 1868 (v nadaljevanju konidijev) četrti dan PI, ter v skupinah okuženih z 10^4 konidijev četrti, sedmi in 16. dan PI, 10^5 konidijev četrti dan PI, 10^6 konidijev četrti in 10. dan PI in 10^8 viabilnih konidijev ml^{-1} 16. dan PI (24% zmanjšanje). Na število mušic, izleglih iz bub, okuženih z glivo *M. brunneum* 1868 v poskusu določanja IC_{50}, je prav tako značilno vplivala 'koncentracija konidijev' ($F_{6, 119} = 3,61$; $p = 0,0026$) in 'čas po izpostavitvi' ($F_{6, 119} = 343$, $p < 0,0001$), ne pa njuna interakcija ($F_{6, 119} = 1,43$; $P = 0,0796$). Okužba bub z 10^8 viabilnimi konidiji glive *M. brunneum* 1868 ml^{-1} je zmanjšalo število izleglih mušic za 8 % 16. dan PI v primerjavi s kontrolno skupino. Število mušic, izleglih iz bub, okuženih z glivo *B. bassiana* (2121) v poskusu določanja IC_{50}, je bilo odvisno od dejavnikov 'koncentracija konidijev' ($F_{6, 119} = 7,93$; $p < 0,0001$) in 'čas po izpostavitvi' ($F_{6, 119} = 246$; $p < 0,0001$) ter tudi njune interakcije ($F_{36, 119} = 2,39$; $P = 0,0002$). Bonferronijevi post testi so pokazali značilno manj izleglih mušic v primerjavi s kontrolo, kjer je bila koncentracija viabilnih konidijev 10^6 ml^{-1} četrti, sedmi, 10. in 16. dan PI in pri koncentraciji 10^8 viabilnih konidijev ml^{-1} četrti dan PI. Okužba bub z 10^8 viabilnimi konidiji glive *B. bassiana* (2121) ml^{-1} je povzročilo 24 % zmanjšanje števila izleglih mušic 16. dan PI v primerjavi s kontrolno skupino. Namakanje bub v 0,1 % raztopino pripravka Naturalis je značilno vplivalo na izleganje mušic četrti dan PI. 16. dan PI je bilo 4 % (neznačilno) manj izleglih mušic v obravnavanju s pripravkom Naturalis v primerjavi s kontrolo (Slika 3).



Slika 3: Število mušic vrste *D. suzukii*, izleglih iz bub, pomočenih v različnih koncentracijah konidijev ali obdelanih z bioinsekticidom, v odvisnosti od časa po izpostavitvi. Zvezdica (*) označuje značilno razlikovanje od kontrolnih vzorcev (P < 0,05). Predstavljena so povprečja ± standardna napaka. Uporabili smo deset bub na ponovitev. Standardne napake so izračunane iz šestih ponovitev kontrolnih vzorcev in treh ponovitev pri ostalih obravnavanjih. Naturalis - bioinsekticid na osnovi izolata glive *Beauveria bassiana* ATCC 74040; PI - po izpostavitvi oz. okužbi.

Figure 3: The number of living *D. suzukii* flies emerged from pupae dipped into various concentrations of fungal conidia or treated with bioinsecticide. Asterisk (*) denotes a significant difference from control samples (P<0.05). Data presented are means ± standard error. Ten pupae per replicate were used. Error bars are drawn from 6 in control and 3 replicates in fungal treatments. Naturalis – bioinsecticide based on *Beauveria bassiana* isolate ATCC 74040. Dnevi PI – days post infection.

4 DISKUSIJA

V poskusih posredne (substratne) in neposredne izpostavitve bub plodove vinske mušice (PVM; *D. suzukii*) smo ugotovili, da glive in bioinsekticida značilno vplivajo na izleganje bub s sočasnim pojavom mikoz na večini neizleglih bub (Slika 1, Slika 2, Tabela 1). Izpostavljenost glivam je, čeprav značilno, sorazmeroma malo zmanjšala število izleglih bub v poskusih z okuženim substratom ter pri poskusih z neposredno izpostavitvijo (izolat glive *M. brunneum* 1154 je povzročil 15 in 8 %, Naturalis pa 5 in 21 % zmanjšanje celokupnega izleganja mušic v poskusih z okuženim substratom oziroma v poskusih z neposredno izpostavitvijo). Naša hipoteza, da bodo talne glive iz rodov *Clonostachys* ali *Trichoderma* v poskusih substratne izpostavitve prekašale entomopatogene glive, je bila zavrnjena, saj je le dobro znana entomopatogena gliva *M. brunneum* (sev H.J.S. 1154) značilno zmanjšala izleganje mušic v okuženem substratu. To gre morebiti pripisati kratkotrajnosti razvojnega stadija bube PVM, ki (entomopatogenim) glivam ne omogoča, da bi v takšnih razmerah dosegle želen učinek (Cini in sod., 2012).

Izpostavljenost glivam je imela tudi posreden vpliv na izlegle mušice, še posebej v poskusih z neposredno izpostavitvijo. Mušice, ki so se izlegle iz bub neposredno okuženih z izolatom glive *M. brunneum* (1154 in 1858) ali bioinsekticidoma Naturalis in Laser 240 SC, so imele značilno manjšo vrednost parametra 'dolgoživost mušic' (Tabela 1). To je lahko povezano z nanosom velikega števila konidijev na posamezno bubo, saj so bili konidiji v poskusu neposredne izpostavitve naneseni neposredno na bube, v primerjavi s poskusom substratne izpostavitve, kjer nismo opazili značilnega vpliva na parameter 'dolgoživost mušic'. Zanimivo pa smo v poskusu substratne izpostavitve opazili, da je izpostavitev bub nekaterim sevom gliv premaknila krivuljo izleganja v levo, torej so se mušice hitreje razvile in izlegle, a tudi hitreje poginile v primerjavi z mušicami v kontrolni skupini (npr. izolata glive *B. bassiana*, seva 2121 in 2122, Slika 1).

Doslej je večina avtorjev testirala komercialne insekticide na podlagi entomopatogenih gliv (EPF) ali pa komercializirane izolate EPF proti jajčecem in/ali mušicam (odraslim osebkom) PVM (Cuthbertson in sod., 2014b; Gargani in sod., 2013; Woltz in sod., 2015), z izjemo Naranjo-Lázaro in sod. (2014), ki so testirali nekomercialne izolate EPF *Isaria fumosorosea* Wize in *Metarhizium anisopliae* (Metchnikoff) Sorokin proti odraslim mušicam. Cuthbertson in sod. (2014b), Woltz in sod. (2015) in Naranjo-Lázaro in sod. (2014) so poročali, da lahko s pršenjem EPF dosežemo značilno povečanje smrtnosti mušic. Izolati glive *I. fumosorosea* Pf21, Pf17 in Pf15 so povzročili 85, 60 oz. 58 % smrtnost 12. dan PI (Naranjo-Lázaro in sod., 2014). Bioinsekticid Naturalis (0,3 % raztopina) je povzročil 44 % smrtnost mušic sedmi dan PI (Cuthbertson in sod., 2014b). Žal pa v teh člankih ne poročajo o vplivu EPF na bube, zato je primerjava rezultatov naših poskusov z objavljenimi rezultati problematična. Če vseeno primerjamo vpliv gliv na izleganje bub z vplivom na odrasle osebke, vidimo, da imajo glive večji vpliv na smrtnost odraslih osebkov (smrtnost med 50 in 85 %) kot na izleganje bub (zmanjšanje izleganja bub za 15 do 21 %). Dodatno smo v tej raziskavi opazili značilen posredni učinek na odrasle osebke prek izračuna parametra 'dolgoživost mušic'. Tako se zdi, da so odrasli osebki bolj dovzetni za glivične okužbe kot bube. Skladno s tem, naši izsledki kažejo na vprašljivo uspešnost potencialne uporabe EPF za obdelavo tal v sadovnjakih z namenom zmanjšanja populacije PVM.

Poskus določanja IC_{50} izleganja bub PVM z namakanjem bub v suspenzijo konidijev gliv *M. brunneum* (1154 in 1868) in *B. bassiana* (2121), je bil neuspešen. Značilno manjše izleganje mušic je bilo opaženo pri več različnih koncentracijah konidijev ob različnih časih PI, vendar ta odziv ni bil odvisen od koncentracije (Slika 3). To je verjetno najlaže pripisati kratkotrajnosti razvojnega stadija bube (Cini in sod., 2012).

5 SKLEPI

Ugotovili smo, da preizkušane glive značilno vplivajo na izleganje bub PVM. Značilno zmanjšanje izleganja smo opazili pri EPF *M. brunneum* in *B. bassiana* (Naturalis), ne pa tudi pri talnih glivah iz rodov *Clonostachys* in *Trichoderma*. Primerjava objavljenih rezultatov z rezultati pridobljenimi v tej študiji kaže, da imajo določeni mikrobni bioinsekticidi in trenutno nekomercialni izolati EPF v nadzorovanih razmerah večji vpliv na smrtnost mušic vrste *D. suzukii*, v primerjavi z nanosom na bube. Posledično bi bilo priporočljivo usmeriti več raziskav v testiranje (neznanih) EPF izolatov proti odraslim mušicam.

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doi:10.14720/aas.2017.109.1.13 Review article / pregledni znanstveni članek

A review of plant protection against the olive fly (*Bactrocera oleae* (Rossi, 1790) Gmelin) and molecular methods to monitor the insecticide resistance alleles

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ABSTRACT

Olive fly (*Bactrocera oleae* (Rossi, 1790) Gmelin) is one of the most important olive pests worldwide. Most plant protection measures are based on insecticides, especially organophosphates, pyrethroids, and recently a spinosad. Insecticides are used as cover sprays or in more environmentally friendly methods in which insecticides are used in combination with attractants and pheromones as bait sprays or for mass trapping. However, due to negative impacts of insecticides to environment, new plant protection methods are constantly developing with the aim to lower the consumption of insecticides or even to eliminate them by biological control with entomopathogenic organisms, sterile insect technique (SIT), or transgenic method RIDL (release of insects carrying a dominant lethal). However, these methods need to be improved in order to guarantee adequate protection. Alternative methods than those traditionally used are required due to long term usage causing the development of resistance to the insecticides, ultimately lowering their effectiveness. Molecular methods for monitoring the frequencies of resistant alleles and the current status of resistance alleles in olive growing countries are reviewed here.

Key words: organophosphates; *ace* gene; resistance alleles; plant protection methods; alternative methods; spinosad; biological control

IZVLEČEK

PREGLED VARSTVA PRED OLJČNO MUHO (*Bactrocera oleae* (Rossi, 1790) Gmelin) IN MOLEKULARNIH METOD ZA SPREMLJANJE ALELOV, ODGOVORNIH ZA RAZVOJ ODPORNOSTI NA INSEKTICIDE

Oljčna muha (*Bactrocera oleae*) je eden najpomembnejših svetovno razširjenih škodljivcev oljke. Večina varstvenih ukrepov temelji na insekticidih, predvsem na organskih fosforjevih estrih, piretroidih in nedavno uvedenem spinosadu. Insekticide se lahko nanese po celotni krošnji. Drugi, okolju prijaznejši način, vključuje uporabo insekticida v kombinaciji z atraktanti in feromoni. Tak pripravek se lahko nanese na del krošnje in deluje kot zastrupljena vaba, ali pa se ga uporabi pri metodi masovnega lovljenja. Zaradi negativnih vplivov insekticidov na okolje se nenehno razvija nove metode varstva, s ciljem zmanjšati porabo insekticidov ali jih celo izločiti. Sem sodijo biološko varstvo z entomopatogeni organizmi, tehnika sterilnih insektov (SIT) ali transgena metoda RIDL (izpust insektov z dominantnimi letalnimi geni). Za doseganje učinkovite zaščite bi bilo omenjene alternativne metode potrebno izboljšati. Alternativne metode so nujne zaradi odpornosti na insekticide, ki se pojavi ob daljši uporabi insekticidov in zmanjšuje njihovo učinkovitost. V okviru članka je bil opravljen pregled molekularnih metod za spremljanje prisotnosti alelov, odgovornih za razvoj odpornosti na insekticide ter pregled stanja prisotnosti rezistentnih alelov v državah, kjer pridelujejo oljke.

Ključne besede: organski fosforjevi estri; *ace* gen; rezistentni aleli; metode varstva rastlin; alternativne metode; spinosad; biološko varstvo

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1 INTRODUCTION

Olive fly (*Bactrocera oleae* (Rossi, 1790) Gmelin) is one of the most important olive pests worldwide (Daane and Johnson, 2010; Malheiro et al., 2015a). Until now, it has not been possible to find cultivars showing clear evidence of resistance or tolerance to this pest (Fabbri et al., 2009). However, not all olive cultivars are equally susceptible to olive fruit fly infestation. Some of the factors related to fruit traits that possibly play a role include fruit size and mass, color, fruit exocarp hardness, surface covering (mainly of aliphatic waxes), phenological stage of the crop, and chemical composition of olive fruits (Daane and Johnson, 2010; Malheiro et al., 2015a). Recently, Malheiro et al. (2016) studied olive fly oviposition preference to the volatiles from olive leaves from different cultivars and they observed correlation between infestation level during olive maturation and the aromatic hydrocarbon toluene. When volatiles from olive fruits were analysed, α-copaene was found as an oviposition promoter (Malheiro et al., 2015b). Garantonakis et al. (2016) observed positive correlation between *B. oleae* infestation and the content of potassium and iron in olive fruits.

However, other factors with impact on olive fly population density have to be considered as well including weather conditions, location, a cultural landscape diversity, crop load, and agronomical technologies. It is known that the development of the olive fly is largely temperature dependent (Daane and Johnson, 2010; Podgornik et al., 2013). Ortega and Pascual (2014) detected a relationship between a population of *B. oleae* and landscape complexity that could occur through the presence of natural enemies of the phytophagous insects in some landscape elements, such as hedgerows or field margins adjacent to land uses with natural or rural vegetation. Regarding agronomical technologies, Burrack et al. (2008) observed more olive fruit flies captured in traps stationed in irrigated trees compared to unirrigated trees. Turning over the soil under the canopies is one of the preventive measures listed in International Olive Council (IOC) guidelines (Jardak et al., 2007) with the aim to disrupt the development of the pupal stage. However, such practice can have negative impacts on beneficial organisms (Herz et al., 2005) and causes soil erosion, loss of organic matter through mineralization, and nutrient leaching in underground water.

2 PLANT PROTECTION AGAINST OLIVE FLY

Since there are no other efficient methods to protect plants from the olive fly, common control methods against B. oleae remain insecticide-based. These are bait sprays, cover sprays, and mass trapping (Haniotakis, 2005). According to the IOC, a treatment threshold for cover sprays should be considered (10 % to 15 % infested fruit intended for oil production and 1 % to 2 % for table olive production), while a poisoned bait should be used before or on the appearance of the first punctures (Jardak et al., 2007). Over the last four decades, organophosphate (OP) insecticides were the most frequently used. Recently, there has been an increased usage of pyrethroids, neonicotinoids, and very recently in a spinosad insecticide (Daane and Johnson, 2010; Knap and Bandelj, 2016; Varikou et al., 2016). Spinosad is available as a bite spray together with a foodstuff attractant in a product named GF-120 (Dow AgroSciences).

Bait sprays have an advantage over cover sprays, because they attract flies to the insecticide using an attractant. This minimizes the impact on natural enemies (Varikou et al., 2014) and reduces the total amount of pesticides used. Bait sprays are generally recognized to be an integral component of integrated pest management (Varikou et al., 2016). Nevertheless, it offers only limited success of protection. Bait sprays consist of hydrolyzed protein (serving as a bait) and of insecticide. In the past, many attempts were made to improve the attractiveness of bait spray solutions (Varikou et al., 2014; Varikou et al., 2015) and to develop new attract-and-kill traps (Potamitis et al., 2014; Yokoyama, 2014b). Varikou et al. (2014, 2015) observed reduced attractiveness in the attractant solution when plant protection products such as pyrethroids (lamba-cyhalothrin, alpha-cypermethrin) or organophosphorous (dimethoate) were added. The best results were obtained with a

combination of all tested proteins and pyrethroids. The only exception was a mixture of Entomela 75 hydrolyzed protein and alpha-cypermethrin, while dimethoate and spinosad solutions displayed weaker attractiveness to *B. oleae*. Although Spinosad did not perform well in this study, its effectiveness is confirmed in large scale trials (Varikou et al., 2015).

Another disadvantage of attractants is their limited effective duration. Attractants do not seem to last more than three to six days in traps and more than three days in bait spray applications (Varikou et al., 2014). More recent results indicate that the ability of tested bait sprays to attract and kill is limited to the first day only with no significant capture observed after the second or third day (Varikou et al., 2015). More research is needed to formulate spraying solutions offering acceptable olive crop protection (Varikou et al., 2015). However, important observations toward reducing the quantity of insecticide in bait spray solutions were provided by Varikou et al. (2016). They observed that one-third or half of the recommended volume of spraying solution, which is 300 ml per tree, was effective as well.

Other than residual bait activity time, other requirements for high efficacy of this method should be considered including area-wide application due to high mobility of adult flies and accurate timing so that the fruit infestation is avoided (Haniotakis, 2005). High mobility of olive flies was reported in a few field studies (Tzanakakis, 2003) and in population genetic analysis of *B. oleae* using microsatellite markers (Ochando and Reyes, 2000; Knap and Bandelj, 2016). In Greece, bait sprays are applied by tractors to almost all olive orchards and these applications are funded by the Hellenic Ministry of Rural Development and Food (Varikou et al., 2013).

Recently, a bait station for attraction and control of oriental fruit fly (*Bactrocera dorsalis* (Hendel, 1912)) was recently implemented (Piñero et al., 2010). Such attract-and-kill bait stations have the advantage over foliar applications, because the insecticidal bait is protected from weather conditions. Bait stations were tested for effectiveness against the olive fly as well (Yokoyama, 2014a). The authors didn't confirm longer toxicity of insecticides due to protection of bait spray from weather (bait sprays and foliage applications were protected from rain in this study). The level of toxicity was lost after one week on bait stations and olive foliage. However, the major advantages of bait stations are that they are protected from rain and reduce the amount of bait spray used in olive orchard.

Traps are used for mass trapping or for olive fly monitoring with the aim to determine the appropriate time for treatments. Burrack et al. (2008) tested different traps (yellow sticky traps, ChamP traps and plastic McPhail traps) and different lures. Mcphail traps baited with torula yeast tablets were the most efficient. Results were confirmed by Varikou et al. (2013) when compared with yellow sticky panels and McPhail traps. Although the number of attracted flies in McPhails traps didn't provide a good estimation of the olive fly density, it was concluded that they can be accurate in determining the timing of spraying against *B. oleae.* McPhails traps are recommended in California. To monitor the olive fly population, Varikou et al. (2013, 2016) used McPhail glass traps with 2 % ammonium sulphate which was replaced by 2 % hydrolyzed protein (Entomela 75 %) at the end of August in the latest research. Traps based on proteins and other sources of ammonia primarily attract female flies because they require source of protein to ensure high fecundity (Hagen and Finney, 1950). Addition of pheromones didn't significantly increase olive fruit fly captures (Varikou et al., 2014). In Greek orchards ammonium salts are still used in McPhail traps during the whole period (summer and autumn), although it was proven that its attractiveness was significantly reduced compared to all tested protein hydrolysates (Haniotakis, 2005; Varikou et al., 2014). In Slovenia, the olive fly is monitored with yellow sticky traps with an added pheromone (Dacotrap®, Isagro S.p.a., Milan, Italy) (Knap and Bandelj, 2016). Rojnić et al. (2015) observed that McPhail traps with hydrolyzed protein were more attractive to olive flies than yellow sticky traps baited with a pheromone. However, the correlation coefficients that were calculated using the cumulative capture of olive flies were high, which proved the comparability of these two trap types. Disadvantages of McPhail traps are their lack of

specificity in that they also attract non-target insects and are not efficient during periods of high humidity. However, since the information about female fecundity can be obtained by dissecting females caught in these traps, a combination of both McPhail traps and sex-pheromone baited traps gives the best population monitoring information (Bueno and Jones, 2002). Gil-Ortiz (2015) exposed the problem with biodegradability in commercial pheromone dispensers made of plastic polymers in which the pheromone is encapsulated and suggested the use of mesoporous materials as an ecological alternative. Some other target devices like plywood rectangles or bags dipped in insecticide, together with attractant and sex pheromone, were tested as well (Bueno and Jones, 2002).

Attempts were made with the aim to find out if there is a relation between captures in traps and infestation of olive fly, but no relation was observed (Varikou et al., 2016).

2.1 Spinosad toxicity in comparison with other insecticides and its impact on beneficial organisms

Akmoutsou et al. (2011) evaluated toxicity of spinosad and deltamethrin on *B. oleae*. Data showed that at the lowest concentrations of 0.05 mg l^{-1} and 0.10 mg l^{-1}, deltamethrin caused significantly higher mortality than spinosad, while at concentrations of 0.50 mg l^{-1} to 4.00 mg l^{-1} the lethal effects were similar. However, high mortality was observed after 72 h of exposure, suggesting a delayed lethal effect and that long periods of application may be needed for high mortality events to occur. Gonçalves et al. (2012) compared efficacy of spinosad and dimethoate in bait sprays and impact on non-target arthropods. Results suggested that spinosad could have the same effectiveness as dimethoate. Recently implemented studies of spinosad effect on non-targeted organisms revealed that it could be related to the meteorological conditions (Gonçalves et al., 2012), however, GF-120 indicated compatibility with the most important groups of natural enemies present in olive groves with the exception of *Orius* spp. and Aphelinidae (Pascual et al., 2014).

2.2 Alternative methods for protection against olive fly

As an alternative to commonly used insecticides, protection against olive fly with kaolin was suggested (Saour and Makee, 2004). However, a few studies reported that kaolin has a negative effect on the arthropod communities at soil level (Pascual et al., 2010; Bengochea et al., 2014).

An environmentally safe alternative to insect pest control is the sterile insect technique (SIT) (Zygouridis et al., 2014). This is a species-specific method of insect suppression in which insects are mass-reared under factory conditions, sterilized by irradiation, and then released (Leftwich et al., 2016). However, due to their adaptation to factory, laboratory, and irradiation, flies have significantly reduced fitness (Leftwich et al., 2016). Zygouridis et al. (2014) observed a substantial loss of variability between F1 and F2-F5 generations in the laboratory, while in F11 a complete adaptation to the new laboratory environment occurs. It was suggested that loss of variability is responsible for the loss of wild characters like low competitiveness of the sterile mass-reared males compared with the wild ones. Loss of variability was shown with microsatellite markers. Authors suggested a solution to refresh a mass-reared colony with wild material at about every five to eight generations (Zygouridis et al., 2014). Efforts to develop a vigorous and efficient mass-reared laboratory olive fly strain is underway. Additionally, a transgenic RIDL (release of insects carrying a dominant lethal) system is trying to overcome the limitations of SIT technology and the first transgenic strains for the olive fly were already developed (Ant et al., 2012; Genç et al., 2016).

Biological protection is the most sustainable method for the environment. A review of biological control attempts was made by Daane and Johnson (2010), concluding that biological control programs previously used did not consistently provide adequate levels of control across the range of climates and olive cultivars commercially grown. Between 2006 and 2013 a trial with a field release of specialized parasitoids, *Psyttalia lounsburyi* (Silvestri, 1913) and *Psyttalia humilis* (Silvestri, 1913), was conducted in California (Daane et al., 2015). However, they

encountered inherent difficulties of establishing parasitoids in the field due to climatic extremes as well as because of periods with low host densities.

Effectiveness of soil applications beneath the tree canopy with entomopathogenic fungus *Metarhizium brunneum* Petch. EAMa 01/58-Su strain, perfectly adapted to Mediterranean soil conditions has been recently evaluated. Two applications were made per year from 2010 to 2015, once in autumn to target larvae that exit from the fruits to the ground to pupate beneath the tree and spend the winter in the pupal stage and once in spring to target the emerging adults. A high reduction (50 % to 70 %) in the *B. oleae* population emerging during the spring from the soil of treated plots was seen compared to controls plots. The authors marked it as efficient biological control method (Yousef et al., 2016).

With the aim to identify new natural enemies of the olive fly, a PCR-based diagnostic assay for detection of *B. oleae* in the gut of insects was developed (Rejili et al., 2016).

New control methods can be developed with new knowledge about microorganisms associated with the olive fly (Malacrinò et al., 2015). One example is the incompatible insect technique (IIT), which employs the cytoplasmic incompatibility (CI) induced by an insect symbiont such as *Wolbachia* (Apostolaki et al., 2011).

3 IDENTIFICATION OF RESISTANCE ALLELS

Since most control programmes against the olive fruit fly have been based on the use of insecticides like OPs, pyrethroids, and spinosad in the last few years, olive flies have developed resistance against them (Vontas et al., 2001; Haniotakis, 2005; Margaritopoulos et al., 2008; Kakani et al., 2010; Daane et al., 2015)). As observed by Kakani et al. (2010) in California where spinosad is the only registered phyto-pharmaceutical product, its exclusive use has led to greater levels of resistance. The intensity of the resistance was shown to be strongly correlated with local history of spinosad use. Five populations from California demonstrated a 9 to 13-fold increase.

3.1 Resistance to organophosphates

One of the earlier studies of resistance to organophosphorus insecticides of *B. oleae* suggested that the resistance is based on increased expression of AChE (acetylcholinesterase) or a gene duplication (Tsakas, 1977). Vontas et al. (2001) showed with biochemical assays that modification of AChE is the dominant factor in organophosphate resistance in *B. oleae* (other metabolic pathways were not found to have major roles in resistance to OPs). AChE terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. It is a key enzyme in the insect nervous system (Mutero et al., 1994). Comparison of the cDNA sequences of *B. oleae* which encode AChE in susceptible and organophosphate resistant *B. oleae* revealed two polymorphisms, resulting in amino acid substitution (I214V and G488S) in the insecticide resistant strains. A combination of I214V and S488G was found in all samples. A field population collected from Attiki, Greece, possessing both mutations, exhibit 16-fold AChE insensitivity compared to susceptible *B. oleae* (Vontas et al., 2002). There were some examples, when mutation 214V was found in the absence of 488S (Hawkes et al., 2005; Nardi et al., 2006). On the contrary, the mutation G488S is almost always accompanied by I214V (Hawkes et al., 2005). Pereira-Castro et al. (2015) observed all samples from Iberia to have both mutations in the same chromosome.

When *ace* (gene for AChE) locus was sequenced by Kakani et al. (2008), a new mutation was discovered, 9 bp deletion (termed Δ3Q) in exon X, which showed a strong correlation with OP-resistance levels. They analyzed olive flies which were previously used by Skouras et al. (2007) in an insecticide assay to study the resistance to dimethoate in *B. oleae* populations from Greece, its islands, and Cyprus. Populations from Crete showed the highest resistance ratio values from 30 to 64 (calculated as a ratio of median effective dose (ED_{50}) of each tested population and ED_{50} of the laboratory susceptible strain), while on Cyprus all populations showed low resistance levels (resistance ratio less than 10). The mutation

resulted in a deletion of three glutamine residues at positions 642-644. Since it was always found as a heterozygous, the authors suggested higher fitness costs of Δ3Q mutation. They suggested that I214V and G488S are the first ones to be selected under the minimum OP pressure, while Δ3Q appears to be associated with resistance at higher OP doses. Δ3Q is located outside the catalytic center of the enzyme and it is suggested that it affects the glycosylphosphatidylinisotol-anchoring efficiency or the stability of the protein. A more detailed account of the role of Δ3Q and two other mutations were discussed by Kakani and Mathiopoulos (2008) and Kakani et al. (2011).

Pereira-Castro et al. (2015) identified a new mutation that causes an alanine to valine substitution at residue 298 (A298V). However, a functional role has not yet been solved.

3.2 Resistance to other insecticides

Regarding the resistance to alpha-cypermethrin, it was indicated that cytochrome P450 monooxygenase could be involved in a resistance mechanism. While no correlation between enzyme activity and resistance was found with carboxylesterase (COE) and glutathione-S-transferase (GST), as well as genetic polymorphism of domain IIS4-IIS6 of the *B. oleae* para-type sodium channel could not be responsible for resistance (Margaritopoulos et al., 2008).

Resistance to insecticides has been studied with NGS technologies as well. A whole transcriptome analysis of spinosad susceptible and resistant flies indicated that several immune system loci as well as elevated energy requirements of the resistant flies might be necessary to lever the detoxification process (Efthimia et al., 2014).

Pavlidi et al. (2013) studied *B. oleae* mRNA and identified at least 132 putative major detoxification genes involved in the metabolism of xenobiotics, such as plant phytotoxins and insecticides.

4 MOLECULAR METHODS FOR MONITORING THE RESISTANCE ALLELES TO OPS

With the aim to easily detect the presence of I214V in exon III (assigned to exon IV by Kakani and Mathiopoulos (2008)) and G488S in exon VI (assigned to exon VII), a simple PCR-RFLP assay was developed (Hawkes et al., 2005). Detection of I214V is based on the fact that I214V mutation creates a site for the restriction enzyme *Acc*I, while detection of G488S is based on associated neutral polymorphism, which destroys a site for *Bss*HII (sequence conservation and the correlation between the two G to A transitions should be confirmed by sequencing of alleles in any studies). Primers Boace3F and Boace3R were designed for amplification of 232 bp fragment within exon III, while Boace6F and Boace6R amplify 106 bp product within exon VI. The presence of resistant alleles is identified with *Acc*I digested two fragments (168 bp and 64 bp) and the presence of full-length (106 bp) resistance-associated exon VI alleles. Susceptible alleles (G488) give bands of 50 and 56 bp.

Nardi et al. (2006) developed primers to amplify two regions that nearly completely include exons III and VI that carry the two mentioned mutations: BoAce_518F and BoAce_1040R; BoAce_1424F and BoAce_1519R, respectively. After primer removal the amplified fragments correspond to 521 bp of 543 bp in exon III and 94 bp of putative 150 bp in exon VI. After PCR reaction, both strands were sequenced and sequences showing double peaks were recorded as heterozygotes and subjected to cloning to resolve and differentiate the two alleles.

Margaritopoulos et al. (2008) developed a new PCR-RFLP diagnostic assay for G488S mutation. The method, previously developed by Hawkes et al. (2005) is based on G488S associated neutral polymorphism, but according to sequences of allele d and f, which were obtained by Nardi et al. (2006), this two mutations are not always present together. Newly developed primers (D6F, D6R) directly target the resistance mutation G448S. A reverse primer, D6R, has been designed to

introduce a base substitution into 100 bp PCR product, which in combination with the resistant allele generates a recognition site for *Mbi*I. Digested PCR product results in two fragment, 31 bp and 69 bp in length.

Kakani et al. (2008) aimed to analyze the nucleotide sequence of the *ace* locus in order to isolate additional mutations and therefore developed five primer pairs for amplification of exons II, III-IV (includes introns), VIII, IX, and X. Forward primer for exons III-IV is labeled as Boace3F, which is the same as the primer developed by Hawkes et al. (2005) but with different sequence, so a caution has to be taken when using this primer. Boace10F and Boace10R primers were used for detection of Δ3Q mutation. PCR of the wild type allele yields a 96 bp product whereas PCR of the mutant allele yields an 87 bp product.

Kakani et al. (2013) developed PCR-RFLP, allele-specific, and *Taq*-Man assay methods for the identification of Δ3Q mutation. In the first method, Boace10F and Boace10R primers were used for the amplification of exon X. PCR product of the wild allele, 96 bp in length, is digested with *Mwo*I enzyme, and results in two fragments, 59 bp and 37 bp. PCR product of the mutant allele, 87 bp in length, remains undigested because the Δ3Q mutation affects restriction site. Digestion enables detection of genotype on a gel with lower resolution compared to undigested fragments. An allele specific method is done with primers Ex10wt3'F-IMP and Boace10R (to test the presence of the wild type allele, 76 bp fragment) or with primers Ex10mut3'F-IMP and Boace10R (to test the presence of the mutant Δ3Q allele, 67 bp fragment). The two Ex10 primers are identical except for the last 3' base that provides the specificity for one or the other allele and introduces internal mismatch in order to increase the specificity. For the detection of I214V and G488S mutations a new duplex qPCR assay was developed.

Pereira-Castro et al. (2015) developed new primers for amplification of different segments of the *ace* gene to achieve a more complete analysis of haplotypes associated with OP-resistance and OP-sensitive *ace* alleles (primers were named Bo12 or Bo14 followed by a dash and a number).

5 FREQUENCY OF RESISTANT ALLELES TO OP ON THE FIELD

5.1 Frequency of I214V and G448S

Hawkes et al. (2005) analyzed samples from Greece, Albania, Italy, France, Spain, and South Africa for the presence of I214V and G488S. 488S was detected in all samples from Greece, almost all field samples from Albania, while high levels were observed in two Italian locations and at lower frequencies in France and Spain. The majority of these 488S individuals also carried 214V. Double mutation haplotype is lower in western Mediterranean regions, which was attributed to lower usage of OPs. Samples from South Africa were homozygous for wild-type for both alterations.

Results of Hawkes et al. (2005) were confirmed by Nardi et al. (2006) where olive flies from Pakistan, Africa, Mediterranean countries, the Middle East, and America were analyzed. No resistant alleles were identified in Pakistan and African samples, low to moderate (50 %) frequency was observed in the Middle East and America samples, while the highest proportion of resistance alleles was observed in the Mediterranean area, where frequencies approach 100 % in Greece and (central/southern) Italy. However, in France and Portugal the frequency of resistance alleles was below 30 % and 0 %, respectively. Nardi et al. (2006) identified 3.4 % samples of chromosomes carrying only I214V (previously identified by Hawkes et al. (2005) in only one French sample). Interestingly, two alleles carrying the mutation I214V were identified (named A and W) which differ by 6 synonymous substitutions. Allele W was found on the island of Sicily, whereas resistant allele A was present at high frequency throughout Greece and south/central Italy. It was suggested that two independent acquisitions of this mutation occurred. Assuming that the mutation most likely happened in an area where the precursor alleles are present, authors hypothesized that allele A arose in the Middle East (the same is suggested for the allele carrying mutation G488S). The high frequency and broad geographic distribution of

allele A compared to that of allele W, would suggest that allele A is older.

Pereira-Castro et al. (2015) analyzed olive flies from the Iberian Peninsula (Portugal and Spain) and both I214V and G488S mutations were found at medium to high frequencies in all locations, demonstrating they are now widespread even in Portugal, while in Andalusia their frequencies vary widely, from 20 % to 90 %. Since complete concordance between the zygosities of I214V and G488S was observed, authors suggested that the chromosome carrying both substitutions was introduced.

Doğaç et al. (2015) studied olive flies collected in 2010 from 12 provinces from Aegean and Mediterranean regions in Turkey and found that resistant forms of exon III and VI had a low to moderate frequency, while they reached the highest frequency, nearly 80 %, in the Aegean populations. This indicates that they were selected in the Aegean coast of Turkey and then spread westward towards Europe. Aegean populations showed a more limited variability of exon III and VI as well.

Hanife (2016) analyzed flies from Çanakkale province, Turkey, for the presence of G488S mutation. Olives sampled in 2006 showed 31.7 % resistant allele homozygosity, 54.14 % were homozygous in 2007, while in 2013, 81.77 % of homozygous flies were observed. Only 3.10 % and 1.10 % of susceptible flies were observed in 2006 and 2007, respectively, while no susceptible flies were identified in 2013. Resistance development is evident in the field as well, since many local farmers complained about inefficiency of their applications.

5.2 Frequency of Δ3Q mutation

The analysis of Δ3Q mutation distribution in the Mediterranean (Israel, France, Cyprus, Greece, Italy, Spain, Portugal and Marocco) revealed the highest frequencies, 12.5 % and 11.1 % found in Greece and Italy, respectively, whereas a gradual decrease of Δ3Q frequency towards the western Mediterranean was also noted. In Portugal no resistant allele was found (Kakani et al., 2013). Results are consistent with the distribution of the other two resistance associated point mutations (Hawkes et al., 2005; Nardi et al., 2006). The absence of Δ3Q in Portugal was confirmed recently by Pereira-Castro et al. (2015). However, they didn't observed Δ3Q mutation in Spain, but Kakani et al. (2013) detected one allele with Δ3Q mutation.

Doğaç et al. (2015) monitored the presence of Δ3Q mutation in the Mediterranean and Aegean regions of Turkey. They observed Δ3Q mutation to be more widespread in the Mediterranean region with frequencies from 6 % to 20 %. In the Aegean region with greater insecticide pressure, lower frequencies of Δ3Q were observed (from 2 % to 8 %, while in some locations it was not even detected). All previous observations of several authors identified Δ3Q to be always in a heterozygous state. However, Doğaç et al. (2015) identified one homozygous sample in the Hatay and one in the Aydin populations.

6 CONCLUSIONS

Genetic studies revealed that OPs resistance loci (I214V, G488S) are now present in all European Mediterranean countries. The first studies suggested (Hawkes et al., 2005; Nardi et al., 2006) that selection caused by insecticide had the greatest impact on the resistance loci expansion, while some other evolutionary forces were suggested as well. However, since both mutations are now widespread in Portugal and Spain with frequencies above 80 %, it could be suggested that new alleles were in the process of introduction in the last few decades. This hypothesis is supported by Pereira-Castro et al. (2015) who found a great difference in resistant associated allele frequencies between locations, only few kilometers apart. The differences were attributed to recent introduction of resistant alleles. However, differences in frequencies of resistant associated alleles were found in Turkey as well (Doğaç et al., 2015), possibly due to local specificity of insecticides use. Another interesting finding was observed by the same authors who identified higher frequency of Δ3Q mutation in a region with lower frequencies of the other two mutations. They explained this

phenomenon with greater fitness disadvantage compared with that of the other two point mutations (Δ3Q offers resistance to only some insecticides pressure). Genetic and biochemical studies showed that high levels of resistance to insecticides from different classes have been developed. In order to reduce the amount of insecticide used in the future and to prevent the development of resistance, further research work on efficient plant protection methods should be continued.

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Učinkovit način dodajanja selena v vsakdanjo prehrano s poudarkom na rastlinskih virih

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IZVLEČEK

Selen je eden izmed esencialnih elementov, potrebnih za človekovo zdravje. Razmere v okolju in kmetijska praksa imajo velik vpliv na vsebnost selena v rastlinah. Gojenje rastlin, obogatenih s selenom, je učinkovit način dodajanja selena v vsakdanjo prehrano tistih, ki tega esencialnega elementa ne prejmejo v zadostnih količinah. Biorazpoložljivost selena je v korelaciji z izvorom in s kemijsko obliko, v kateri je ta prisoten v živilu. S prehranskega vidika so s selenom obogatena živila potencialni dodatni vir selena, ki se vnaša v organizem preko prehranske verige in se s kompleksnimi pretvorbami na molekulski ravni pretvarja iz anorganskih v bolj razpoložljive organske oblike. V članku so zbrani rezultati sistematičnega raziskovanja vsebnosti selena v posameznih rastlinskih vrstah in različnih načinov dodajanja selena za dosego večje koncentracije letega v pridelku. V Sloveniji so tla s selenom revna in je možnost pomanjkanja selena v prehrani nekaterih skupin prebivalstva velika. Zato je v članku večji poudarek namenjen pregledu objavljenih del domačih strokovnjakov, ki so vsebnost selena spremljali ob simulaciji sprememb okoljskih dejavnikov, kar je dodatno vplivalo na fiziološke lastnosti in pridelek rastlin. Upoštevana je pozitivna vloga selena v presnovi človeka ter njegov vpliv na zdravje. Omenjeni so tudi negativni vplivi zaradi pomanjkanja ali presežka selena v hrani, ki opozarjajo na to, da je treba priporočila v zvezi z vnosom selena v naš organizem temeljito spremljati in premišljeno prilagajati referenčne vrednosti.

Ključne besede: selen; rastlinski viri; prehranski vnos; dodatek selena

ABSTRACT

PLANT RESOURCES BASED SELENIUM SUPPLEMENTATION IN DAILY NUTRITION

Selenium is one of the essential elements that has a direct effect on human health and disease. Environmental conditions and agricultural practice have a profound influence on the selenium content in plants. Cultivation of plants enriched with the selenium has an effective potential for selenium supplementation in diets for population which is exposed to selenium deficiency. Bioavailability of selenium compounds from food is in strong correlation with the source and its chemical form. The selenium of different sources and forms can become a part of human consumption when entering the food chain, wherein the inorganic forms of selenium are metabolized and converted to more available organic forms. Numerous results of systematic research of the selenium content in individual plant species as well as various techniques for producing selenium enriched foodstuffs is reviewed. The soil in Slovenia is selenium-poor and may concern a part of population which is potentially sensitive to selenium status. The merits of selenium effect, either alone or in combination with different environmental changes on plant production published by Slovenian authors are thus closely considered. Controversies continue to prevail regarding adequate amounts for selenium for health and disease prevention. Thus, general and individualized recommendations for selenium intake and supplementation in the future need to be cautiously followed and the reference values continually revised.

Key words: selenium; plant resources; dietary intake; selenium supplementation

1 UVOD

Za prvega, ki je l. 1817 izoliral in kemijsko opisal selen, znanost priznava švedskega kemika Jönsa Jakoba Berzeliusa, čeprav obstajajo viri, da je ta kemijski element kot rdeče žveplo, ostanek v peči po uparitvi žvepla, opisal že Arnold de Villanova v 13. stol. (Reilly, 2006).

Selen je eden izmed približno 60 esencialnih hranil, potrebnih za človekovo zdravje (Hatfield s

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sod., 2012). V 200 letih od njegova odkritja so strokovnjaki temeljito proučevali njegovo vlogo z biokemijskega, molekulskega in genetskega zornega kota. Številne študije obravnavajo vpliv pomanjkanja ali presežka selena v prehrani na živalskih modelih ali s kliničnimi študijami pri ljudeh. Primerna preskrbljenost s selenom je nujno potrebna za človekovo zdravje, predvsem zaradi njegovih antioksidativnih lastnosti in prisotnosti v selen vsebujočih beljakovinah (selenobeljakovinah). S stališča presnove selena in vpliva selena na zdravje v zadnjem času raziskovalci opozarjajo tudi na razlike med moškim in ženskim spolom.

Identifikacija in koncentracija določene kemijske oblike, v kateri je selen prisoten, sta ključni za opis molekulskih mehanizmov biološke aktivnosti tega elementa in opis specifičnih presnovnih procesov v celicah in tkivih. Omenjeni procesi so predmet medicinskih, fizioloških in prehranskih raziskav predvsem z vidika:
- vpliva selena na zdravje in razvoj bolezni,
- presnove in aktivnosti terapevtskih molekul in nanodelcev, ki vsebujejo selen,
- živil in krmil, obogatenih s selenom in/ali pridobljenih z biotehnološkimi postopki,
- biološke uporabnosti zaužitega selena.

2 SELEN V OKOLJU

V kemijskih lastnostih je selen podoben žveplu (Reilly, 2006). V okolju se nahaja v anorganski obliki (elementarni selen: Se, selenid: Se^{-}, selenit(IV): SeO_3^{2-}, selenat(VI): SeO_4^{2-}) in v organski obliki: predvsem kot metilirane selenove spojine, seleno-aminokisline in selenobeljakovine v bioloških sistemih (Pyrzyńska, 2002; Uden s sod., 2004). Frost (1972) opisuje dinamično ravnotežje in pretvarjanje med anorganskimi in organskimi oblikami selena.

Anorganski selen je v zemeljski skorji geografsko zelo neenakomerno razporejen in je v koncentracijah od skoraj nič do 1250 mg/kg (Hatfield s sod., 2016). Z izjemo območij, kjer sežigajo fosilna goriva, kjer je postavljena steklarska industrija ali obsežna proizvodnja kemikalij in elektronike, je vnos selena in s tem vpliv na človekovo zdravje preko inhalacijskih poti neznaten (Wang in Gao, 2001).

Povprečna koncentracija selena v zemlji je mnogo večja na območjih s sedimentnimi kamninami v primerjavi s tistimi z vulkansko podlago. Področja, kjer je koncentracija selena v tleh zelo velika, so v delih Wyominga, Severne in Južne Dakote v Ameriki, v nekaterih predelih Kitajske, Rusije, Irske, Kolumbije in Venezuele. Johnson s sod. (2010) poroča o koncentraciji preko 600 mg v kilogramu črnega skrilavca. Določene rastline lahko iz s selenom zelo bogate podlage akumulirajo tudi do 3 mg tega elementa na gram rastline. Take rastline so potencialno toksične za pašno živino (Combs, 2001).

Dodajanje natrijevega selenita umetnim gnojilom ali živalski krmi je običajna praksa v državah, kjer so tla revna s selenom (Wang s sod., 1998; Watkinson, 1983). To velja predvsem za severozahodni Oregon, Finsko, Novo Zelandijo, centralno Srbijo in velik del afriške celine (Ngo s sod., 1997). Pirc in Šajn (1997) navajata vsebnosti selena v tleh v Sloveniji. Po njunih podatkih le-ta znaša od manj kot 0,1 do 0,7 mg/kg, kar pomeni, da so tudi v Sloveniji tla revna s selenom.

V isti državi, tak primer je npr. Kitajska ali Avstralija, pa se lahko koncentracije selena v tleh tudi regionalno zelo razlikujejo.

Pitna voda z običajnimi količinami selena le malo doprinese k dnevnemu vnosu (Deveau, 2010). Največjo biorazpoložljivost pripisujejo predvsem vodotopnemu anorganskemu selenatu in selenitnim ionom. Vrednosti selena v pitni vodi variirajo od 0,12 do 0,44 µg/l (Cutter, 1989). Izjeme so zabeležili v ruralnem okolju jugovzhodnega Kolorada zaradi izrazite suše leta 1975 kot tudi v vodnih izvirih osrednje-zahodnega dela Združenih držav Amerike, pri čemer so bile vrednosti med 50 in 300 µg/l (Hatfield s sod., 2012). Tudi Vincenti s sod. (2010) navaja, da so v severni Italiji leta 1990 določili neobičajno velike količine selena v vodovodni vodi. V podtalnici ali površinski vodi so količine selena lahko zelo variabilne – geografsko pogojene, in sicer od 0,06 do 400 µg/l, v nekaterih primerih celo do 6000 µg/l (Hatfield s sod., 2016). Ameriška regulativa oz. zvezni standardi v pitni

vodi dopuščajo koncentracijo do 50 µg/l (Hatfield s sod., 2012; Hatfield s sod., 2016), ki je v primerjavi z Nemčijo (in drugimi evropskimi državami), kjer je zgornja meja v ustekleničeni vodi ali vodovodni vodi le do 10 µg/l, bistveno večja (Hatfield s sod., 2016). Svetovna zdravstvena organizacija (WHO) je za selen v pitni vodi določila priporočeno vrednost 10 µg/l, ki je izračunana na 10 % delež vnosa z vodo (NIJZ, 2014). V slovenskem Pravilniku o pitni vodi je selen uvrščen v Prilogo I, del B, kjer je določena mejna vrednost v pitni vodi 10 µg/l (UL RS, 2004). Koncentracije v pitni vodi so različne in so geografsko pogojene; koncentracije so večje pri nizkem ali visokem pH zaradi večje topnosti v takem okolju (NIJZ, 2014).

V morski vodi zasledimo selen le v manjših količinah (od 0,09 do 0,11 µg/l). Živa bitja, vključno s prokariontskimi celicami, z algami, morsko travo, nevretenčarji in vretenčarji, so sposobna ta mineral akumulirati. Tako so ti organizmi vir selena za človeka, ki ga človek vnese v telo preko prehranske verige (Hatfield s sod., 2012).

3 POTREBE PO SELENU IN POSLEDICE ODSTOPANJ OD PRIPOROČENIH VREDNOSTI DNEVNEGA VNOSA

Selen je esencialni element za mnogo živalskih vrst, tudi za človeka. Aktivnost je odvisna od kemijske oblike. Z vgradnjo v različne beljakovine vpliva na rast in razvoj organizma ter je vključen v zaščito tkiv pred oksidativnimi procesi in zaščito pred okužbami. Premalo zaužitega selena (manj kot 13 do 19 µg/dan) pri človeku sproži resne posledice in vodi v motnje in bolezni, kot so vrtoglavica, slabost, izguba apetita, srčno popuščanje, srčne aritmije in povečanje srca. Bolezen, ki prizadene predvsem otroke in nosečnice, so poimenovali Keshanova bolezen. Druga poznana bolezen, ki je povezana s premajhnim dnevnim vnosom selena, je Kashin Beckova bolezen. To je bolezen povečanih sklepov oziroma vrsta revmatoidnega stanja-osteoartritisa. Posledice bolezni so šibkost udov, okornost, otekanje in bolečine v prstnih členkih, povečanje sklepov in atrofija nekaterih progastih mišic. Drugi znaki pomanjkanja selena pri ljudeh so poškodbe srčne mišice, trebušne slinavke, mišična distrofija, izguba lasnega in kožnega pigmenta (Reilly, 2006; Hatfield s sod., 2012; Hatfield s sod., 2016).

Čeprav je v mikro količinah nujno potreben, lahko v velikih koncentracijah škoduje zdravju ljudi. Dolgotrajna izpostavljenost velikim koncentracijam pri ljudeh (nad 1000 µg/dan) ima genotoksične in kancerogene učinke, pri več kot 3200 µg/dan pa povzroča selenozo (Reilly, 2006). Toksičnost se v hujših oblikah odraža v nenormalnem delovanju živčnega sistema in žlez z notranjim izločanjem (predvsem jetra), v moteni sintezi ščitničnih in rastnih hormonov kot tudi v porušeni presnovi inzulinu podobnega rastnega faktorja (Reilly, 2006).

Potrebe po selenu so različno določene, vendar precej podobne, v Nemčiji je npr. ocenjen ustrezni vnos med 30-70 µg/dan. V Evropi je ocenjen dejanski vnos med 30 – 90 µg/dan. Po Evropski agenciji za varnost hrane (EFSA; European Food Safety Authority) je dopusten dnevni vnos preko živil za selen za odrasle 300 µg, za mlajše sorazmerno manj, odvisno od telesne teže (NIJZ, 2014).

V Sloveniji je podatke o dnevnem vnosu selena v domovih starejših občanov zbiral Pokorn in sod. (1991). Ob energijski vrednosti celodnevnih obrokov 8,12 MJ so preiskovanci zaužili 40 µg selena oz. 30 µg na dan, če je bila energijska vrednost obrokov manjša (7 MJ).

Smrkolj in sod. (2005a) so analizirale 20 dnevnih vojaških obrokov in ugotovile, da je povprečni vnos selena 87 µg (od 34 do 163 µg/dan) ob povprečni energijski vrednosti obroka 15,8 MJ.

Ameriški Zvezni urad za hrano in zdravila (FDA) je v okviru projekta Total Diet Study v letih od 1973 do 2010 redno spremljal podatke o koncentraciji selena glede na potrošniško košarico. Merjenje vsebnosti različnih analitov v istem živilu je na ta način omogočalo vpogled v morebitne interakcije selena z ostalimi hranili ali s toksičnimi snovmi (Hoffman-Pennesi s sod., 2015). Ugotovitve kažejo na smiselnost nadaljevanja

meritev koncentracij selena in ponoven pregled referenčnih oz. priporočenih vrednosti njegovega dnevnega vnosa. Kriterij za določitev optimalne preskrbljenosti s selenom je zasičenost oz. koncentracija selenoproteina P v plazmi. Na osnovi več študij so privzete vrednosti po različnih regijah glede na starost in spol zbrane v preglednici 1.

Preglednica 1: Priporočen dnevni vnos (PDV), največji dopusten vnos (NDV) in primeren vnos (PV) selena v µg/dan (Kipp s sod., 2015; Hatfield s sod., 2016).

Table 1: Recommended dietary allowance (PDV), tolerable upper intake (NDV) and adequate intake (PV) levels of selenium in µg/day (Kipp et al., 2015; Hatfield s sod., 2016).

Starost		Nemčija, Avstrija, Švica		Severna Evropa		Nova Zelandija, Avstralija		ZDA		Združeno kraljestvo	
		PDV	NDV	PDV	NDV	PDV	NDV	PDV	NDV	PDV	NDV
Dojenčki (starost v mesecih)	0-3	10		-			45	15	45	10	
	4-6	15						15		13	
	~7-12	15		15				20	60	10	
Otroci (starost v letih)	1-2	15		20		25	90	20	90	15	
	2-4	15		25		25	90	20	90	15	
	~4-8	20		30		30	150	30	150	20	
	~8-10	30		30		50	280	40	280	30	
	~10-13	43		40		50	280	40	280	45	
	~13-15	60		60		70	400	55	400	45	
Moški	15-18	70				70	400	55	400	70	
	18-70	70	400	60	300	70	400	55	400	75	450
	70+	70	400	60	300	70	400	55	400	75	450
Ženske	15-18	60		50		60		55		60	
	19-70	60	400	50	300	60	400	55	400	60	450
	70+	60	400	50	300	60	400	55	400	60	450
Nosečnost		60		60		65	400	60	400		
Doječe matere		75		60		75	400	70	400	75	

4 PREHRANSKI VIRI SELENA

4.1 Hrana rastlinskega izvora

Hrana je primarni vir, s katerim človek v svoje telo vnaša selen. Največ selena zaužijemo z žiti, mesom in ribami (Combs, 2001). Žita in žitni izdelki na Kitajskem, kjer so tla revna s selenom, prispevajo kar 70 % vsega selena v prehrani človeka. V Indiji je ta delež 40-50 %, v Združenem kraljestvu pa 18-24 % (Tamás s sod., 2010). Golubkina in Alfthan (1999) sta z raziskavo v 27 pokrajinah Rusije ugotovila visoko statistično značilno korelacijo med selenom v serumu preiskovancev in vsebnostjo selena v pšenični moki, iz česar sklepata, da je pšenica pomemben vir selena med rusko populacijo.

Absolutne vrednosti koncentracij selena v pšenici so zelo variabilne. Wolf in Goldschmidt (2007) sta raziskovala vsebnost selena v vzorcih pšenic. Ugotovila sta, da je prevladujoča oblika selena v pšenici selenometionin (okrog 55 %), v znatnih količinah (do 20 %) pa je tudi selenocisteina in selenita/selenata. Pomemben zaključek iz analiz je, da je skupna vrednost selena zaradi geografskih razlik ali načina gnojenja variirala kar za faktor 500, delež selenometionina pa je v vseh primerih ostajal konstanten, t.j. okrog 55 % glede na skupno vrednost selena. Avtorja nakazujeta, da so v rastlinah mehanizmi, ki regulirajo tip in količino določene kemijske oblike selena.

Biološka uporabnost ali biorazpoložljivost pove, kolikšen delež hranila pride v krvni obtok. Biorazpoložljivost selena je v korelaciji z izvorom in s kemijsko obliko, v kateri je ta prisoten v živilu (Finley, 2006; Reeves s sod., 2005). Razmere v okolju in kmetijska praksa imajo velik vpliv na vsebnost selena v mnogih rastlinah. Gojenje rastlin, obogatenih s selenom, je učinkovit način dodajanja selena v vsakdanjo prehrano tistih, ki tega esencialnega elementa ne prejmejo v zadostnih količinah.

Botanično ajda ni žito, vendar je po nekaterih lastnostih le-temu podobna. Notranjost trirobega ploda je podobna žitnemu meljaku. Iz nje pridelujemo kašo, zdrob, moko in kosmiče (Kreft, 1995). Pregled elementov v sledovih (Se, Zn, Fe, Co, Ni, Rb, Sb, Ag, Hg, Cr, Sn) in njihovo razporeditev v rastlinah in mlevskih frakcijah navadne (*Fagopyrum esculentum* Moench) in tatarske ajde (*Fagopyrum tataricum* Gaertn.) so objavili Bonafaccia in sod. (2003). Številne raziskave o količini selena pri navadni in tatarski ajdi kažejo na sposobnost teh rastlin za nalaganje selena kot tudi njegov vpliv v kombinaciji z nekaterimi drugimi okoljskimi dejavniki na biokemijske procese v rastlini. O povečani vsebnosti selena v rastlinah ajde, zraslih na tleh, ki so bila pognojena z različnimi koncentracijami vodnih raztopin natrijevega selenata, poročajo Golob in sod. (2015). Večji vpliv dodatka selena in možnost vzgoje ajde v smislu varnega funkcionalnega živila se je izkazal pri tatarski ajdi.

Tudi z listnim škropljenjem oz. dodajanjem selena v obliki natrijevega selenata različnih koncentracij v času cvetenja se je koncentracija selena v različnih delih ajde povečala, v največji meri v zrnju (Vogrinčič s sod., 2009; Stibilj s sod., 2004; Golob s sod., 2016c), glede na vrsto pa v tatarski ajdi (Golob s sod., 2015). O večji koncentraciji selena v potomkah rastlin tatarske ajde, ki je bila v prvi generaciji škropljena z natrijevim selenatom, poročajo Golob in sod. (2016a).

Listno škropljenje v času cvetenja ajde v kombinaciji s pomanjkanjem vode, z obsevanjem rastlin z UV-B žarki ali s sočasnim listnim škropljenjem z natrijevim sulfatom, je vplivalo tako na vsebnost selena v rastlinah kot tudi na presnovne procese v rastlinah (Smrkolj s sod., 2006b; Kreft s sod., 2013) in posledično na biomaso (Breznik s sod., 2005; Tadina s sod., 2007; Golob s sod., 2016b).

Tretji način za obogatitev rastlin ajde s selenom, opisan v literaturi, je z namakanjem semen pred setvijo v različnih koncentracijah natrijevega selenata-Se(VI), natrijevega selenita-Se(IV) in selenometionina-SeMet. Privzem selena je odvisen od kemijske oblike selena in koncentracije v raztopini. Glede na obliko selena si rezultati za privzem selena sledijo v vrstnem redu: Se(VI) > SeMet > Se(IV) (Ožbolt s sod., 2008; Cuderman s sod., 2010).

Vpliv dodanega selena na fiziološke lastnosti, kot so fotokemična učinkovitost fotosistema II in respiratorni potencial, ter pridelek, so Germ in sod. (2014) raziskovali v fižolu, Smrkolj s sod. (2006a) pri grahu, Germ s sod. (2007b) pri radiču, Germ in Osvald (2007) v rukoli, Germ s sod. (2007a) pri krompirju, Stibilj s sod. (2004), Smrkolj s sod. (2005b) ter Germ in sod. (2005) pri bučah. S prehranskega vidika so vsa ta živila potencialni vir selena, ki se vnaša v organizem preko prehranske verige in se s kompleksnimi pretvorbami na molekulskem nivoju pretvarja iz anorganskih v organske oblike.

Enako, kot v že omenjeni študiji Wolfa in Goldschmidta (2007), ki sta pri pšenici ugotovila, da je prevladujoča oblika selena selenometionin, potrjujejo tudi raziskave v različno tretiranih vzorcih ajde (93 % - Smrkolj s sod., 2006b), buč (81 % - Smrkolj s sod., 2005b), ječmena in rži (70-83 % - Stadlober s sod., 2001).

Sadje in zelenjava, pridelana na podlagi z majhno vsebnostjo selena, vsebujeta le neznatne količine selena, npr. paradižnik vsebuje manj kot 0,1 µg/100 g tega elementa, šparglji 2,3 µg/100 g in limski fižol 7,2 µg/100 g. Nekatere rastline so sposobne nalagati selen. Edmonds in Morita (2000) sta pri čebuli, divjem poru, česnu in brokoliju določila kar 50-kratno večjo vsebnost selena v rastlinah, gojenih na podlagi, obogateni s selenom. Fox in sod. (2005) poročajo o velikih koncentracijah selena v česnu, ki je po njihovih analizah znašala kar 1355 µg/100 g.

Različni viri (Hatfield s sod., 2012) navajajo, da lahko prevladujočo obliko selena, ki je selenometionin, zamenja večja koncentracija selen-

metilselenocisteina in γ-glutamil-selen-metilselenocisteina. Več kot 40 % selen-metilselenocisteina zasledimo v brokoliju. Med rastlinskimi viri, ki lahko akumulirajo večje količine selena, so še različne vrste alg, križnice (družina Brassicaceae) in brazilski oreščki. Ti vsebujejo kar 1470-1917 µg/100 g, pri čemer je večina v obliki selenometionina. Glive, kot so gobe in kvasovke, lahko selen nalagajo v večjih količinah in v več kot 20 različnih seleno-spojinah, anorganskih ali organskih, kot so npr. selenometionin, selen-metilselenocistein, selenocistein in selen-adenozilselenohomocistein (Lobinski s sod., 2000).

4.2 Hrana živalskega izvora in prehranski dodatki

Relativno velik delež selena človek v telo vnese tudi z mesom, mesnimi izdelki in ribami, mlečnimi izdelki ter jajci. Nekaj podatkov o vsebnosti selena v hrani živalskega izvora je v članku omenjenih zgolj zaradi primerjave z njegovo vsebnostjo v rastlinskih virih. Govedina vsebuje približno 20-35 µg/100 g, piščančje meso 10-24 µg/100 g, jagnjetina 20-30 µg/100 g in svinjina 20-40 µg/100 g tkiva. Tkivo drobovine vsebuje bistveno več selena kot tkivo mišic. 100 g ledvic goveda ali prašiča vsebuje kar 100-311 µg selena v 100 g tkiva. Selenometionin je oblika selena, ki v živalskih virih prevladuje, čeprav viri (Hatfield s sod., 2012) poročajo, da se lahko ta prednost zmanjša v korist selenocisteina, kadar so kot dodatek krmi živalim dodajali anorganski obliki selena - selenit in selenat.

Vpliv dodanega selena v krmo na vsebnost selena v mesu so proučevali Smrkolj in sod. (2003). Krmni obrok je vseboval različne količine selena (0,4 mg Se/dan oz. 4,4 mg Se/dan). Ob manjšem dodatku selena h krmi so določili od 3,3 do 3,9 µg selena in od 13 do 15 µg selena (v obeh primerih merjeno na 100 g svežega vzorca), ko je bila krma bogatejša s selenom.

Smrkoljeva in sod. (2005a) so ugotavljali tudi vsebnost selena v ribah, kjer ga je 15,3-68,6 µg/100 g, v piščančjem mesu 9,7-15,4 µg/100 g) in v puranjem mesu 9,9-11,6 µg/100 g.

Jajca v povprečju vsebujejo 26 µg selena v 100 g vzorca. Z dodatkom selena h krmni mešanici za kokoši je možno vsebnost selenometionina in selenocisteina v jajcih močno povečati (Sun in Feng, 2011).

Selen je v različnih oblikah prisoten tudi v izdelkih, ki so označena kot prehranska dopolnila. Eno izmed njih je pripravljeno z rastjo pivskega ali pekovskega kvasa (*Saccharomyces cerevisiae* Meyen ex E.C. Hansen) v gojišču z dodanim selenom in v razmerah, ki omejujejo privzem žvepla. Izolaciji sledi liofilizacija in stiskanje kvasa v tablete (Power, 1995). Rayman (2004) poroča, da v različnih izdelkih iz kvasa, obogatenega s selenom, prevladuje selenometionin (60-84 %). Poleg dopolnil s samim selenom je le-ta pogosto vključen tudi v multivitaminske in multimineralne izdelke. Po oceni dostopnih baz o prehranskih dopolnilih (Zhao in sod., 2009) lahko z dnevnim odmerkom zaužijemo med 10 in 200 µg selena, največkrat v obliki selenometionina. Zgornja vrednost je tako skoraj štirikrat večja od priporočenega dnevnega vnosa za odraslega človeka in obenem predstavlja polovico največjega dopustnega vnosa glede na Preglednico 1.

5 ZAKLJUČEK

Za veliko snovi velja, da so v prehrani v manjših količinah nujne, v večjih pa škodljive. Za element selen je ta lastnost še posebej značilna. Ime, ki ga je element dobil po grški boginji meseca Seleni, je zanj v tem smislu zelo prikladno, saj tako kot luna kaže svojo svetlo in temno stran. Rezultati številnih raziskav potrjujejo, da je selen esencialni element v sledovih, ki je navzoč praktično povsod v okolju, vendar v različnih količinah glede na geografsko območje. Znanstvene objave, vključene tudi v tem pregledu, kažejo na velik napredek v znanju in razumevanju biološke vloge selena in njegove pomembnosti v prehrani človeka. Selen vstopa v prehransko verigo iz zemlje, posledično vpliva na rast rastlin ter s tem tudi na kakovost rastlinskih in živalskih izdelkov. Obstaja vrsta načinov, kako na učinkovit način obogatiti prehranske vire s selenom, pri čemer je zaradi ozke

meje med premajhnim in prevelikim vnosom selena potrebna skrajna previdnost.

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