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Physiological and agronomic responses of maize (*Zea mays* L.) cultivars to plant population and defoliation at post-anthesis in the humid rainforest

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ABSTRACT

Variations in response pattern of maize (*Zea mays*) grown at plant populations, defoliated at post-anthesis in the rainforest were tested. Two field trials were conducted at Abeokuta, (Longitude 3^{0}25'E, Latitude 7^{0}15'N; 144 m a.s.l) and Ibadan (3^{0}56'E, 7^{0}33'N: 168 m a.s.l), Nigeria in 2015. The trials consisted of maize variety {2009 TZE-W DT STR [open pollinated variety (OPV)] and TZEI 124 × TZEI 25 (hybrid)]} in the main plot, plant population (71111, 80000 and 106666 plant ha^{-1}) in sub plot and defoliation (+ defoliation and – defoliation) as sub-sub plot. It was laid out in a split-split plot arrangement fitted into randomised complete block design with three replicates. OPV had significantly higher assimilatory surface, rate of current photosynthesis, reduced dry matter translocation efficiency, reduced days to 50 % anthesis and more 1000 grain massthan the hybrid maize, with similar grain yields. Both locations experienced increased leaf area index with increased plant population. Reduced 1000 grain massat both locations when maize was defoliated suggested a disruption in source:sink balance.

Key words: defoliation; open pollinated maize; plant population; current rate of photosynthesis; efficiency of photosynthesis

IZVLEČEK

FIZIOLOŠKI IN AGRONOMSKI ODZIV SORT KORUZE (*Zea mays* L.) NA GOSTOTO POSEVKA IN DEFOLIACIJO PO ANTEZI V RAZMERAH VLAŽNEGA DEŽEVNEGA GOZDA

V raziskavi so bile preiskuševane spremembe v odzivih koruze (*Zea mays* L.) na gostoto posevka in defoliacijo po antezi v razmerah vlažnega deževnega gozda. Izvedena sta bila dva poljska poskusa v Abeokuti (ZD 3^{0}25'E, ZŠ 7^{0}15'N; 144 m a.s.l) in Ibadanu (3^{0}56'E, 7^{0}33'N: 168 m a.s.l) v Nigeriji, leta 2015. Poskusi so obsegali sorto koruze {2009 TZE-W DT STR [tujeprašno sorto (OPV)] in hibrid TZEI 124 × TZEI 25} na glavni ploskvi, gostoto posevka (71111, 80000 in 106666 rastlin ha^{-1}) na podploskvah in defoliacijo (+ defoliacijain – defoliacija) na nadaljnih podploskvah. Poskus je bil zasnovan kot popolni naključni bločni poskus z deljenkami s tremi ponovitvami.Tujeprašna sorta (OPV) je imela značilno večjo asimilacijsko površino, večjo fotosintezo, zmanjšano sposobnost translokacije suhe snovi, zmanjšano število dni do 50 % anteze in večjo maso 1000 zrn kot hibridna sorta koruze, a podoben pridelek zrnja. Na obeh lokacijah se je indeks listne površine povečal z gostoto posevka. Zmanjšana masa 1000 zrn na obeh lokacijah v primeru defoliacije nakazuje motnje v ravnovesju med virom in ponorom asimilatov.

Ključne besede: defoliacija; tujeprašna koruza; gostota posevka; velikost fotosinteze; učinkovitost fotosinteze

1 INTRODUCTION

Maize (*Zea mays* L.) is the most widely cultivated crop and the most important staple food in sub-Saharan Africa, accounting for up to 70 % of the daily human calorie intake (Martin et al., 2000). One of the management factors that have contributed to the increased performance of maize in recent past was increased plant population. An appropriate plant stand may help in harnessing all the renewable and non-renewable resources in a more efficient manner towards higher crop yields (Sarlangue et al., 2007). Unfortunately, there is no single recommendation for all conditions, because the optimum plant population varies depending on environmental factors such as soil fertility, moisture supply and genotype (Gonzalo et al.,

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2006). This management factor was able to increase productivity per unit area, albeit with reduced yield per plant. This observation could be attributed to the fact that increased plant population per unit area could predispose maize crop to increased competition for available growth resources, especially light and water (Lemcoff and Loomis, 1994).

It was also observed that with high plant population there could be a change in canopy architecture, attenuation of radiant energy incident on leavesand alternation in light spectrum(Maddonni et al., 2001). This could be accompanied by a reduction in red/far red ratio, increased leaf senescence and a reduction in canopy apparent photosynthesis (Sangoi, 2001). Suggestions had been made that defoliation of maize plant could reduce competition for light under increased plant density per unit area (Liu et al., 2015). This suggested technique would increase interception of light into the canopy thus increase yield per plant under high density (Liu et al., 2015). Defoliation of the two uppermost leaves in maize plant three days after silking had been reported in China to have resulted in significant improved performance of maize at high plant population (Liu et al., 2015). The efficacy of this technique is dependent on its timing, intensity, genotype used and environment (Ahmadi and Joudi, 2007; Yin et al., 1998; Zhenlin et al., 1998) and the intensity of defoliation. Defoliation could alter source-sink balance as manifested in a reduction in carbon assimilation process and subsequently reduced assimilate availability (Rajcan and Tollenaar, 1999). Assimilate availability could be mediated through remobilization of earlier formed assimilate to the sink especially during the grain filling period. This could result in increased kernel mass and increased maize productivity at high plant population. However, the possibility of increasing leaf senescence is high especially at the grain filling stage if maize crop experiences nitrogen deficiency (Borrás et al., 2003). Furthermore rate, efficiency and contribution of remobilization to assimilate availability under a compromised source-sink relationship could be further complicated by the type of maize involved. Generally it had been indicated that maize has low remobilization efficiency compared to soybean and wheat (Kiniry et al., 1992). There is paucity of information in the literature on the response of hybrid and open pollinated (OPV) maize to defoliation at grain filling stage and its implication on assimilate availability. Apart from the effect of defoliation on assimilate availability at the grain filling stage, it was reported that this could be further confounded by the amount of radiant energy available at this growth stage (Borrás et al., 2004). This is more germane in the transitory rainforest, where there had been a remarkable variation in weather pattern compared to what was obtainable in recent past. The implication of this environmental factor on maize (hybrid and OPV) types is still unknown. Maize hybrids are known to require high inputs to attain their yield potential. How this requirement would act in combination with the contribution of remobilization on the grain yield of hybrid maize compared with OPV on senescence at high plant density still needs further investigation.

This investigation tested the hypothesis that post-anthesis defoliation of the two uppermost leaves could ameliorate the negative impact of increased plant population among maize cultivars; that there would be variation in the performance of these maize cultivars under these treatments in the rainforest transitory agroecology. Our findings would increase our understanding of the physiological mechanisms that underpins maize cultivar responses to defoliation at increasing plant population and would provide a technique of increasing plant population with minimal negative implication on their performance.

2 MATERIALS AND METHODS

2.1 Description of location and experimental site

The experimental fields were sited at the Teaching and Research farm, Federal University of Agriculture, Abeokuta (FUNAAB), Ogun state, (Longitude $3^0$25'E, Latitude $7^0$15'N,; altitude 144 m a.s.l). and National Horticultural Research Institute, Ibadan (NIHORT) ($3^0$56'E, $7^0$33'N: altitude 168 m a.s.l), Nigeria. Agrometeorological data were collected from the agrometeorological station of FUNAAB and NIHORT. Soil particle size distribution was determined using the hydrometer method (Bouyoucos, 1962). Soil pH was determined in soil: water suspension (1:1) using glass electrode pH-meter(McLean, 1982). Soil organic carbon was determined using the wet oxidation method of Walkley and Black (Allison, 1965). Total nitrogenwas determined using the modified micro Kjeldahl digestion technique (Jackson, 1962). Available phosphorus was evaluated based on Bray-1 method (Bray and Kurtz, 1945) and determined colometrically according to protocol devised Murphy and Riley, (1962). Exchangeable cations were determined by extracting the cation with 1N ammonium acetatebuffered at pH7. Potassium in the extract was determined by flame photometry.At Abeokuta soil organic carbon was 0.53 mg kg^{-1}, with 0.8 g kg^{-1}total nitrogen, 6.42 mg kg^{-1} available phosphorus and K^+ was 0.35 cmol kg^{-1}.At Ibadan the soil consisted of 0.46 mg kg^{-1} of organic carbon, 0.6 g kg^{-1}total nitrogen, 5.65 mg kg^{-1} of available phosphorus and 0.18 cmol $kg^{-1}$of K^+.The soil textural class of both locations was sandy, while the pre-planting soil pH at both locations was similar, slightly acidic (5.20 and 5.30 at Ibadan and Abeokuta respectively).

2.2 Treatments and design

The treatment consisted of maize cultivars, plant population and post-anthesis defoliation. These were arranged in split-split plot fitted into randomised complete block design. The main plot consisted of maize cultivars [TZEI 124 × TZEI 25 (hybrid) and 2009 TZE-W DT STR (open pollinated)]. Both maize cultivars belong to the early maturity class (90-95 days to reach physiological maturity). The sub-plot was made of plant population (106,666 plants ha^{-1}, 80, 000 plants ha^{-1} and 71, 111 plants ha^{-1}), while the sub-sub plot consisted of post-anthesis defoliation (control and defoliated maize plant).

2.3 Cultural practices

Planting material was sourced from International Institute of Tropical Agriculture, Ibadan. Ploughing was done twice and harrowing was conducted once. Sowing of maize seed was done on a flat land surface.Planting was conducted manually at a depth of about 20mm on 24th and 25th of May, 2015 at Abeokuta, 3rd and 4th of June, 2015 at Ibadan. Planting was conducted at a spacing of 0.75 × 0.25 m at two plants per stand constituting 106, 666 plants ha^{-1}, 0.75 × 0.50 m at three plants per stand constituting 80, 000 plants ha^{-1} and 0.75 × 0.75 m at four plants per stand constituting 71, 111 plants ha^{-1}. Each gross plot measured 3 × 4 m with a net plot of 2 × 2 m. Plots were separated from each other by 0.5 m path and each block was separated by 1 m walk way.

Missing stands were supplied at 1 week after planting (WAP). Weeding was done manually 3 and 6 WAP. A recommended rates of 140 kg N ha^{-1} and 70 kg N $ha^{-1}$for hybrid and open pollinated maize respectively were applied in two splits at planting in form of NPK 15- 15-15 and urea fertilizer at 4 weeks after planting as top dressing (Aduayi et al., 2002). Defoliation of two uppermost leaves was conducted 4 days after silking (DAS) as described by Liu et al., (2015).

2.4 Sampling and data collection

Soil samples were randomly collected from the sites before planting for the determination of pre-planting soil physico-chemical properties. Agronomic, phenological, physiological as well as yield and yield component variables were taken at 4WAP (vegetative stage), 8WAP (reproductive stage) and 12WAP (physiological maturity stage). Five plants were tagged randomly from the net plot. Plant height was determined from the soil surface to the tip of the last formed leaf. Leaf area was evaluated using the formula Leaf Area = length × widest width × 0.75. The correction factor was 0.75 (Dwyer and Stewart, 1986) and leaf area index was calculated as the ratio of total leaf area to land area: $\frac{Leafarea\ (cm^2)}{Landarea\ (cm^2)}$

Phenological variables (days to 50 % anthesis, and days to 50 % silking) were determined by standard procedures. Yield and yield component variables were determined by standard agronomic procedures at harvest maturity.Above the ground biomass was sampled at 4DAS (days after silking) and R6 (physiological maturity). These was later packed in an envelope and placed in an electric oven at 60˚C until constant mass was obtained. The samples were removed, cooled for about 30 minutes and weighed. Post-silking source–sink ratio(PSSR) was determined as the change in above-ground biomass dry massduring 4 DAS and at physiological maturity.Dry matter translocation (DMT) (rate of remobilization) (kg ha^{-1}) = dry matter at anthesis – dry mass at maturity (all vegetative parts except grains). While dry matter translocation efficiency (DMTE) (Remobilization Efficiency)(%) = dry matter translocation / dry matter at anthesis× 100.Rate of current photosynthesis (RCP) was calculated using the formula: grain yield (kg ha^{-1}) – dry matter translocation (kg ha^{-1}) and efficiency of current photosynthesis (ECP): rate of current photosynthesis (kg ha^{-1}) / dry massof vegetative organs at maturity stage (kg ha^{-1}) (Papakosta and Gayianas, 1991; Van Sanford and Mackown, 1987).

2.5 Statistical analysis

Data collected were subjected to mixed model analysis of variance (ANOVA), at 5 % probability level and means of significant treatmentwere separated using least significant difference (LSD). The statistical package used was Genstat 12th Edition.

3 RESULTS

At Abeokuta maximum amount of rainfall (165 mm) during the cropping season was observed in June, 2015, while no precipitation was recorded in January of that year. Similar pattern was observed on relative humidity, except that the maximum relative humidity was observed in July. Temperature was in the range of 30.2 °C and 26.8 °C for the months of March and June respectively (Table 1). Ibadan had similar rainfall pattern as Abeokuta except for the quantity of rainfall observed for the months during the cropping season. Maximum amount of rainfall (321.9 mm) was observed in May with the minimum (6.6 mm) recorded in January. Consequently higher relative humidity was observed in Ibadan ranged between 92 % to 88 % in August and February respectively. However, February and March had similar relative humidity during the cropping season. The highest temperature (29 °C) was observed in February while the least (25 °C) was observed in August (Table 2).

Table 1: Means of agrometeorological observations, Abeokuta

Months	Rainfall (mm)	Relative Humidity (%)	Mean temperature (°C)
January	0.0	47.7	27.8
February	51.0	61.4	29.5
March	67.0	60.4	30.2
April	69.0	62.8	29.0
May	60.0	61.9	28.3
June	165.0	70.8	26.8
July	66.0	73.0	27.2
August	29.0	70.3	26.2

Source: Department of Agrometeorological and Water Management, Federal University of Agriculture, Abeokuta.

Table 2: Means of agrometeorological observations, Ibadan

Months	Rainfall (mm)	Relative Humidity (%)	Mean temperature (°C)
January	6.6	90.0	28.5
February	28.4	88.0	29.0
March	189.6	88.0	28.0
April	246.3	89.0	28.5
May	321.9	89.0	28.0
June	233.7	90.0	28.5
July	157.9	82.0	26.0
August	139.4	92.0	25.0

Source: Weather station, National Horticultural Institute of Nigeria (NIHORT).

3.1 Growth response

Plant height was similar between the maize cultivarsat each period of investigation in each location(Tables 3 and 4). There was no significant ($P > 0.05$) varietal variability on leaf area and leaf area index at Abeokuta, except at 4 WAP. Variety 2009 TZE-W DT STR had larger leaf area and leaf area index than TZEI 124 X TZEI 25.Leaf area index significantly ($P < 0.05$) increased as plant population increases across all periods of investigation at both locations (Tables 3 and 4).

At 12WAP defoliated maize cultivar was significantly shorter than non-defoliated at Abeokuta (Table 3).

Table 3: Effect of plant population and post-anthesis defoliation on the means of growth of maize variables at 4, 8 and 12 WAP, Abeokuta

Treatments	Plant height (cm)			Leaf area (cm²)			Leaf area index		
Variety (V df 1)	4 WAP	8 WAP	12 WAP	4 WAP	8 WAP	12 WAP	4 WAP	8 WAP	12 WAP
TZEI 124 × TZEI 25	116.57	199.74	209.52	392.29	635.3	642.22	0.13	0.21	0.21
2009 TZE-W DT STR	119.15	202.68	217	457.94	660.13	675.18	0.15	0.22	0.22
LSD (5 %)	NS	NS	NS	43.12*	NS	NS	0.01*	NS	NS
Plant Population (PP df 2)									
106666 plants ha^{-1}	126.41	206.03	218.42	456.71	671.79	669.24	0.24	0.36	0.36
80000 plants ha^{-1}	116.58	198.33	213.17	434.44	640.97	658.98	0.12	0.18	0.17
71111 plants ha^{-1}	110.58	199.28	208.20	384.19	630.37	647.87	0.07	0.12	0.11
LSD (5 %)	NS	NS	NS	NS	NS	NS	0.03**	0.02**	0.03**
Defoliation (D df 1)									
No Defoliation		203.48	217.91		649.94	664.20		0.22	0.21
Defoliated		198.94	208.61		645.49	653.19		0.21	0.21
LSD (5 %)		NS	8.63*		NS	NS		NS	NS
V × PP (df 2)		NS	NS		NS	NS		NS	NS
V × D (df 1)		NS	NS		NS	NS		NS	NS
PP × D (df 2)		NS	NS		NS	NS		NS	NS
V × D × PP (df 2)		NS	NS		NS	NS		NS	NS

* indicates significance at $P < 0.05$ probability level. WAP: Weeks after planting, NS: Not significant, df- degree of freedom

Table 4: Effect of plant population and post-anthesis defoliation on the means of growth variables of maize varieties at 4, 8 and 12 WAP, Ibadan

Treatments	Plant height (cm)			Leaf area (cm²)			Leaf area index		
Variety (V df 1)	4 WAP	8 WAP	12 WAP	4 WAP	8 WAP	12 WAP	4 WAP	8 WAP	12 WAP
TZEI 124 × TZEI 25	72.44	142	159.56	173.96	491.5	445.64	0.06	0.16	0.15
2009 TZE-W DT STR	71.29	147.28	171	188.23	501.65	474.04	0.06	0.16	0.16
LSD (5 %)	NS	NS	NS	NS	NS	NS	NS	NS	NS
Plant Population (PP df 2)									
106666 plants ha^{-1}	72.68	150.26	170.69	202.78	468.78	493.06	0.11	0.25	0.26
80000 plants ha^{-1}	70.93	144.5	165.97	167.07	499.76	453.59	0.04	0.13	0.12
71111 plants ha^{-1}	71.99	139.17	159.18	173.44	521.18	432.86	0.03	0.1	0.08
LSD (5 %)	NS	NS	NS	NS	NS	NS	0.01**	0.02**	0.01**
Defoliation (D df 1)									
No Defoliation		143.36	164.22		493.67	467.77		0.17	0.16
Defoliated		145.92	166.34		499.47	451.91		0.16	0.15
LSD (5 %)		NS	NS		NS	NS		NS	NS
V × PP (df 2)		NS	NS		NS	NS		NS	NS
V × D (df 1)		NS	NS		NS	NS		NS	NS
PP × D (df 2)		NS	NS		NS	NS		NS	NS
V × D × PP (df 2)		NS	NS		90.59*	NS		NS	NS

* indicates significance at $P < 0.05$ probability level. WAP: Weeks after planting, NS: Not significant, df degree of freedom

3.2 Dry matter translocation, current photosynthesis, phenology, yield and yield components

At both locations there were no significant varietal differences on translocation and post silking source sink variables except on dry matter translocation efficiency at Ibadan (Table 5). Dry matter was more efficiently remobilised in maize variety TZEI 124 × TZEI 25 than variety 2009 TZE-W DT STR. With increase plant population dry matter was more remobilised in plant population of 80000 plants ha^{-1}.Thereafter, a significant depression was observed at Ibadan. Defoliation had no significant effect on the aforementioned variables at both locations (Table 5). Significant ($P < 0.05$) varietal variability was observed on days to 50 % anthesis at both locations (Tables 6 and 7). Variety 2009 TZE-W DT STR attained 50 % anthesis earlier than variety TZEI 124 × TZEI 25.Significant ($P < 0.05$) varietal variability was observed on rate of current photosynthesis at Abeokuta (Table 6). Maize variety 2009 TZE-W DT STR had significantly higher rate of current photosynthesis than maize variety TZEI 124 × TZEI 25.Plant population had significant ($P < 0.05$) effecton rate of current photosynthesis at Abeokuta and Ibadan. Plant population of 80000 plants ha^{-1} and 71111 plants ha^{-1} had the highest rate of current photosynthesis while plant population of 106666 plants ha^{-1} and 80000 had significantly the least rate of current photosynthesis at Abeokuta and Ibadan respectively.Increasing plant population significantly ($P < 0.05$) decreased number of kernels per row at Abeokuta (Table 6). Significant varietal variability was observed in number of kernels per row at Ibadan where variety TZEI 124 × TZEI 25 had significantly higher kernels per row than 2009 TZE-W DT STR (Table 7). Significant varietal difference ($P < 0.05$) was observed on one thousand grain massat Abeokuta (Table 6). Heavier one thousand grain masswas found in variety 2009 TZE-W DT STR compared with variety TZEI 124 × TZEI 25.Defoliation had significant effect on ($P < 0.05$) on one thousand grain mass across the locations. Non-defoliated maize produced heavier 1000 grain massthan defoliated maize. Grain yield differ significantly ($P < 0.05$) among plant populations at Ibadan (Table 7). The order of increase in grain yield obtained from plant population treatments was 106666 plants ha^{-1}> 71111 plants ha^{-1}> 80000 plants ha^{-1}.

Table 5: Effect of plant population and post –anthesis defoliation on the means of physiological variables of maize varieties (Abeokuta and Ibadan)

Treatments	Abeokuta			Ibadan		
	Translocation variables			Translocation variables		
	DMT (kg ha^{-1})	DMTE (%)	PSSSR	DMT (kg ha^{-1})	DMTE (%)	PSSSR
Variety (V df 1)						
TZEI 124 × TZEI 25	2847.09	9.26	-0.12	3028.59	6.35	-23.93
2009 TZE-W DT STR	783.79	-4.22	0.38	153.98	-22.38	-21.86
LSD (5 %)	NS	NS	NS	NS	50.68*	NS
Plant population (PP df 2)						
106666 plants ha^{-1}	3426.91	6.21	-0.05	3530.97	13.08	-29.95
80000 plants ha^{-1}	638.3	-2.91	0.3	3711.13	23.5	-17.87
71111 plants ha^{-1}	1381.11	4.26	0.13	-2468.26	-60.62	-20.86
LSD (5 %)	NS	NS	NS	2952.95*	NS	NS
Defoliation (D df 1)						
No Defoliation	3190.91	10.34	-0.28	1138.99	-22.25	-22.19
Defoliated	439.96	-5.3	0.53	2043.57	6.21	-23.6
LSD (5 %)	NS	NS	NS	NS	NS	NS
V × PP (df 2)	9299.98*	NS	NS	NS	NS	NS
V × D (df 1)	NS	NS	NS	NS	NS	NS
PP × D (df 2)	NS	NS	NS	NS	61.54*	NS
V × D × PP (df 2)	NS	NS	NS	NS	NS	NS

* indicates significance at $P < 0.05$ probability level and ** indicates significance at $P < 0.01$ probability level. DMT: Dry matter translocation, DMTE: Dry matter translocation efficiency, PSSSR: Post silking source sink ratio, df: degree of freedom

Table 6: Effect of plant population and post-anthesis defoliation on the means of phenology, current photosynthesis, yield and yield components of maize varieties, Abeokuta

Treatments	50 % Anthesis	50 % Silking	ECP kg kg^{-1}	RCP kg ha^{-1}	Number of kernels per row	1000 grain mass (g)	Grain mass kg ha^{-1}
Variety (V df 1)							
TZEI 124 × TZEI 25	44.89	47.72	0.17	2222.25	30.47	146.67	5069.33
2009 TZE-W DT STR	44.00	47.33	0.33	5014.22	27.04	186.06	5798.01
LSD (5 %)	0.632*	NS	NS	14058.51*	NS	14.722**	NS
Plant Population (PP df 2)							
106666 plants ha^{-1}	45.17	48.17	0.15	1947.58	25.73	164.33	5374.49
80000 plants ha^{-1}	44.17	47.17	0.35	5092.83	29.92	174.67	5731.13
71111 plants ha^{-1}	44	47.25	0.24	3814.29	30.6	160.08	5195.4
LSD (5 %)	NS	NS	NS	2077.45*	2.604**	NS	NS
Defoliation (D df 1)							
No Defoliation	44.33	47.61	0.16	2455.69	28.24	173.78	5646.6
Defoliated	44.56	47.44	0.33	4780.77	29.26	158.94	5220.74
LSD (5 %)	NS	NS	NS	NS	NS	10.377**	NS
V × PP (df 2)	NS	NS	0.72*	12553.18**	4.299*	NS	NS
V × D (df 1)	NS	NS	NS	NS	NS	NS	NS
PP × D (df 2)	NS	NS	NS	NS	NS	NS	NS
V × D × PP (df 2)	NS	NS	NS	NS	NS	NS	NS

* indicates significance at $P < 0.05$ probability level. WAP: Weeks after planting, NS: Not significant, ECP: Efficiency of current photosynthesis, RCP: Rate of current photosynthesis, df: degree of freedom

Table 7: Effect of plant population and post-anthesis defoliation on the means of phenology, current photosynthesis, yield and yield components of maize varieties, Ibadan

Treatments	50 % Anthesis	50 % Silking	ECP kg kg^{-1}	RCP kg ha^{-1}	Number of kernels per row	1000 grain mass (g)	Grain mass kg ha^{-1}
Variety (V df 1)							
TZEI 124 X TZEI 25	49.78	53.61	0.44	2446.54	27.63	150.44	5475.13
2009 TZE-W DT STR	48.94	53.33	0.75	4815.39	22.29	166.78	4969.36
LSD (5 %)	0.82*	NS	NS	NS	4.033*	NS	NS
Plant Population (PP df 2)							
106666 plants ha^{-1}	50.00	54.08	0.31	2592.73	22.69	152.17	6123.70
80000 plants ha^{-1}	49.08	53.17	0.13	590.94	26.10	163.58	4302.08
71111 plants ha^{-1}	49.00	53.17	1.35	7709.23	26.09	160.08	5240.97
LSD (5 %)	NS	NS	0.77*	3206.01**	NS	NS	1364.50*
Defoliation (D df 1)							
No Defoliation	49.33	53.56	0.80	3944.68	24.34	169.67	5083.67
Defoliated	49.39	53.39	0.39	3317.25	25.58	147.56	5360.82
LSD (5 %)	NS	NS	NS	NS	NS	11.59**	NS
V × PP (df 2)	NS	NS	NS	NS	NS	19.80*	NS
V × D (df 1)	NS	NS	NS	NS	NS	NS	NS
PP × D (df 2)	NS	NS	0.88*	NS	NS	NS	NS
V × D × PP (df 2)	NS	NS	NS	12101.19*	NS	NS	NS

* indicates significance at $P < 0.05$ probability level. WAP: Weeks after planting, NS: Not significant, ECP: Efficiency of current photosynthesis, RCP: Rate of current photosynthesis, df: degree of freedom.

4 DISCUSSION

The open pollinated maize variety (2009 TZE-W DT STR) displayed better growth variables than the hybrid maize (TZEI 124 × TZEI 25) at Abeokuta. The higher leaf area and leaf area index could increase the assimilatory surface of this variety for the interception of light and increased transpiration. These facts combined could have aided the photosynthetic capacity of OPV as reflected in the significantly higher rate of current photosynthesis in this variety than the hybrid maize at Abeokuta. Reduced dry matter translocation efficiency at Ibadan suggested that there could be enough assimilate available to sustain the growth of OPV. Availability of sufficient assimilate at Ibadan could be linked to precipitation pattern observed during the cropping season. It had earlier been reported that increasing dry matter translocation efficiency could predispose crops to increased rate of leaf senescence especially under nitrogen deficiency (He et al., 2003), subsequently compromising crop performance. Shorter days to 50 % anthesis could have increased duration of reproductive structures in the absence of production constraints. This was reflected in the significantly higher 1000 grain mass observed in OPV than the maize hybrid at Abeokuta. However, in Abeokuta despite the presence of more macronutrients than Ibadan rainfall distribution could constraint their availability. Water plays a significant role in nutrient availability, especially the solubility of mobile nutrients and their movement to the rhizosphere. This environmental constraint could have suggested that OPV could not optimally utilise the available growth resources. A shorter phenology could have enabled OPV to escape this production constraint. As a consequence this could have affected its performance, which was not significantly different from that of the hybrid maize at both locations despite the morphological and physiological advantages earlier displayed. The significant depression in the number of kernels per row in Ibadan might be linked with the reduced dry matter translocation efficiency observed in OPV than hybrid maize. This pattern would have a profound effect on the grain yield of OPV at Ibadan if current photosynthesis was not adequate enough to sustain assimilate availability at the grain filling growth stage. However, the rate of current photosynthesis and 1000 grain mass for this variety was higher than the hybrid maize though not significant at Ibadan. This could have implied a kind of compensatory relationship between the grain mass and number kernel. Such relationship had been reported in the cereals in the past, especially under stressful condition (Squire, 1990).

Increased plant population at both locations resulted in increased LAI at all periods of investigation. This increased assimilatory surface could predispose maize variety to increased interception of light and transpiration especially under vapour pressure deficit. Increased transpiration could have been more pronounced at Abeokuta, where there was higher temperature with reduced relative humidity than Ibadan. With the rainfall pattern observed at Abeokuta there could be possibility of subjecting maize variety to soil moisture deficit. This response could compromise photosynthetic rate. However, the pattern of RCP at both locations with increasing plant population indicated that maize variety at Abeokuta was able to acclimatise to increasing plant population than those at Ibadan, though they both displayed depression in RCP albeit at different plant populations. That could be partly explained by the distribution of nitrogen in the canopy. High plant population had been reported to have resulted in increased light attenuation and distribution of nitrogen along the strata vertically (Maddonni et al., 2001). This pattern could be further accentuated by deficiency of nitrogen in the soil thereby compromising canopy photosynthetic capacity through increased senescence that could have resulted from increased remobilisation of assimilates from other organs in maize plant. However the evidences available at Ibadan supported the hypothesis of increased remobilisation of assimilates with high plant population as indicated in the increased dry matter translocation when maize population attained population of 80000 plants ha^{-1} with converse pattern on rate of current photosynthesis and efficiency of current photosynthesis. Senescence could further be simulated by reduced red to far red ratio observed with increasing light attenuation in the canopy at both locations (Varlet-Grancher and Gautier, 1995). At Abeokuta the depression observed in the number of kernels per row with increasing plant population could have been linked to the assimilate availability. Increasing plant population at this location reduced RCP which could have compromised assimilate availability. This observation was corroborated by Tollenaar and Daynard, (1982). Maize plant had been observed to have reduced remobilisation efficiency in post-anthesis source-sink manipulation. The remobilisation efficiency of maize was evaluated to be 0.26 g of seed g^{-1} of stored carbohydrate, which was significantly lower than that of wheat and soybean (Kiniry et al., 1992). At Ibadan increased grain yield with increasing plant population would have been explained by the response pattern observed in RCP and efficiency of current photosynthesis. Carbon supply through the increased photosynthetic variables observed at high plant population at Ibadan especially at the critical growth stage of maize could increase nitrogen uptake by the root. This is even more germane at Ibadan where soil nitrogen content is lower than Abeokuta. But with increased root growth and higher precipitation the possibility of mining this nutrient is very high. Uhart and Andrade, (1995) had earlier posited that availability of assimilates at the grain filling period could increase root growth and possibly increase the grain yield. This observation was further corroborated by Moll et al.(1994).

At both locations reduced 1000 grain mass could have been responsible for the performance of defoliated maize at post anthesis. However there were no significant differencesin the performances of defoliated and non-defoliated maize at both locations. This could have suggested that other yield components could have contributed to the performance of defoliated and non-defoliated maize apart from the number of reproductive structures. It had earlier been posited that if a significant effect in the alternation of source sink ratio was observed on the seed dry mass it could be as a result of source limitation (Borrás et al., 2004). This could be linked to the change in canopy architecture through a change in plant height as observed in reduced plant height in defoliated maize crop compared to the non-defoliated at post anthesis period in Abeokuta. This reduced plant height could limit the penetration of both direct and diffuse light into the canopy. Reduced light into the canopy could compromise canopy photosynthesis and subsequently availability of the assimilates to the reproductive structures, especially at the grain filling stage. Maize had been reported to change seed mass to assimilate availability more than wheat especially under high solar radiation and temperature during the seed filling period, however it is highly sensitive under reduced assimilate availability (Borrás et al., 2004). The limited availability of assimilates due to source restriction would compromise the grain yield in maize if considered in the context of comparatively reduced remobilisation efficiency of maize compared to other grain legumes and cereals (Kiniry et al., 1992). This observation is in contraction of the results obtained by Liu et al., (2015). In their trial defoliation resulted in increased grain yield. The increased grain yield under defoliation was premised on higher canopy apparent photosynthesis, reduced senescence and reduced source:sink ratio than non-defoliated maize crop. Other observations that contributed to increased grain yield in defoliated maize crop than non-defoliated were increased harvest index, increased kernel mass and ear mass in their trial. It could be suggested that the high plant population used in their trial within the context of prevailing temperature in their condition resulted in sink limitation of maize crop performance. It had been reported that the performance of maize could be both source or sink limited (Thomas, 1992). This limitation is based on the threshold of assimilate availability, above or below which the performance of maize crop could be compromised. The differences in the genetic make-up of the varieties used in both experiments could have accounted for the differences in the results obtained. Same argument could be used when considering the environment and the management techniques in place when both trials were carried out, which were considerably different. However, the underlying mechanism that underpins differences in physiological and agronomic responses in both trials needs further research.

5 CONCLUSION

The investigation demonstrated that varietal differences observed between the maize varieties was reflected in their growth, phenology and assimilate translocation variables. The significantly higher assimilatory surface of OPV than the hybrid maize could have supported the interception of more radiant energy and gas exchange processes as indicated in rate of current photosynthesis, reduced dry matter translocation efficiency and bigger 1000 grain mass. However, the prevailing rainfall pattern at Abeokuta could have compromised the positive physiological responses of OPV. Thus the reduced days to 50 % anthesis observed could have reduced the potential utilisation of growth factors to attain yield potential. These evidences taken together would have suggested that OPV could attain similar grain yield to the hybrid under these agroecological conditions . This could be of economic benefit taken into consideration the cost implication of obtaining hybrid seed especially among resource challenged farmers. Both locations experienced increased LAI with increased plant population. However, response pattern of rate of current photosynthesis differs with increasing plant population at both locations. At Ibadan at population of 80000 plants ha^{-1} maize variety experienced reduced rate of current photosynthesis and efficiency of current photosynthesis with concomitant increase in high dry matter translocation probably to support grain set and grain filling as reflected in high grain yield at plant density of 106666 plant ha^{-1}. Contrarily, at Abeokuta the highest rate of current photosynthesis was attained at the plant density of 80000 plant ha^{-1}. This response could have made assimilate available to support reproductive structures as indicated in the grain yield at that plant population. Further increase in the plant population could have resulted in mutual shading in the canopy. These discrepancies in the response pattern to plant population could have been explained by the differences in the intensity of rainfall and its effect on nutrient availability especially at Abeokuta that experienced reduced intensity of rainfall. Reduced 1000 grain mass at both locations when maize varieties were defoliation suggested a disruption in source:sink balance through source constraint. This was validated at Abeokuta with a decrease in plant height that could have affected maize canopy architecture, though maize had similar grain yield at both locations under this treatment. It could be concluded that defoliation of two uppermost leaves had no significant effect on the performance of maize varieties in this agroecology.

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Assessment of toxicity on the basis of total phenolic content in oleander leaves (*Nerium oleander* L.) against *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae)

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ABSTRACT

Nerium oleander is an evergreen flowering shrub or small tree distributed widely in the Mediterranean region. It is also a source of polyphenols and cardenolides ?? with insecticidal effect which could be a safe alternative of chemical control of insect pests. In the present work, five concentrations (0 %, 1 %, 2.5 %, 5 %, and 10 %) of ethanolic extract from *Nerium oleander* leaves were evaluated for its insecticidal effect against 3 to 4 days old *Myzus persicae* individuals under laboratory conditions. Obtained results showed a significant insecticidal effect with 70 % of mortality at the highest concentration (10 %). Total phenolic content of leaf ethanolic extract of this plant was 1721.36 mg gallic acid equivalent 100 g^{-1} dry matter. The results obtained suggest that we could make bioinsecticides based on leaves ethanolic extracts from *N. oleander* which rich in polyphenols for use eventually in integrated pest management.

Key words: *Nerium oleander; Myzus persicae;* insecticidal effect; total phenolic content

IZVLEČEK

OVREDNOTENJE STRUPENOSTI ETANOLNIH IZVLEČKOV FENOLOV IZ LISTOV OLEANDRA (*Nerium oleander* L.) ZA SIVO BRESKOVO UŠ (*Myzus persicae* (Sulzer, 1776), Hemiptera: Aphididae)

Oleander (*Nerium oleander*) je vednozelen grm, ki je razširjen v celotnem mediteranskem območju. Je tudi vir polifenolov in kardenolidov z insekticidnimi učinki, ki bi lahko bili varna alternativa kemijski kontroli škodljivih žuželk. V raziskavi so bili ovrednoteni insekticidni učinki etanolnih izvlečkov oleandrovih listov v petih koncentracijah (0 %, 1 %, 2,5 %, 5 %, in 10 %) na smrtnost 3 do 4 dni starih sivih breskovih uši v laboratorijskih razmerah. Dobljeni izsledki so pokazali značilne insekticidne učinke s 70 % smrtnostjo pri največji koncentraciji izvlečka (10 %). Celokupna vsebnost fenolov etanolnega izvlečka oleandrovih listov je bila 1721,36 mg, izražena kot ekvivalent galne kisline na 100 g^{-1} suhe snovi. Dobljeni rezultati nakazujejo, da bi etanolne izvlečke na polifenolih bogatih oleandrovih listov lahko uporabili v integriranem uravnavanju škodljivcev.

Ključne besede: *Nerium oleander; Myzus persicae;* insekticidni učinek; celokupna vsebnost fenolov

1 INTRODUCTION

Chemical pesticides used in plant protection have hazardous effects on human health and environment. Phytochemicals and plant extracts have long been a subject of research in an effort to develop alternatives to conventional insecticides but with reduced health and environmental impact (Dancewicz et al., 2011). Most plant species which are used for plant protection contain ingredients which inhibit the development of insects,

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hinder their feeding (antifeedants) or act as repellents and confusants (Rojht et al., 2009, Laznik et al., 2010, Rojht et al., 2012.). Polyphenols are a reputed and large phytochemical family with many interesting properties such plant resistance to insect pests (Bennett, 1996, Ding et al., 2000), antioxidant activity (Emmons and Peterson, 2001), and organoleptic properties (Es-Safi et al., 2003).

The green peach aphid, *Myzus persicae* (Sulzer, 1776) is found throughout the world. In addition to attacking plants in the field, the aphid readily infests vegetables and ornamental plants grown in greenhouses (Capinera, 2011). It is also an insect model for many studies (Ji et al., 2016). Its management is generally based upon the use of synthetic insecticides (Ciarla et al., 2005). Consequently, this study aims to assess the toxicity of *Nerium oleander* L. ethanolic leaf extract against *M. persicae* and determine the content of phenolic compounds of this extract due to its richness in these molecules which implicated in pest control in order to valorize the role of this plant in this field.

2 MATERIALS AND METHODS

N. oleander leaves were collected in April 2014 at the flowering stage from Batna in the East of Algeria. It is located at 35° 61´ N, 6° 24´ E with an elevation of 641 meters above sea level. Leaves were dried at 50 °C and ground to a fine powder in a mortar grinder (Retsch RM 200). The air-dried and finely ground leaves (80 g) were extracted by successive extraction three times. Indeed, the powder was stirred with 400 ml of ethanol at room temperature for 30 min. The mixture was filtered three times to obtain three filtrates. These three filtrates were mixed and brought together in a sand bath (N'guessan et al., 2009). These series of operations resulted in a concentrated solution. The extract was kept at +4 C° until toxicity test on the aphid and determination of total phenolic content.

2.1 Aphid collecting and rearing

Last stage larvae of green peach aphid have been collected collected from Biskra in the South East of Algeria at 34° 52´ N, 5° 45´ E in April 2014 where they were living on *Malva* sp. Mass rearing of the green peach aphid was started on broad beans (*Vicia faba* L.) in a greenhouse. Each plant was inoculated with an apterous adult when emerging in the morning. Aphids were collected after 10 days by brushing them carefully from the leaves.

2.2 Preparation of dilutions

Five dilutions have been made: 0, 1, 2.5, 5 and 10 %. These were prepared by adding the methanol according to the protocol of Singh et al. (2012).

2.3 Bioassay test

To assess the insecticidal effect of *N. oleander* leaf extract, 15 *M. persicae* larvae (3 to 4 days old) per treatment were placed in a Petri dish (90 mm) containing three leaves of *Vicia faba* soaked in these different concentrations with three replications. The experiment was carried out under laboratory conditions (25 ± 1 °C, 50 ± 5 %). The mortality was determined after 24 h from the beginning of exposure. When no leg or antennal movements were observed, insects were considered dead (Salari et al., 2010).

2.4 Total phenolic content

The total phenolic content of the ethanolic extract of *N. oleander* L. was measured by the method described by Juntachote (2007). 0.5 ml of the extract was added to 5 ml of distilled water and vortexed for 1 min using a vortex mixer (Janke & Kunkel IKA, Model: VF2, Germany). 1 ml of Folin and Ciocalteu's Phenolic reagent was added and mixed well. After 5 min, 1 ml of saturated sodium carbonate solution was added and the mixture was vortexed again. The sample was allowed to develop a blue colour for 1 h. The absorbance was measured at 640 nm using a spectrophotometer (UV-120-01; Shimadzu Co., Kyoto, Japan). A standard curve was prepared at the same time with gallic acid (Sigma-Adrich GmbH, Sternheim, Germany) at concentrations ranging from 0 to 0.2 mg ml^{-1}. The quantity of total phenolic content in the sample was calculated as gallic acid equivalent by using the standard curve.

2.5 Statistical analysis

Logistic regression analysis was employed to predict the probability that the augmentation of ethanolic extract concentrations would increase mortality of *M. persicae* individuals. The predictor variable was ethanolic extract concentrations (1 %, 2.5 %, 5 %, and 10 %). The Chi-square value will determine whether there is a difference between the current model and the intercept-only model. We used the statistical program Statistica 8 (StatSoft, Inc., Tulsa, OK) for all analyses.

3 RESULTS AND DISCUSSION

Nerium oleander (common oleander) is potentially lethal plant after ingestion for human beings, all parts of this plant are toxic (Bandara et al., 2010). It has antimicrobial properties (Huq et al., 1999; El Sawi et al., 2010), antioxidant activity (Mohadjerani, 2012) and also insecticide effect (Bagari et al., 2013).

Statistical analysis indicated the presence of dependence between the mortality and the different concentrations ($p < 0.01$). The Chi-square value for the difference between the current model and the intercept-only model is highly significant. Thus, we can conclude that mortality is related to ethanolic extract concentrations. In fact, Table 1 shows the logistic regression coefficient, Wald test and odds ratio. Using a 0.05 criterion of statistical significance showed that all concentrations had significant effect (Table 1).

Table 1: Logistic regression predicting mortality from concentrations and lethal concentration (LC_{50} and LC_{90} %)

χ^2	B	Wald χ^2	*P*	Odds Ratio	LC_{50} (%)	LC_{90} (%)
74.81	-2.6	62	0.000	1.43	7.2	9.85

A concentration of 10 % caused 73 % of mortality on green peach aphid (Figure 1) with a LC_{50} of 7.2 % while LC_{90} was 9.85 %.

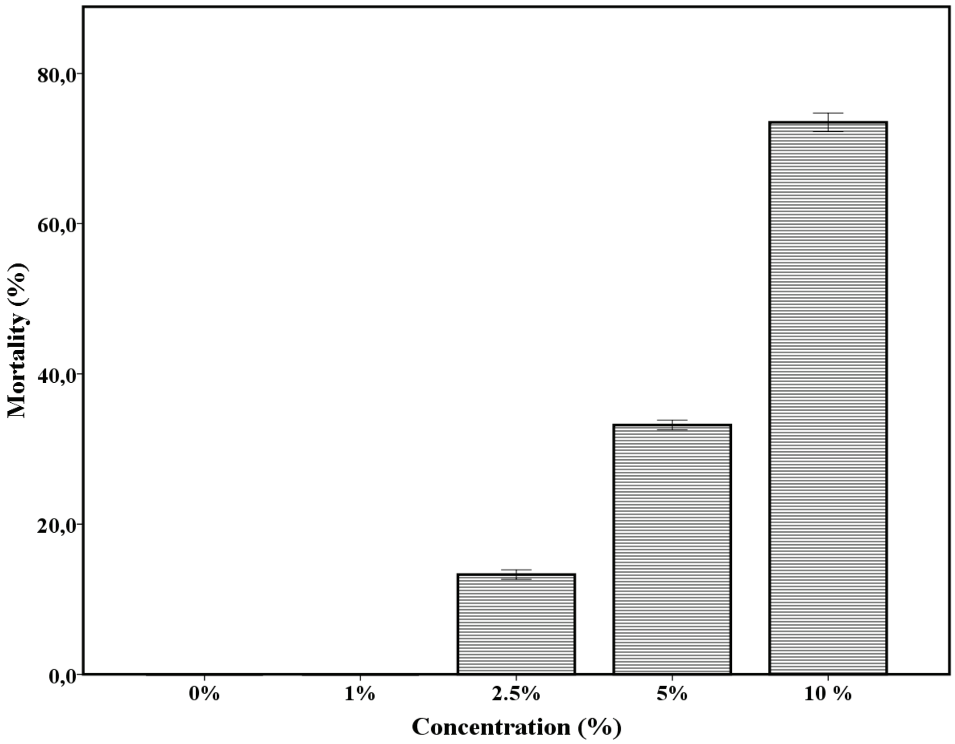

Figure 1: Average mortality (% ± standard error) of *Myzus persicae* larvae with leaf extracts from *Nerium oleander* with several concentrations after 24 hours

The toxicity of 2 % crude phenol extract of the leaves of *N. oleander* on larvae and adults of *Bemisia tabaci* (Gennadius, 1889) (Hemiptera: Aleyrodidae) reached 82.63 % and 60.45 % respectively (Rathi and Zubaidi, 2011). The extract hydro-alcoholic of its leaves administred to larvae of the tribe Rhizotrogini (Coleoptera: Scarabaeidae) had an effect on the protein content of the haemolymph and acetylcholine esterase activity (Madaci et al., 2008).

In the literature, many authors had mentioned the richness of *N. oleander* (Madaci et al., 2008; Rathi and Zubaidi, 2011; Siddiqui et al., 2012).

Based on the absorbance values of phenolic extract reacting with the Folin-Ciocalteu, and compared to the standard gallic acid solution, the result of the quantitative analysis of total phenolic compounds was 1721.36 mg gallic acid equivalent 100 g^{-1} of DR (dry matter) (Figure 2).

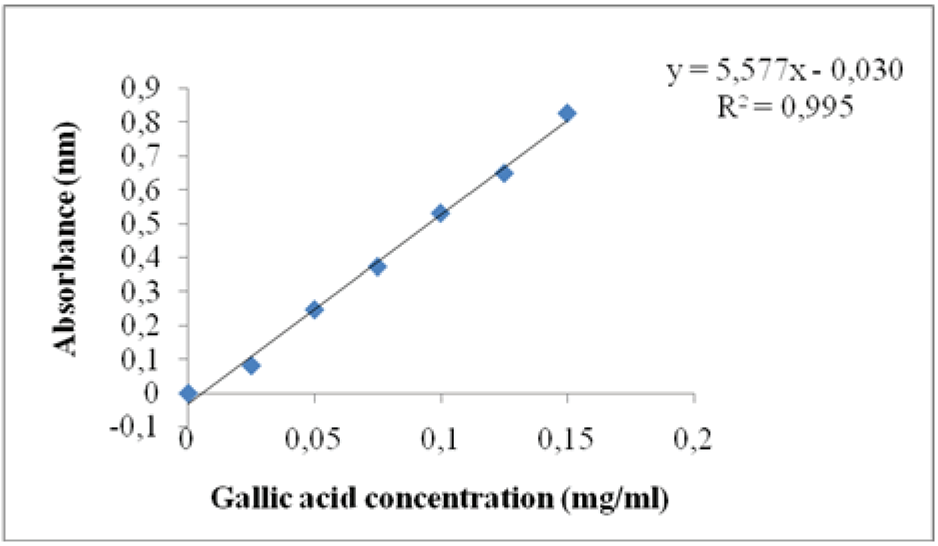

Figure 2: Total phenolic content in leaves of *Nerium oleander*

Our results and those obtained by Zibbu (2012) and Mohadjerani (2012) who found 48.94 mg equivalent catechol 100 mg^{-1} dry matter and 4.54 µg gallic acid 100 $µg^{-1}$ dry matter respectively. Also, Srivastava et al. (2013) found 30.10 mg EAG 100 g^{-1} but in fresh mass indicated the high amounts of these molecules contained in leaves of common oleander.

High toxicity against green peach aphid individuals was due probably to the high amounts of phenolic compounds present in the extract. Indeed, many authors have highlighted the importance of polyphenols for aphid control which support our findings. Indeed, Laznik (2010) tested cinnamic acid against *Aphis pomi* (De Geer, 1773) and found that this molecule has showed aphicidal properties. Also, two flavanoles and one flavanone were found to be active as aphicid against the woolly apple aphid, *Eriosoma lanigerum* (Hausmann, 1802) by Ateyyat et al. (2012). In fact, larval mortality was higher than that obtained against apterous adults. The increase in the concentration of polyphenols has resulted in a remarkable augmentation in the larval mortality rate. However, works on aphicidal activity of *N. oleander* are very scarce. Goławska (2012, 2008) reported the effectiveness of polyphenols against *Acyrthosiphon pisum* (Harris, 1776). El-Akhal et al. (2015) tested the ethanolic extract of *N. oleander* on culicid mosquitoes and noticed toxic effects on their larvae. Indeed, the lowest concentration necessary to achieve 100 % mortality of *Culex pipiens* (Linné, 1758) larvae was evaluated at 160 mg ml^{-1}.

4 CONCLUSIONS

Leaf ethanolic extract of *N. oleander* was effective against green peach aphid and this efficacy is probably due to the high content of this extract on polyphenols. Thus, the results obviously show that will be possible to develop new biopesticides based on high content of these molecules in integrated pest management programs to reduce the use of conventional insecticides. Nevertheless, further research is needed on the phytotoxicity of these molecules and toxicity against non-target species.

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Response of sunflower to organic and chemical fertilizers in different drought stress conditions

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ABSTRACT

The main objectives of this research were to determine the effects of applying organic and chemical fertilizers under different irrigation regimes on sunflower (*Helianthus annuus* L.) morphological traits, yield components, grain yield and grain quality. The experiment was conducted as spilt plots based on a randomized complete block design with three replicates. Irrigation treatments at three levels (well-irrigated, mild and severe drought stress) were allocated to main plots and eight fertilizer treatments (urea (F1), urea + composted cattle manure (F2), zeocompost (F3), vermicompost (F4), zeolite-amended chicken manure (Z-ACM) (F5), zeocompost + vermicompost (F6), zeocompost + Z-ACM (F7) and vermicompost + Z-ACM (F8)) were randomized in sub-plots. The results showed that irrespective of the drought stress intensity, organic fertilizer treatments produced more dry matter, heavier and greater grain than did chemical treatments. In well-irrigated plots, the highest grain yield was obtained from F6, F7 and F8 treatments. Under drought stress conditions, the highest grain yield was obtained from the high zeolite content organic fertilizers i.e. F3, F5 and F7. We concluded that amending soil with organic fertilizers in combination with zeolite can be a beneficial approach for decreasing chemical fertilizer application rates and improving the sustainability of agricultural systems.

Key words: drought stress; zeolite; soil fertility; grain quality; sunflower

IZVLEČEK

ODZIV SONČNICE NA ORGANSKA IN MINERALNA GNOJILA V RAZMERAH RAZLIČNEGA SUŠNEGA STRESA

Glavni namen te raziskave je bil določiti učinke uporabe organskih in mineralnih gnojil v različnih režimih namakanja na morfološke lastnosti, komponente pridelka, pridelek zrnja in njegovo kakovost pri sončnici (*Helianthus annuus* L.). Izveden je bil poskus z deljenkami kot popolni naključni bločni poskus s tremi ponovitvami. Obravnavanja so obsegala namakanje na treh ravneh na glavnih ploskvah (dobro namakano, blagi in veliki sušni stres) in osem načinov gnojenja na podploskvah (urea (F1), urea + kompostiran goveji gnoj (F2), zeokompost (F3), vermikompost (F4), zeolite z dodatkom kokošjega gnoja (Z-ACM) (F5), zeokompost + vermikompost (F6), zeokompost + Z-ACM (F7) in vermikompost + Z-ACM (F8). Izsledki so pokazali, da je ne glede na jakost sušnega stresa obravnavanje z organskimi gnojili dalo več suhe snovi, težja in večja zrna kot gnojenje z mineralnimi gnojili. Na dobro namakanih ploskvah je bil dosežen največji pridelek zrnja pri obravnavanjih F6, F7 in F8. V razmerah sušnega stresa je bil dosežen največji pridelek zrnja pri obravnavanjih, kjer so organska gnojila vsebovala veliko zeolita, obravnavanja F3, F5 in F7. Zaključili smo, da je dodadajanje organskih gnojil z zeolitom primeren pristop k zmanjševanju uporabe mineralnih gnojil pri izboljševanju trajnosti agroekosistemov.

Ključne besede: sušni stres; zeolite; rodovitnost tal; kvaliteta zrnja; sončnica

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1 INTRODUCTION

Water and nutrients availability and their interactions commonly impact crop growth and yield (Jing et al., 2012). The impact of industrial farming practices on soil and water quality is now a global concern, and much recent research has focused on management options for reducing nutrient waste, improving soil quality and increasing crop yield (Edmeades, 2003). Alternative management systems, such as organic and integrated farming, are being promoted because they are more environmentally benign and enhance soil and water quality relative to industrial farming practices (Delate & Cambardella, 2004; Gholamhoseini et al., 2013). Nitrogen is one of the most important elements, playing a key role in achieving desired yield and quality of crop production. In sustainable agroecosystems, N cycle is sustainably managed to reduce the risk of N leaching to groundwater (Basso & Ritchie, 2005). Therefore choosing the right amount of the most suitable N fertilizer source is critical to plant growth as well as livestock, human and environmental health.

Historically, organic materials such as manure have been mixed with soil to improve water and nutrient retention (Bigelow et al., 2004). Organic matter affects crop growth and yield directly by supplying nutrients and indirectly by modifying soil physical properties that can improve the root environment and stimulate plant growth (Bandyopadhyay et al., 2010). Vermicompost is a product of biodegradation of organic materials using various species of earthworms and microorganisms (Yang et al., 2015). Vermicomposts are rich in all essential plant nutrients, humic acids and vitamins as well as enzymes and plant growth regulators (Singh et al., 2011). Applications of vermicompost individually or in combination with either other organic fertilizers or mineral fertilizers have been proved effective to improve growth and yield of various crops (Javaad & Panwar, 2013; Singh et al., 2011; Simsek-Ersahin, 2011).

Zeocompost is made by composting cattle manure mixed with zeolite. Zeolites are crystalline, hydrated aluminosilicates, characterized by an ability to lose and gain water reversibly and to exchange their constituent elements without a major change of structure (Leggo et al., 2006; Gholamhoseini et al., 2012). Zeolites have high cation exchange capacity (200-300 $cmol_c\ kg^{-1}$), selective absorption and structure stability over the long term (Baerlocher et al., 2001). Although the effects of various organic fertilizations have been studied in several crops (Yolcu, 2010; Rokhzadi & Toashih, 2011), there is little information regarding the effect of zeolite and composted cattle manure on sunflower production.

Chicken manure is the organic waste from poultry composed of mainly feces and urine of chickens and spilled feed and feathers. The mixture of chicken manure with zeolite as bedding materials is referred as zeolite-amended chicken manure (Z-ACM). Therefore Z-ACM is organic manure enriched with many major plant nutrients like N, P, K and many trace elements like Zn, Cu, As etc. In a study, Leggo (2000) investigated the response of wheat to chicken manure amended by zeolite and found out that crop faced a better growth rate when zeolite was applied in chicken manure, and reported that the increase of growth and yield is due to N availability by zeolite.

Considering the significant role of organic fertilizers in providing N in sustainable production of oil seed crops, an experiment was conducted to study the effect of different organic fertilizers on growth, grain yield, oil and protein content of sunflower grown under different drought stress conditions.

2 MATERIALS AND METHODS

The field experiment was conducted at Agricultural Research Farm, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran in 2012. The experiment was laid out as split plot in a randomized complete block design with three replicates. Irrigation treatments at three levels (irrigation after depleting 30, 50 and 70 % of field capacity as well-irrigated (I1), mild drought stress (I2) and severe drought stress (I3), respectively) were allocated to main plots and eight fertilizer treatments (urea (F1), urea + composted cattle manure (F2), zeocompost (F3), vermicompost (F4), Z-ACM (F5), zeocompost + vermicompost (F6), zeocompost + Z-ACM (F7) and vermicompost + Z-ACM (F8)) were randomized in sub-plots. Soil samples were collected before fertilizer application at 0-30 cm depth to assess soil physical and chemical properties. Soil analyses were conducted using procedures described by soil and plant analysis council (1999). The soil texture was sandy loam based on the textural triangle classification (Table 1). Potassium and P were not applied during the growth season because the soil had adequate levels of these elements (Table 1). The values of moisture contents at field capacity, permanent wilting point and available water were 21. 9 and 12 %, respectively. Time domain reflectometry (TDR model Trime-FM) was used to measure soil volumetric water at a soil depth of 0–60 cm (two soil layers, at 30 cm intervals). The following equation was used to calculate management allowed depletion (MAD) or maximum water depletion percentage (Allen et al., 1998).

$$MAD\ (\%) = 100 \times \frac{1}{n} \sum \frac{FCi - \theta i}{FCi - PWP}$$

Where MAD: management allowed depletion; n: number of soil layers at the depth of root development; FCi: field capacity at i layer; θi: soil water volumetric percentage before irrigation at i layer and PWP: permanent wilting point.

Required water for each plot was calculated according to the following equation (Allen et al., 1998).

$$Vd = \frac{MAD(\%) \times (FC - PWP) \times Rz \times A}{100}$$

Where Vd: required water (m^3); MAD: management allowed depletion (%); Rz: depth of root development (m); A: plot area (m^2)

Table 1: Soil physiochemical properties*

Soil depth (cm)	soil texture	pH ($CaCl_2$)	EC (dS m^{-1})	C_{org} (%)	N_t (%)	N_{av} (%)	P	K
							(mg kg^{-1})	
0-30	sandy-loam	7.6	2.28	1.01	0.09	0.001	24	375

C_{org} – organic carbon; N_t – total N; N_{av}– available N

A polyethylene pipeline and a counter were installed to control irrigation. According to sunflower N requirement (120 kg N ha^{-1}) and soil available N content (~0, Table 1), required N was calculated. The N quantity to supply crop needs from organic fertilizers was calculated as follow (Sabahi, 2007).

Vermicompost, zeocompost and Z-ACM were supplied from Tehran University Horticultural Research Centre. Chemical analysis of the organic fertilizers is shown in table 2. After preparing the plots (3 m width and 4 m length consisted of 6 rows) certain amount of organic fertilizers (Table 3) were spread onto the plots and then incorporated into the soil with shovel. There were 2 m gaps between the blocks, and a 1 m alley was established between each plot to prevent lateral water movement and other interferences. Sunflower seeds (‘Azargol’, Seed and Plant Improvement institute, Karaj, Iran) were sown by hand at depths of 5 cm on the rows 25 cm apart. At first, the experimental plots were over-seeded and then thinned at the three-leaf stage to achieve the recommended plant density of 80,000 plants ha^{-1}. The first irrigation was performed immediately after sowing. Weeds were controlled manually during growing season. The crop was harvested at physiological maturity by cutting the plants off at ground level.

The plants were analyzed into laboratory, being determined the following traits: plant height, stem diameter, dry matter and grain yield as well as yield components including head diameter, number of grains per head and 1000-grain mass. Final yield was calculated as the grain yield at 10 % moisture content and expressed in kg ha^{-1}. Harvest index was calculated as the ratio of grain yield to above ground biomass yield expressed as a percentage. Grain oil and protein percentage were determined using Inframatic (Inframatic 8620 Percor) and Kjeldahl methods, respectively. The data were subjected to analysis of variance (ANOVA), to determine the variability of each measurement. The means of treatment were compared according to Fisher's LSD (0.05).

Table 2: Organic fertilizers chemical properties

Characteristics	Zeocompost	Z-ACM	Vermicompost	composted cattle manure
N (%)	1.25	4.2	1.4	1.1
Available nitrogen (%)*	0.85	2	0.75	0.85
P (%)	0.39	1.2	0.5	0.6
K (%)	1.7	0.8	0.7	2.4
Organic carbon (%)	20.2	---	9.6	25.4
pH	5.8	9.7	2.7	9.1
EC (ds m^{-1})	8.14	---	8.2	21.4
Cu (mg kg^{-1})	7.26	35.9	80.8	21.2
Zn (mg kg^{-1})	101.4	110.8	16.7	117
Fe (mg kg^{-1})	1245	550	89.1	6525
Mn (mg kg^{-1})	237	430	620	289

* Available nitrogen = 95% of NH_4^+ + 25% of organic N (Tarkalson et al., 2006)

Table 3: Amount of applied fertilizers to supply 120 kg N ha^{-1}

Treatments	urea (kg ha^{-1})	composted cattle manure (t ha^{-1})	Zeocompost (t ha^{-1})	Z-ACM (t ha^{-1})	Vermicompost (t ha^{-1})	Zeolite (kg ha^{-1})
F1	261	0	0	0	0	0
F2	70	7	0	0	0	0
F3	0	0	14	0	0	2100
F4	0	0	0	0	16	2400
F5	0	0	0	6	0	900
F6	0	0	7	0	8	1050+1200
F7	0	0	7	3	0	1050+450
F8	0	0	0	3	8	450+1200

3 RESULTS AND DISCUSSION

The results indicated that the main effects of irrigation and fertilizer treatments were significant on all studied traits except for harvest index (Table 4). In addition, dry matter yield, grain number per head and final grain yield as well as oil and protein percentage were significantly affected by interaction between irrigation and fertilizers treatments (Table 4).

3.1 Dry matter yield

Under well-irrigation conditions, increased N availability due to organic fertilizers application has led to increased vegetative growth and eventually increased dry matter production (Fig. 1). Among organic fertilizer treatments, zeocompost + vermicompost, zeocompost + Z-ACM and vermicompost + Z-ACM treatments caused the maximum dry matter yield, among which vermicompost + Z-ACM showed the maximum value of 6563 kg ha^{-1} (Table 5). Increase in dry matter production may be due to enhanced soil N content on account of Z-ACM application during growing season and also improved soil microbial activity through applying vermicompost (Salehi et al., 2016). In drought stressed plots, organic fertilizers in which zeolite was applied led to increased dry matter (Fig. 1). Under drought stress conditions, the maximum dry matter yield was obtained from Z-ACM and zeocompost + Z-ACM treatments (Fig. 1). By contrast, the minimum yield was related to urea and urea + composted cattle manure treatments (Fig. 1). It has been reported that zeolite application improves soil water retention capacity (Xiubin & Zhanbin, 2001; He et al., 2002).

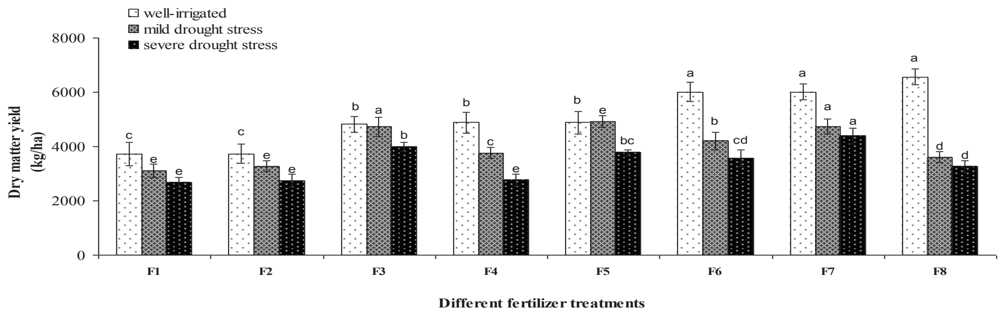

Figure 1: Interaction effect of irrigation treatments × fertilizer treatments on sunflower dry matter yield. In each irrigation treatments, means followed by the same letter are not significantly different ($P \leq 0.05$). Vertical bars indicate standard deviation (n=3)

Table 4: Analysis of variance on yield, yield components, morphology and qualitative characteristics of sunflower as affected by irrigation regimes and fertilizers

S.O.V	df	Dry matter yield	Grain yield	Harvest index	Grain number per head	1000 grain mass	Plant height	Stem diameter	Head diameter	Grain oil percentage	Grain protein percentage
Replication	2	ns	ns	ns	ns	ns	ns	ns	**	**	ns
Irrigation	2	**	**	ns	**	**	**	**	**	**	**
Error	4	177307.37	73088.64	56.03	2227.30	13.68	249.25	2.32	9.31	2.76	5.39
Fertilizer	7	**	**	ns	*	**	**	**	**	**	*
Interaction	14	**	**	ns	**	ns	ns	ns	ns	**	*
Error	42	147854.33	54126.67	52.61	553.34	6.64	224.83	1.54	8.52	2.36	3.07
C.V (%)		9.20	13.30	17.00	5.70	5.00	11.23	6.00	13.30	3.50	8.10

ns: non-significant, *: significant at 0.05 and **: significant at 0.01 probability level

Table 5: Main effects of irrigation regimes and fertilizers systems on sunflower yield and yield components

Treatments	Dry matter ($kg\ ha^{-1}$)	Grain yield ($kg\ ha^{-1}$)	Harvest index (%)	Grain number per head	1000 grain mass (g)
Irrigation regimes					
I1	5075 a	2084 a	41.8 a	516 a	57.3 a
I2	4045 b	1632 b	40.3 a	402 b	50.8 b
I3	3397 c	1533 c	45.4 a	299 c	46.5 c
Fertilizers systems					
F1	3176 d	1342 d	45.5 a	348 d	45.8 c
F2	3245 d	1332 d	41.6 a	376 c	46.5 c
F3	4513 b	1773 bc	39.7 a	422 b	49.6 b
F4	3805 c	1579 c	40.9 a	384 c	50.8 b
F5	4527 b	1967 ab	44.3 a	411 b	51.3 b
F6	4594 b	1957 ab	43.0 a	412 b	56.4 a
F7	5051 a	2141 a	42.6 a	472 a	54.8 a
F8	4466 b	1909 b	43.6 a	424 b	57.0 a

Means in columns followed by the same letters are not significantly different at $P \leq 0.05$ using LSD test.
I1: Irrigation after depleting 30 %, I2: Irrigation after depleting 50 %, I3: Irrigation after depleting 70 % of field capacity. F1: urea, F2: urea + composted cattle manure, F3: zeocompost, F4: vermicompost, F5: Z-ACM, F6: zeocompost + vermicompost, F7: zeocompost + Z-ACM and F8: vermicompost + Z-ACM

3.2 Grain yield

There was a significant difference among irrigation treatments in terms of grain yield (Table 5). The maximum grain yield (2084 kg ha^{-1}) was obtained from well irrigated plots. Grain yield decreased by 22 and 29 % due to mild and severe drought stress, respectively (Table 5). Sunflower grain yield strongly depends on water availability in the soil and would decrease with increasing water shortage level (Sezen et al., 2011). The final grain yield reduction due to drought stress was mainly through low grain mass and grain number per head and also head diameter. In this study, there were positive and significant correlations between grain yield and yield components i.e. grain number per head, grain mass and head diameter. Drought stress, like other environmental stresses, reduces grain filling period through reducing photosynthesis and assimilates transport into the grains. The maximum grain yield was achieved when plants were well irrigated and treated with zeocompost + vermicompost, zeocompost + Z-ACM or vermicompost + Z-ACM treatments (Fig. 2). By contrast, the minimum grain yield was related to urea followed by urea + composted cattle manure treatments (Fig. 2). It seems that the application of organic fertilizers accompanied by zeolite in the present experiments decreased N leaching because organic fertilizers improved the physicochemical conditions of the soil (Gholamhoseini et al., 2013) and increased the activity and penetration of plant roots (Evanylo et al., 2008). In addition, the most important process by which zeolite application decreases N leaching arises from the unique properties of this natural mineral. In zeolites, the canals are so large that cations such as ammonium can fit therein, but bacteria, particularly nitrifying bacteria, cannot access the zeolite canals (Baerlocher et al., 2001). Therefore, when ammonium is available in organic fertilizers or soil, zeolite selectively absorbs ammonium (Gholamhoseini et al., 2012) and renders it unavailable to nitrifying bacteria, which are active in well-aerated sandy soils. Thus, the transformation of ammonium to nitrate (the latter of which is prone to leaching) will decrease with zeolite addition, therefore decreasing N leaching. Any change in N availability will have significant effect on grain yield as N availability during growing season affects assimilates allocation among vegetative and reproductive organs. Increase in grain yield on account of organic fertilizers application may be due to the role of composts in improving soil N content over growing season (Bandyopadhyay et al., 2010). As can be seen from table 2, Z-ACM is rich in N, P and K as well as Mg. Nitrogen is released quickly from Z-ACM during early growth stage (Sabahi, 2007) and help plants to establish rapidly and grow strong root systems. In the rest of the growth season, N is released gradually from vermicompost throughout the growth stages of the crop. On the other hands, it appears that increase of grain yield in organic treatments, especially F8, results from a proper balance between available soil N and plant N requirements. Under mild and severe drought stress conditions the maximum grain yield was obtained from those treatments in which more zeolite was applied i.e. zeocompost, Z-ACM and zeocompost + Z-ACM treatments. By contrast, the minimum grain yield was observed in chemical and combined treatments. Zeolite provides more moisture by improving soil water retention capacity and promotes sunflower grain yield. Generally, zeolite can decrease the bulk density and increase total porosity, which consequently increase soil water content (Nakhli et al., 2017). Its application changes the inter-particle porosity of soil. Zeolite is a porous medium with open pore network channels into its structure, which can also play an important role in water retention. Additionally, it seems that adding zeolite to organic fertilizers prevents the N loss from the soil due to absorption and subsequent release of the N by the zeolite. In this way, organic fertilizers amended by zeolite can act as a slow-release fertilizer to supply N to the crop gradually. A direct relationship between N supply and crop dry matter has been reported by Hermanson et al. (2000). Thus, it is to be expected that that organic fertilizers with zeolite (especially F6 and F8) will increase sunflower grain yield.

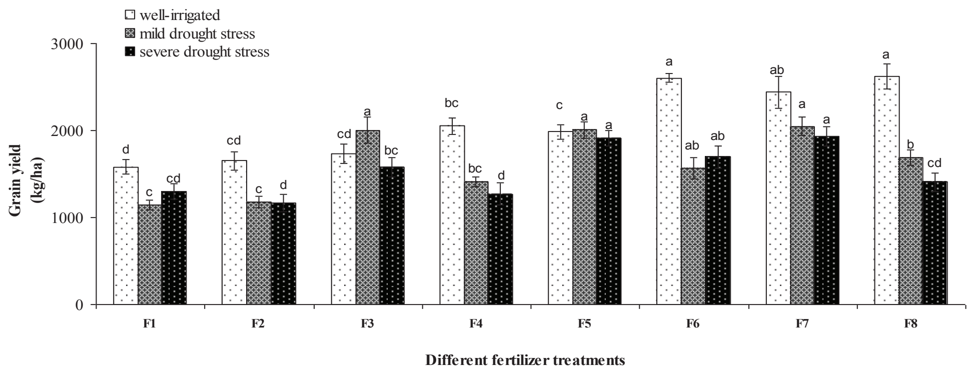

Figure 2: Interaction effect of irrigation treatments × fertilizer treatments on sunflower grain yield. In each irrigation treatments, means followed by the same letter are not significantly different ($P \leq 0.05$). Vertical bars indicate standard deviation (n = 3)

3.3 Harvest index

The harvest index was not affected by irrigation regimes and fertilizer treatments (Table 4). According to Hay and Porter (2006) harvest index has been reported to have lower variances so that in some crops the value has reached to its maximum. In a nutshell, any factor that reduces the biological yield can lead to reduction in grain yield and finally will causes weaker variation in harvest index.

3.4 Yield components

According to the results application of combined organic fertilizers (zeocompost, vermicompost, zeocompost + Z-ACM and vermicompost + Z-ACM) under well irrigation conditions produced more grain per head compared with other treatments (Fig. 3). It appears that the availability of N and other nutrients supplied from composts improves photosynthesis and increases assimilate production during reproductive growth stage. Nitrogen and other nutrients are released gradually from organic fertilizers and nourish plants during growing season. Slow release reduces the risk of nutrients leaching into waterways and support plants until the end of growing season. Under mild and severe drought stress the maximum grain number per head were found in those treatments in which more zeolite was applied (zeocompost, Z-ACM and zeocompost + Z-ACM) (Fig. 3). Zeolites can modify soils water content by altering the bulk density and total and aeration porosity (Nakhli et al., 2017). Bulk density is a basic soil physical property that can have an effect on the total porosity and topsoil stability, such that the bulk density of light-textured soils can be lowered with the application of zeolites (Ramesh et al., 2011). Furthermore, in sandy soils, zeolites application can lead to higher water holding capacities, which can be attributed to zeolites high pore volumes that enable them to hold more water in their structures (Ramesh et al., 2011). Other researchers have also found water retention or soil water contents to be greater in soils to which zeolite was applied. Bigelow et al. (2001) mixed 10 % zeolite with putting green sand and noted a 20 % increase in volumetric water content during the first year after putting green establishment as compared with unamended sand. Al-Busaidi et al. (2008) applied zeolite to sand at a rate of 5 kg m^{-2} (5 % by mass), reporting an increase in soil water content of approximately 2.5 % to 4.8 % (by mass), depending on water source, as compared with a control. In addition, zeolites can modify the soil hydraulic conductivity, a physical property of soil showing the easiness of water movements within the soil, due to the existence of channels within their structure (Nakhli et al., 2017). In heavy-textured soils, zeolites are able to increase the hydraulic conductivity, while in light-textured soil, they lower the hydraulic conductivity (Razmi & Sepaskhah, 2012). In a study clinoptilolite zeolite was added to four different soil textures including clay, loam, loamy sand, and sand. The results indicated that application of zeolite decreased the hydraulic conductivity of sandy and loamy soils. On the contrary, it increased the hydraulic conductivity of clay soil (Gholizadeh-Sarabi & Sepaskhah, 2013). Change in the hydraulic conductivity was highly attributed to change the average particle size of the soil (Mahabadi et al., 2007).

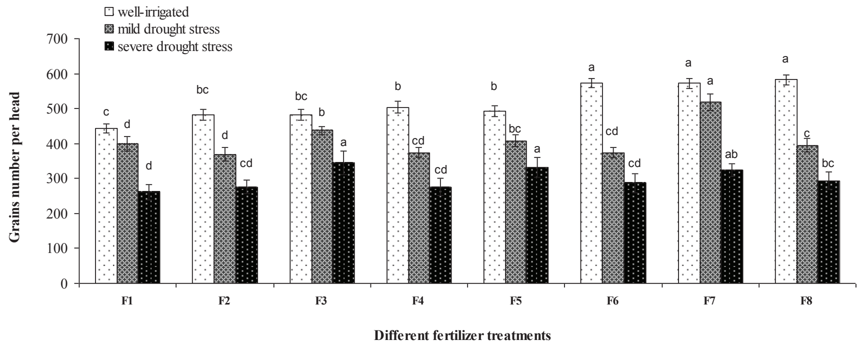

Figure 3: Interaction effect of irrigation treatments × fertilizer treatments on sunflower grain number per head. In each irrigation treatments, means followed by the same letter are not significantly different ($P \leq 0.05$). Vertical bars indicate standard deviation (n = 3)

Grain mass as an important yield component was significantly affected by irrigation treatments (Table 4). The maximum and minimum grain mass were related to well irrigated and severe drought stress plots, respectively (Table 5). Shorter grain filling period due to water deficit stress is the main reason for decreased grain mass (D' Andria et al., 1995). Among fertilizer treatments, combined organic treatments and chemical treatments produced the maximum and minimum grain mass, respectively (Table 5). Increase in grain mass has been found to be correlated with increase in nutrients uptake (Efeoğlu et al., 2008). According to Hay and Porter (2006) consistent N availability increases dry matter production and leaf area duration. So it is not surprising that organic fertilizers play a major in increasing grain mass.

3.5 Morphological characteristics

Drought stress significantly reduced plant height so that there were 18 and 31 % reduction when control treatment was compared with mild and severe drought stress treatments, respectively (Table 6). Reduction in plant height may be due to lower turgor pressure and diminished cell division on account of water deficit stress (Ogbonnaya et al., 2003). Similar results have been found by Kazi et al. (2002). From the results the tallest and shortest plants were observed when combined organic fertilizers (zeocompost + vermicompost, zeocompost + Z-ACM and zeocompost + Z-ACM) and combine chemical treatments (urea and urea + cattle manure) were applied, respectively (Table 6). Plant height is an index of vegetative growth. Considering this fact that N is constituent of proteins, N availability plays a key role in plant growth and cell expansion. The positive effect of N in stem elongation in sunflower has been reported by Khaligh and Cheema (2005). In addition, increase in plant height might be due to application of different organic fertilizers at the same time. For example, Z-ACM provides required N during early growth stages and vermicompost improves growth through increasing vitamins and plant growth regulators into the soil. On the other hands, the application of organic fertilizer improves soil physical and chemical properties and increases root activity, leading to enhancement of plant N absorption (Gholamhoseini et al., 2013).

Stem diameter decreased due to mild and severe drought stress (Table 6). Similar results have been reported by Kazi et al. (2002). Stem diameter was at the minimum limit when urea was applied (Table 6). From the other side, the maximum stem diameter was observed when vermicompost + Z-ACM treatment was used (Table 6). Nutrients availability in combined organic treatments improves photosynthesis and assimilates production which finally leads to more vegetative growth. Stem diameter affect yield and yield components so that plants with thicker stems produce more grain yield.

The results indicated that the maximum and minimum head diameters were related to well-irrigated and drought stressed plots (Table 6). Head diameter is not only controlled by environmental factors such as soil moisture, but also by genetic properties. Water availability during reproductive stage has critical role in increasing yield via increasing assimilates production and sink size such as grain size and head diameter (Tarantino & Alba, 1979). According to the results application of combined organic fertilizers could increase head diameter (Table 6).

Table 6: Main effects of irrigation regimes and fertilizers systems on sunflower morphological and qualitative characteristics

Treatments	Plant height (cm)	Stem diameter (mm)	Head diameter (cm)	Grain oil (%)	Grain protein (%)
Irrigation regimes					
I1	159 a	23.5 a	24.4 a	45.7 a	19.6 c
I2	130 b	20.5 b	21.9 b	44.2 b	21.6 b
I3	109 c	19.1 c	19.3 c	41.9 c	23.5 a
Fertilizers systems					
F1	103 c	18.3 d	19.2 c	42.3 de	22.1 b
F2	105 c	19.9 bc	19.8 c	41.9 e	23.2 a
F3	124 b	19.7 c	22.0 ab	44.3 bc	22.2 ab
F4	127 b	20.6 bc	21.1 bc	43.7 cd	21.4 b
F5	129 b	20.9 b	21.3 ab	44.8 bc	21.2 bc
F6	174 a	22.5 a	22.8 ab	43.6 cd	19.6 c
F7	137 a	23.0 a	24.5 a	45.3 ab	22.1 ab
F8	164 a	23.4 a	22.7 ab	45.9 a	20.9 bc

Means in columns followed by the same letters are not significantly different at $p < 0.05$ using LSD test. I1: Irrigation after depleting 30 %, I2: Irrigation after depleting 50 %, I3: Irrigation after depleting 70 % of filed capacity. F1: urea, F2: urea + composted cattle manure, F3: zeocompost, F4: vermicompost, F5: Z-ACM, F6: zeocompost + vermicompost, F7: zeocompost + Z-ACM and F8: vermicompost + Z-ACM

3.6 Grain quality

The maximum grain protein content was obtained from severe drought stress treatment, whereas the minimum value was found in well-irrigated plots (Fig. 4). It has been reported that higher temperature and lower soil moisture content during growing season decrease grain mass and oil percentage (Mirales, 1997). It has been reported that oil content would increase with increasing soil available water content (Wang et al., 2003). Reduction in oil percentage because of drought stress might be due to alterations in seeds metabolisms and/or assimilates transfer into the seeds. In fact, drought stress, especially at seed filling stage, reduces oil percentage but increases protein percentage. Under well-irrigation conditions, grain oil percentage was higher in plants which were treated with organic fertilizer containing vermicompost (vermicompost, vermicompost + zeocompost and vermicompost + Z-ACM) so that oil percentage in these treatments increased by 12, 12 and 16 %, respectively, compared with chemical treatment (Fig. 5). From one side, slow release of N and other nutrients from vermicompost and from the other side improving soil physical properties by organic fertilizers could improve plant growth, photosynthesis and finally oil synthesis. Similar results have been found by Arancon et al. (2007). Liu et al. (2004) has reported that organic fertilizers increase seed oil percentage through extending seed filling period and increasing leaf area duration. The results revealed that the minimum oil percentage was related to chemical treatment in which urea was individually applied. Under mild and severe drought stress, the maximum oil percentage was obtained from treatments in which more zeolite was applied i.e. zeocompost, Z-ACM and zeocompost + Z-ACM (Fig. 5). It has been reported that zeolite improves water retention capacity and provides more water for the plants (Gholamhoseini et al., 2013).

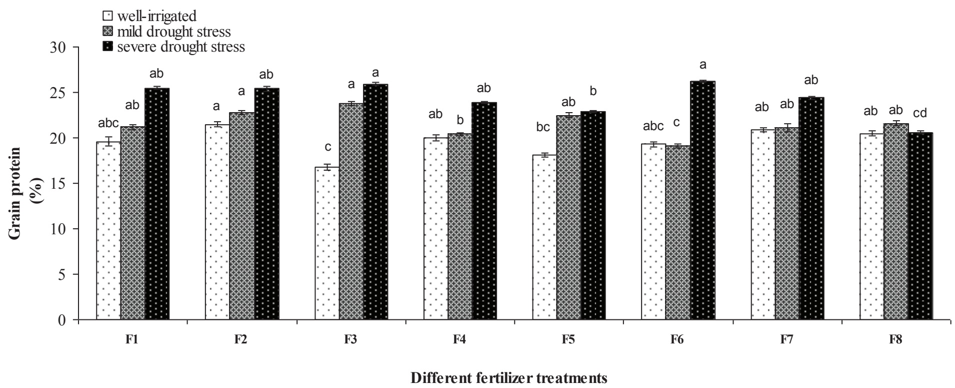

Figure 4: Interaction effect of irrigation treatments × fertilizer treatments on sunflower grain protein content. In each irrigation treatments, means followed by the same letter are not significantly different ($P \leq 0.05$). Vertical bars indicate standard deviation ($n = 3$)

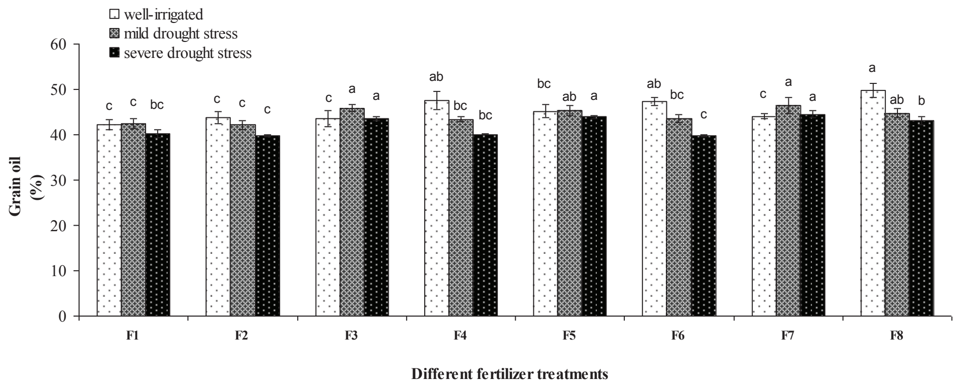

Figure 5: Interaction effect of irrigation treatments × fertilizer treatments on sunflower grain oil content. In each irrigation treatments, means followed by the same letter are not significantly different ($P \leq 0.05$). Vertical bars indicate standard deviation ($n = 3$)

4 CONCLUSIONS

This study compared the effects of different irrigation regimes associated with different fertilizer treatments under semiarid climatic conditions on various aspects of sunflower production. The results herein clearly indicate that the application of combined organic fertilizers with 1600-2200 kg ha^{-1} zeolite, F6 and F8 treatments, was considerably more effective than the chemical treatment (F1) or the integrated treatment without zeolite (F2) for improving most quantitative and qualitative sunflower traits. According to the obtained results, combined organic fertilizers, especially zeocompost + vermicompost, zeocompost + Z-ACM or vermicompost + Z-ACM, are recommended to use under well-irrigation conditions in sunflower production. Moreover, under drought stress conditions, application of zeolite amended organic fertilizers is highly recommended. Overall we concluded that soil amending with combined organic fertilizers and zeolite can be a beneficial approach for decreasing chemical fertilizer application rates and improving the sustainability of agricultural systems.

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Comparative growth and physiological responses of tetraploid and hexaploid species of wheat to flooding stress

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ABSTRACT

Present study is aimed to comparatively investigate the response of two ploidy levels of wheat including a tetraploid (*Triticum turgidum* L.) and a hexaploid (*Triticum aestivum* L.) wheat to different durations of flooding stress. Wheat seedlings were exposed to flooding stress for 0, 3, 6 and 9 days. Results showed that all flooding treatments significantly decreased the shoot and root length, and chlorophyll content of both species of wheat. The decrease in chlorophyll content of tetraploid wheat was more than that of hexaploid one. In both species, ADH activity of root was significantly increased under flooding stress, where the increase was more in hexaploid wheat. Flooding stress did not significantly affect root and shoot water content, root porosity, and shoot protein content of any wheat species. Tetraploid and hexaploid wheat used different mechanisms for better tolerance of flooding condition, where tetraploid wheat increased the proline content but in hexaploid wheat, an increase in soluble sugar content was observed.

Key words: flooding stress; polyploidy; soil water; waterlogging

IZVLEČEK

PRIMERJALNI RASTNI IN FIZIOLOŠKI ODZIVI TETRAPLOIDNE IN HEKSAPLOIDNE PŠENICE NA POPLAVNI STRES

V raziskavi je bil primerjalno preučevan odziv tetraploidne (*Triticum turgidum* L.) in heksaploidne pšenice (*Triticum aestivum* L.) na različno trajanje poplavnega stresa. Sejanke pšenice so bile izpostavljene poplavnemu stresu za 0, 3, 6 in 9 dni. Rezultati kažejo, da so vsa obravnavanja s poplavitvami značilno zmanjšala dolžino poganjkov in korenin ter vsebnost klorofila pri obeh vrstah pšenice. Zmanjšanje klorofila je bilo večje pri tetraploidni kot pri heksaploidni pšenici. Pri obeh vrstah se je v razmerah poplavnega stresa aktivnost alkohol dehidrogenaze (ADH) v koreninah značilno povečala, a bolj pri heksaploidni vrsti. Poplavni stres ni značilno vplival na vsebnost vode v koreninah in poganjkih, niti na poroznost korenin in vsebnost beljakovin pri obeh vrstah. Tetraploidna in heksaploidna pšenica sta uporabili različne mehanizme tolerance na poplavne razmere, tetraploidna s povečanjem vsebnosti prolina, heksaploidna s povečanjem vsebnosti topnih sladkorjev.

Ključne besede: poplavni stres; poliploidija; talna voda; zalitje z vodo

1 INTRODUCTION

Waterlogging is defined as prolonged soil saturation with water, at least 20 % higher than the field capacity (Aggarwal et al., 2006). Anaerobic root respiration can induce an accumulation of potentially toxic metabolites such as ethanol, lactic acid, acetaldehyde and cyanogenic compounds. For instance, the accumulation of lactic acid can induce cytosolic acidosis, resulting in the cell death (Kozlowski, 1997; Liao and Lin, 2001; Jackson, 2002; Ashraf, 2012). Since gas diffuses about 4 times more slowly through water than through air, a reduced supply of oxygen during waterlogging may result in halting of growth and survival of many species (Drew, 1997). This is because most tissues of higher plants could not survive under anaerobic condition (Taiz and Zeiger, 2010).

Plants respond to flooding stress by different mechanisms. They have genetic differences in term of the tolerance to waterlogging (Fausey et al., 1985; Vantoai et al., 1988; Davies and Hillman, 1988). Short-

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term acclimation including biochemical mechanisms may decrease the negative impacts of flooding (Vartapetian and Jackson, 1997). However, some plants such as wetland species are adapted to flooding due to the specialized structures such as aerenchyma and structural barriers to prevent O_2 diffusion outward to the soil (Vartapetian and Jackson, 1997; Bacanamwo and Purcell, 1999; Taiz and Zeiger, 2010). Acclimation to the anaerobic conditions may involve the expression of anaerobic stress genes, mainly the enzymes of the glycolytic and fermentation pathways such as alcohol dehydrogenase (Taiz and Zeiger, 2010).

Wheat is one the most important crops that was domesticated approximately 10,000 years ago. Wheat is a polyploid plant, originated by hybridization between different species (allopolyploidy) (Dubcovsky and Dvorak, 2007). Hexaploid wheat was formed by hybridization of a domesticated form of allotetraploid wheat (*Triticum turgidum* L.) and a diploid goat grass *Aegilops tauschii* Coss. With an increase in ploidy level of plants, their physiological performance and their tolerance to biotic and abiotic stresses may be improved (Yang et al., 2014). For instance, it has been reported that hexaploid wheat has greater physiological and ecological plasticity than its tetraploid and diploid progenitors (Dubcovsky and Dvorak, 2007).

The results of present study may have great significance for wheat farming in frequently waterlogged areas. Beside, these results could be used in further agricultural studies on the response of plants to anaerobic condition. Present study aimed to comparatively investigate the response of two ploidy levels of wheat including a tetraploid and a hexaploid wheat to different durations of flooding stress.

2 MATERIALS AND METHODS

2.1 Plant material and treatment conditions

Seeds of a tetraploid (*Triticum turgidum* 'Oshnavie') and a hexaploid (*Triticum aestivum* 'Sardari') wheat were obtained from the Agricultural Research Center of Urmia, West Azarbayjan, Iran. Seeds were surface sterilized with 5 % (v/v) sodium hypochlorite solution for 10 min (Mousavi Kouhi et al., 2014), rinsed with tape water, and imbedded for 12 h. The seeds were placed in Petri dishes containing moist filter paper and were kept in a dark incubator for 72 h at 27 °C. Then, 8 seedlings were sown in each plastic pot (13 × 16 cm) containing vermiculite. The seedlings were grown in a growth chamber under controlled condition (26 ± 2 and 24 ± 2 C° in condition of 16 h of light and 8 h of dark, respectively). Seedlings were independently flooded for 0, 3, 6 and 9 days. For this purpose, plastic pots were water-filled up to 18 mm above the bed. Control plants were normally irrigated with tap water according to the field capacity (Jamei et al., 2008). At the end of each treatment the roots and shoots of seedlings were harvested separately and then the growth criteria and biochemical and physiological changes of the roots and shoots tissues were investigated.

2.2 Determination of root porosity and water content of root and shoot

Root air space was measured by a pycnometer method (Jensen et al., 1969). Water content of root and shoot was determined as WC = FM - DM/ FM in which WC, FM, and DM indicate water content, fresh mass, and dry mass, respectively.

2.3 Determination of chlorophyll

Chlorophyll a and b content were measured in the fresh shoots after extraction with 80 % acetone, according to the spectrophotometric method (Lichtenthaler and Wellbum, 1983). Chlorophyll content was calculated as mg chlorophyll per g fresh leaf.

2.4 Determination of proline and protein content of shoot

Proline content was determined according to the Bates et al. (1973). Protein content was measured as recorded by Lowery et al. (1951). Two separate curves were prepared by different concentrations of proline and egg albumin for determination of proline and protein content, respectively.

2.5 Determination of soluble sugar

Phenol-sulfuric method was used to determine soluble sugars. After homogenizing 0.5 g of roots or shoots, 2 ml from each sample was taken, and then 1 ml 5 % phenol and 5 ml 98 % sulfuric acid was added. After coolness and complete color emergence of the solutions, sugar contents were maintained spectrophotometrically at 485 nm (Hsu et al., 2000).

2.6 Assay of alcohol dehydrogenase (ADH) activity of root

After preparing enzyme extract using 0.1 M Tris-HCl (pH = 8), 0.1 M NADH, 1 mM EDTA, 5 mM sodium borate, 5 mM dithiothreitol, and 10 % glycerol (Hoffman et al., 1986) ADH activity were determined in a 3 ml reaction mixture including 1 ml of 0.5 M Tris-

HCl (pH = 8), 0.5 mM NAD, 40 mM ethanol, and 15 µl enzyme extract (Donaldson et al., 1985).

2.7 Statistical Analysis

A factorial experiment was conducted as completely randomized design with three replicates. Significant differences (at $p \leq 0.05$) and means comparison by Duncan's multiple range test was determined using Mstat-C software.

3 RESULTS

3.1 Growth traits and water content of root and shoot

Results showed that all flooding treatments significantly decreased the shoot and root length of both tetraploid and hexaploid wheat (Figure 1a & b). In both species, root growth was more affected by flooding than shoot growth. For instance, while under 9 days flooding the root length of *T. turgidum* and *T. aestivum* was decreased by 55 and 51 %, respectively, their shoot length was decreased by 17 and 23 %, respectively, compared to their controls. However, shoot and root length of both wheat showed a similar response to flooding stress. Results showed that root and shoot water content of both ploidy levels of wheat was not significantly affected by any level of flooding, compared to their controls (Figure 1c & d).

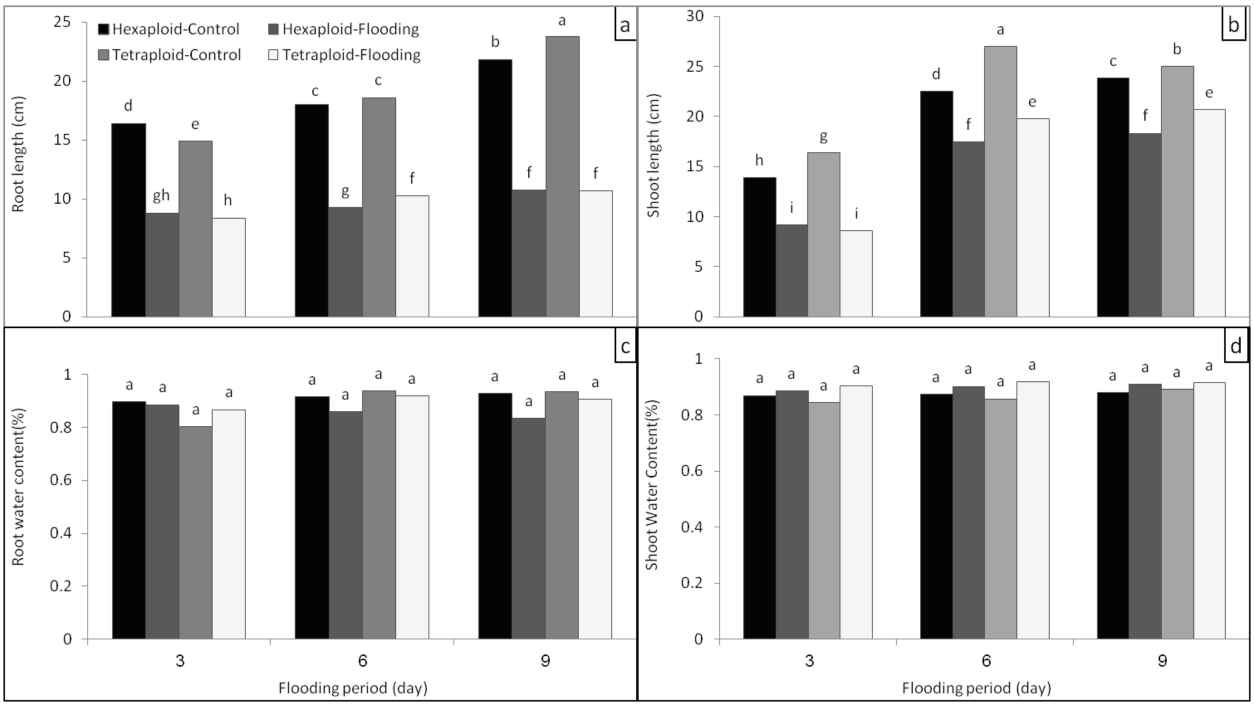

Figure 1: The effect of flooding stress on root (a) and shoot (b) length, and water content of root (c) and shoot (d) of the seedlings of tetraploid and hexaploid wheat. Different letters indicate significant differences ($P < 0.05$).

3.2 Chlorophyll a and b content

The pattern of changes in chlorophyll a and b (mg per g fresh mass) in response to different periods of flooding stress was similar. The flooding stress significantly reduced chlorophyll a and b content of both species during all flooding periods (Figure 2a & b). However, the decrease in chlorophyll a and b content of tetraploid wheat was more than that of hexaploid one. For instance, while chlorophyll a content of tetraploid wheat under 3, 6, and 9 days flooding was decreased by 61.50, 46.53, and 55.28 %, respectively, that of hexaploid wheat was decreased by 34.42, 22.40, and 23.87 %, respectively.

3.3 ADH activity and root porosity

In root of both ploidy levels of wheat, ADH activity was significantly increased under all periods of flooding, compared to the control (Figure 2c). The increase in ADH activity in root of hexaploid wheat was more intensive than that of tetraploid one. For instance, while under 3, 6, and 9 days flooding the root ADH activity of hexaploid wheat showed a 38-, 37-, and 62-fold increase over control, respectively, that of tetraploid was increased 33-, 51-, and 57-fold relative to control, respectively. Flooding stress did not also significantly affect root porosity of any species of wheat, when each treatment compared with its control (Figure 2d).

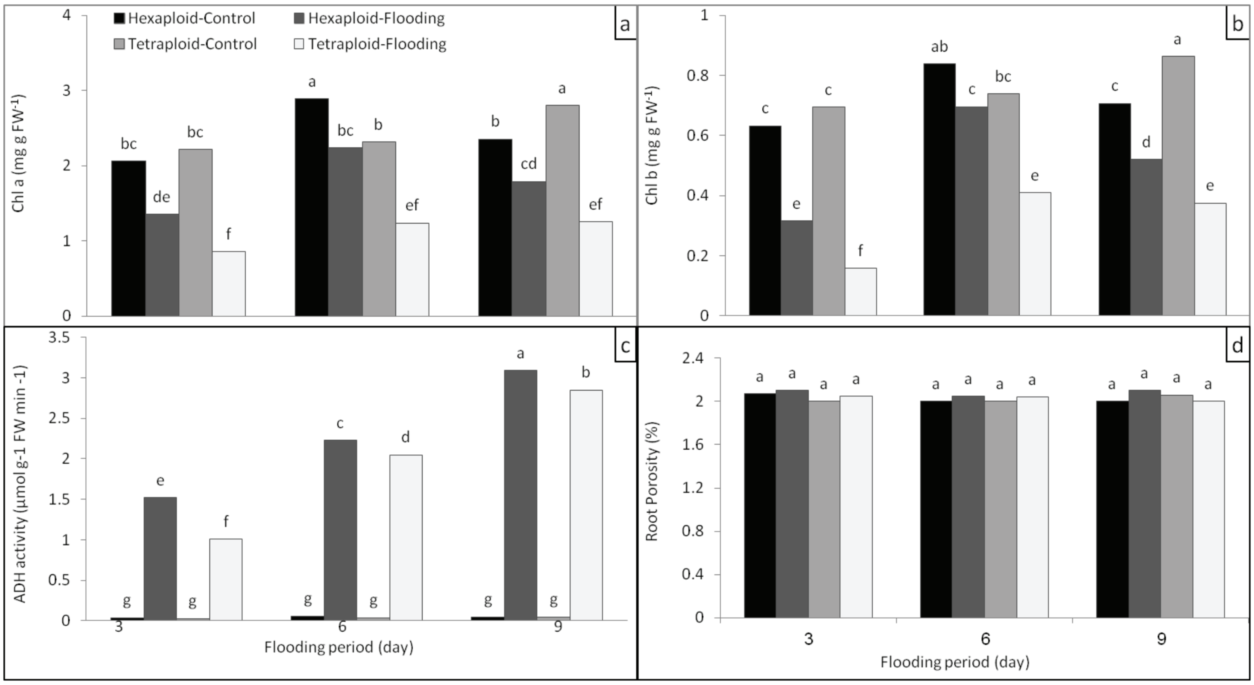

Figure 2: Changes in chlorophyll a (a) and chlorophyll b (b) content, ADH activity of root (c), and root porosity (d) of the seedlings of tetraploid and hexaploid wheat under flooding stress. Different letters indicate significant differences ($P < 0.05$).

3.4 Proline and protein content of shoot

Flooding stress did not significantly affect shoot protein content of tetra- and hexaploid (Figure 3a). In contrast to hexaploid that proline content of shoot did not show any significant change compared to control, in tetraploid wheat it was significantly increased under all periods of flooding (Figure 3b). Shoot of tetraploid seedlings grown under 9 days flooding had the highest amount of proline relative to other treatments so that it showed a 42 percent increase relative to its control.

3.5 Soluble sugars content

The soluble sugar concentration in shoots of both tetraploid and hexaploid wheat was significantly decreased compared to control during all flooding treatments, except for that of hexaploid wheat under 9 days flooding. Under 3 days flooding, the concentration of soluble sugars in roots of both species was increased that was significant in case of hexaploid. Soluble sugar content in root of tetra- and hexaploid was not significantly changed under 6 and 9 days flooding, except that it was decreased in tetraploid under 6 day flooding (Figure 3c & d).

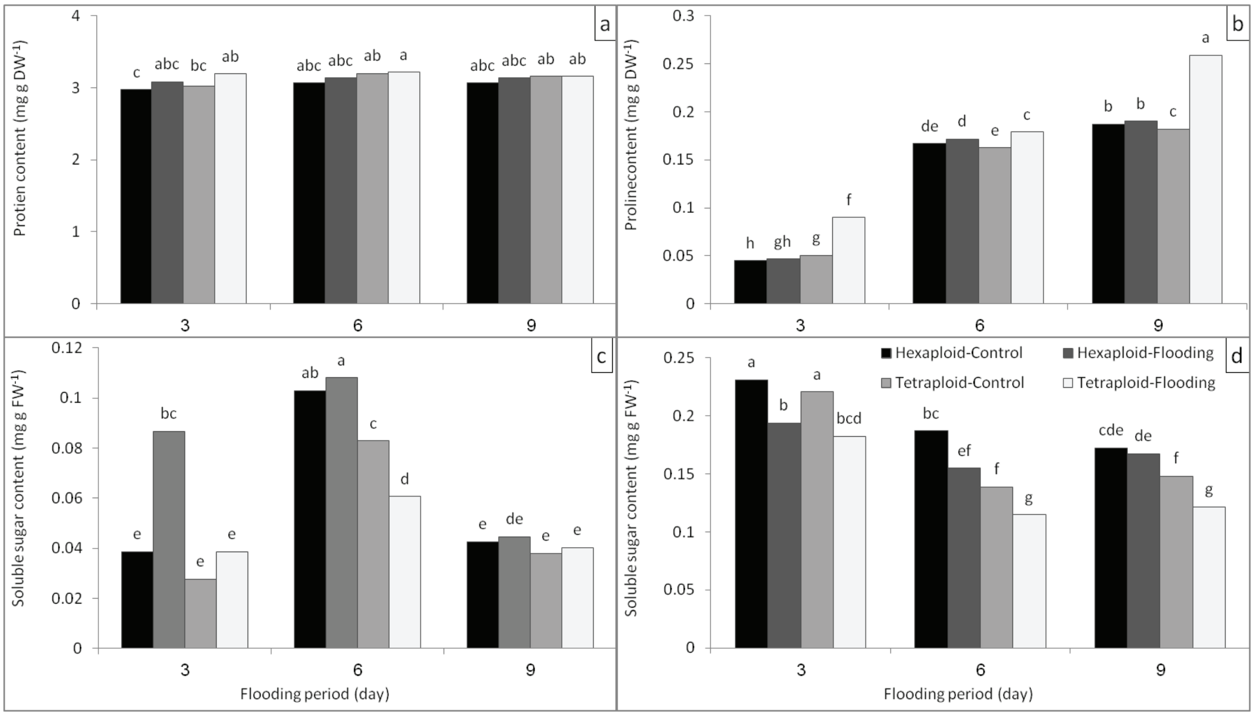

Figure 3: Changes in protein (a) and proline (b) content of shoot, and soluble sugar content of root (c) and shoots (d) of the seedlings of tetraploid and hexaploid wheat. Different letters indicate significant differences ($P < 0.05$)

4 DISCUSSION

Changes in the growth under flooding condition were reported in many plants such as pea, maize, and winter wheat (Przywara and Stepniewski, 2000; Lizaso et al., 2001; Huang et al., 1994). Under flooding condition, aerobic respiration and cellular ATP production can be reduced resulting in lack of energy to support physiological processes led to the reduced growth and crop yield (Fukao and Bailey, 2004; Taiz and Zeiger, 2010). It was reported that some plants such as wheat failed to absorb nutrient elements by roots and transport them to the shoot, resulting in mineral deficiencies that can subsequently led to the disrupting plant growth and development (Taiz and Zeiger, 2010).

Under flooding condition, net photosynthesis may also be suppressed by several mechanisms such as decrease in chlorophyll content that was observed in present study, resulted in a decrease in growth and development. In a study, barley plants exposed to the flooding for 3 days showed a remarkable decrease in photosynthesis and dry matter accumulation (Yordanova and Popova, 2001). Flooding can generally accelerate leaf senescence due to the chlorophyll degradation by a peroxidase-mediated mechanism (Hurng and Kao, 1994).

Under moderate flooding conditions, the concentration of soluble sugars in roots of hexaploid wheat was increased. This may be one of the mechanisms related to its relatively greater waterlogging-tolerance than tetraploid one. A genotype with a sufficient root sugar levels has a better chance of survival under anaerobic condition (Sairam et al., 2008). Under anaerobic condition, maintaining an adequate concentration of fermentable sugars in roots can help plants for long-term survival. In water-saturated soils, plants capable of converting the residual starch to fermentable carbohydrates are generally more tolerant to flooding stress (Guglielminetti et al., 2001; Ismail et al., 2009). However, the reduction of soluble sugars in shoot sugar content of both wheat species could be related to the decline in photosynthesis under flooding condition. Decline in photosynthesis under anaerobic condition has been observed in other cultivars of wheat (Huang et al., 1994).

Developing aerenchyma, a tissue having gas-filled spaces, in roots of wetland plants can help them to tolerate anaerobic condition. However, aerenchyma can be developed in the stem base and newly developing roots (Taiz and Zeiger, 2010). Nevertheless, in present study the root porosity of any species of wheat was not affected by flooding. This may be indicate that formation of aerenchyma is not a tolerating mechanism under flooding or may be due to this fact that present study were conducted in the seedling stage so that plants did not have sufficient time to develop aerenchyma. Verifying later speculation, in a study on two wheat genotypes, root porosity was increased after treating plants under flooding for 21 days (Huang and Johnson, 1995). Flooding-tolerant species such as *Rumex* sp. tend to develop root porosity (Laan et al., 1989). Flooding - tolerant species of clover such as *Trifolium fragiferum* L. have more root porosity than sensitive ones such as *T. glomeratum* L. (Gibberd et al., 1999).

Shoot proline content of tetraploid wheat was significantly increased under flooding stress. Proline is one of the compatible solutes that its synthesis increased under different stresses. This amino acid has a high degree of solubility (Buchanan et al., 2000). Besides having a role in osmotic adjustment, proline can act as a protective factor against stress by directly or indirectly interaction with macromolecules and maintaining their natural shape and structure under stress condition. Compared to other osmoprotective solutes, proline is more efficient under stress condition. Proline also has an indirect role against stress due to its antioxidative function (Aubert et al., 2004). Proline act as an antioxidant factor via suppressing the hydroxyl radicals and some of compounds produced under stress condition that disturb electron transfer in chloroplast and mitochondria (Sousa and Sodek, 2002).

Increase in ADH activity under anaerobic condition was reported to be due to an early increase in the lactate concentration as a signal in the early stage of flooding condition. This interaction between lactic and ethanolic fermentation can lead to the cytoplasmic pH (Roberts et al., 1984). Consistent with this, accumulation of ethanol was reported in the endosperm of castor bean under anaerobic condition (Donaldson et al., 1985). Induction of ADH activity in roots of barely under hypoxia (Good and Crosby, 1989) and in that of soybean under anoxia (Neuman and Van Toai, 1991) has been reported.

Different responses of wheat species with different ploidy levels to environmental stresses have been reported by many researchers. It was known that, hexaploid *T. aestivum* has better tolerance to different environmental stresses such as salt, low pH, and aluminum toxicity, and also better resistance to several pests and diseases, compared to tetraploid wheat. This is because of the fact that in the new allopolyploid species different genomes with potential of adaption to different environments are converged, leads to the potential of new emerged cultivars for tolerating a wide range of environmental conditions (Dubcovsky and Jan Dvorak, 2007). Li et al. (2017) have investigated photosynthetic response of tetraploid and hexaploid wheat to water stress and concluded that the effects of water stress on the photosynthetic performance were species dependent. Their results showed the improved performance of tetraploid wheat ears under water stress, attributed by researchers to its more efficient water utilization. Consistent with our study, in another work on different responses of hexaploid and tetraploid wheat to drought stress it was showed that under drought stress, proline accumulation in hexaploid wheat was more than that of tetraploid one (Chandrasekar et al., 2000).

5 CONCLUSIONS

In summary, flooding stress induced some adaptive responses in physiological levels such as increase in proline content, soluble sugar, and ADH activity of the investigated wheat species in which some responses were different. In tetrapoid wheat, an increase in proline content seemed to be one of the mechanisms for tolerating flooding stress. In hexaploid wheat, the increase in soluble sugar was a defense mechanism for better tolerance to moderate flooding stress.

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Allelopathic effect of two medicinal plants on seed germination, seedling growth and grain production of purslane *(Portulaca oleraceae* L.) weed

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ABSTRACT

A laboratory factorial (2 × 5) experiment was carried out based on completely randomized block in four replications to evaluate the effect of plant species and concentration of their allelopathic extracts on seed germination and seedling growth of purslane weed (*Portulaca oleraceae* L.). The first factor studied was plant species (*Artemisia sieberi* and *Salvia syriaca*) and the second one the concentration percentage of plant ethanol extract (0.0, 5 %, 10 %, 15 % and 20 %). The effect of *Artemisia* on germination reduction of purslane was stronger compared to *Salvia.* The results indicated that higher extract concentration led to decreased germination percentage, germination speed and seedling growth indices of purslane including leaf length, leaf number, leaf width, leaf dry mass, stem mass and stem length. The effect of *Artemisia* was higher than that of *Salvia*. In a complementary experiment, the effect plant residues of *Artemisia* and *Salvia* (0.0, 1, 2, 3 and 4 g kg^{-1} soil) were evaluated in a factorial experiment base on completely randomized block design with three replications. The results of field experiment showed that plant residues significantly ($P \leq 0.01$) reduced capsule number per plant, seed number per capsule, seed production and shoot dry mass, while its effect on 1000-seed mass was not substantial. The effect of plant species and the interaction of plant species and their allelopathic extracts concentration had no major impact on the above-mentioned properties. The increase in plant residues in soil, led to the reduction of growth and seed production of purslane induced by plant number per unit area and capsule per plant. *Artemisia sieberi* Bess*.* and *Salvia syriaca* L. residues can be successfully used for non-chemical control of purslane weed.

Key words: allelopathy; *Artemisia sieberi* Bess.; *Salvia syriaca* L.; germination speed; seedling growth; seed production

IZVLEČEK

ALELOPATSKI UČINEK DVEH ZDRAVILNIH RASTLIN NA KALITEV SEMEN, RAST SEJANK IN PRODUKCIJO SEMEN NAVADNEGA TOLŠČAKA *(Portulaca oleraceae* L.)

Za ovrednotenje učinka dveh rastlinskih vrst in koncentracije njunih alelopatskih izvlečkov na kalitev semen in rast sejank navadnega toščaka (*Portulaca oleraceae* L.) je bil izveden laboratorijski bločni faktorski poskus s štirimi ponovitvami. Prvi preučevani dejavnik sta bili rastlinski vrsti (*Artemisia sieberi* in *Salvia syriaca*), drugi koncentracija njunega etanolnega izvlečka v odstotkih (0,0, 5 %, 10 %, 15 % in 20 %). Učinek pelina (*Artemisia*) na zmanjšanje kalitve tolščaka je bil močnejši v primerjavi z učinkom kadulje (*Salvia*)*.* Izsledki so pokazali, da so večje koncentracije izvlečka vodile k zmanjšanemu odstotku kalitve, manjši hitrosti kalitve in k zmanjšanju rastnih parametrov sejank tolščaka kot so dolžina in širina lista, število listov, suha masa listov in stebla in dolžina stebla.V komplementarnem poljskem poskusu so bili učinki ostankov obeh vrst (0,0, 1, 2, 3 in 4 g kg^{-1} tal) ovrednoteni v faktorskem bločnem poskusu s tremi ponovitvami. Rezultati tega poljskega poskusa do pokazali, da so ostanki obeh vrst značilno ($P \leq 0.01$) zmanjšali število glavic na rastlino, število semen na glavico, produkcijo semena in suho maso poganjkov tolščaka, a vpliv na maso 1000-semen ni bil značilen. Učinek rastlinske vrste in njene interakcije s koncentracijo alelopatskih izvlečkov na zgoraj navedene parametre v tem poskusu ni imel večjega vpliva. Povečevanje ostankov obravnavanih vrst v tleh je vodilo k zmanjšani rasti in zmanšani produkciji semena navadnega tolščaka zaradi zmanjšanja števila rastlin na enoto površine in zmanšanega števila glavic na rastlino. Ostanki obeh vrst (*Artemisia sieberi* Bess., *Salvia syriaca* L.) bi se lahko uspešno uporabljali za nekemično zatiranje navadnega tolščaka.

Ključne besede: alelopatija; *Artemisia sieberi* Bess.; *Salvia syriaca* L.; hitrost kalitve; rast sejank; produkcija semena

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1 INTRODUCTION

Weeds compete with crops for environmental resources such as solar radiation, soil moisture and nutrient, leading to the reduction of crop growth and yield (Cavigelli et al., 2009; Gherekhloo et al., 2010). Therefore, the initial goal of weed management in agroecosystems is reducing their negative effects on crop plants (Holander et al., 2007). Although chemical control is a simple and effective weed control measure, increased weed resistance to herbicides, public health and negative consequences of herbicides uses on environment such as surface water pollution and harmful to other organisms, concern the increased use of herbicides in production systems as well (Demden & Liewellyn, 2006; Yuan-Quan et al., 2012). For this reason, an effort to eliminate the use of synthetic herbicides has been a growing trend. However, utilizing some non-chemical methods have limitations. Use of mechanical methods, rely heavily on the availability of machinery, whereas hand weeding cannot be implemented in all plants and needs a lot of labor as well (Kropff & Walter, 2000). The use of a method that can reduce competitive ability and growth and development of weed seedlings at early growth stages has been emphasized (Holander et al., 2007). Employing allelopathic properties of medicinal plants is a way to achieve this goal (Ghomi & Tavili, 2012).

Allelopathic compounds, which can be applied as non-synthetic chemical control of weeds, provide the possibility of introducing a new generation of weed inhibitors, reducing the costs of crop production (Ghorbanli et al., 2008). According to International Allelopathy Association, allelopathy is defined as any process in which the secondary metabolites produced by plants affect the growth and development of biological systems (Gholami et al., 2011). The leaves and roots are important sources of allelopathic compounds (Jefferson & Pennachio, 2003). However, all parts of the plant may have allelopathic compounds. For example, Samad et al., 2008 observed that the extracts of all parts of five weed species had allelopathic effect on maize and reduced its growth and yield (Samad et al., 2008).

Portulaca oleraceae L., hereafter referred to as 'purslane', is a summer weed from Portulacaceae family that is able to grow in different environmental conditions. In some crop plants, such as sesame, bean and mung bean whose early seedling growth is slow, purslane damage is high, indicating the importance of timely purslane control. Purslane population can be reduced through allelopathic effects of medicinal plants. *Salvia syriaca* L., hereafter referred to as 'salvia', is a biennial herbaceous plant which has allelopathic compounds (Hassanpour & Azizi, 2007). *Artemisia sieberi,* Bess, hereafter referred to as 'artemisia', is a perennial herb from Asteraceae family with the height of 25-50 cm whose secondary metabolites prevent it from grazing by livestock (Moghimi, 2005). It has been reported that many herbaceous species are not able to grow in the vicinity of *Artemisia sieberi* (Ghorbanli et al., 2008).

Researches have shown that medicinal plants have allelopathic properties. Ghomi & Tavili (2012) reported that *Salvia sclarea* L. extract decreased germination and seedling growth of blue grass (*Bromus tectorum* L.), where higher concentration of plant extract showed stronger inhibitory effect on the weed growth. They also indicated that allelopathic effect of roots was higher compared to shoot extract. Behdad et al., (2008) showed that the reduction of *Bromus* germination resulted from allelopathic effects of *Artemisia sieberi*. Jafarpour et al., (2011) evaluated the allelopathic effect of residue powder of *Salvia syriaca* and *Artemisia sieberi* on bean and observed that bean germination and seedling growth were reduced. Germination percentage and speed as well as stem length and root length of orchard grass (*Dactylis glomerata* L.) were also negatively affected by *Artemisia sieberi* extract (Gholami et al., 2011).

The present experiment aimed at assessing the allelopathic effects of extract and whole plant remains of *Artemisia sieberi* and *Salvia syriaca* on germination, seedling growth and grain production of purslane.

2 MATERIALS AND METHODS

The current study was conducted in both laboratory and field. In the laboratory test, *Artemisia sieberi* and *Salvia syriaca* extract was prepared in the concentrations of 0.0 (control), 5.0 %, 10.0 %, 15.0 % and 20.0 % and their effects on germination properties of purslane weed (including germination speed, germination percentage and seedling growth) were evaluated. A completely randomized design with four replications was employed. After collecting and drying, the samples (whole plant parts of *Artemisia sieberi* and *Salvia syriaca*) were ground by electrical mill. Afterwards, 100 grams of ground sample were placed in a two-liter beaker and 1000 ml of ethanol (96.5 %) were added to it and stirred with a shaker for 24 hours. The obtained solution was passed through a filter paper and its alcohol was evaporated by a rotavapor. Next, different concentrations (according to experimental samples) were prepared using distilled water and kept refrigerated (Samadani & Baghestani, 2001).

Seeds of purslane were disinfected with sodium hypochlorite solution (1 %) to prevent fungal damage. Subsequently these seeds were rinsed three times with distilled water for 20 minutes. To evaluate seed germination under different concentrations of *Artemisia sieberi* and *Salvia syriaca* extracts, 25 seeds were placed in petri dishes containing a filter paper (every replication included 100 seeds in four petri dishes). Five ml of different concentrations were added to each petri dish. Distilled water was used as control treatment. The samples were then placed in a growth chamber under the conditions of darkness and 25 °C temperature. The seeds were classified as germinated when root protrusion occurred as much as 2 mm. Germination speed was determined, using the following equation:

$$R = \frac{\sum n}{\sum (D.n)}$$

Where, n is the number of germinated seeds on day D, and D is the number of days from the start of the test. Seedling grew for 7 days after germination occurrence. To evaluate the effect of two medicinal plant extracts on seedling growth properties of purslane, leaf length, leaf width, leaf number, leaf mass, stem length and stem mass were measured.

Supplementary experiment was carried out in an experimental field in Shushtar (Latitude 32°30/N, Longitude 48°20/E, and altitude 150 m above sea level), Iran during 2014-2015 growing season. The experiment was factorial (2 × 5) and based on randomized complete block design with four replications. The treatments included medicinal plant species (*Artemisia sieberi* and *Salvia syriaca*) and the amount of residue mixed with soil (0.0, 1.0, 2.0, 3.0 and 4.0 g kg^{-1} soil). The previous crop of experimental site was wheat. After harvesting and field plowing, wheat remains were removed from the field. Purslane seeds (6 plants m^{-2}) were sowed in the depth of 0.5 cm. Each plot contained 6 rows with the length of 2 m. The distance between two consecutive rows was 20 cm (Farahmand, 2011). After four months, purslane was harvested. For this purpose, 15 plants were harvested from middle rows. The plants were placed in a plastic bag and were transported to laboratory. After that, shoot dry mass, plant number per unit area, seed number per capsule, capsule number per plant and seed production (kg ha^{-1}) were determined.

Analysis of variance of the data and mean comparison (according to Duncan's multiple range test) were carried out, using MSTATC statistical software.

3 RESULTS AND DISCUSSION

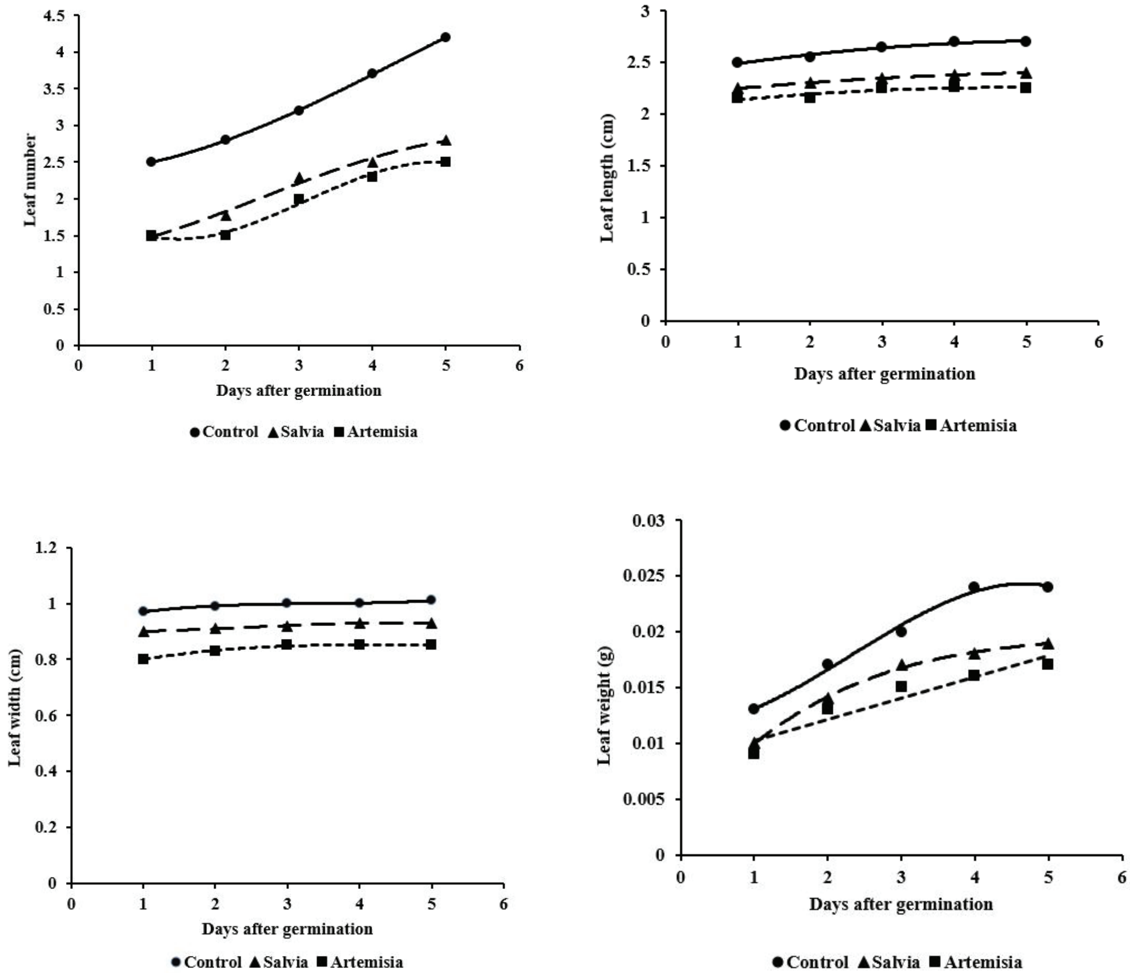
The results of the laboratory experiment showed that the main effects of plant species and concentration of their whole plant extracts on seed germination and early seedling growth of purslane were significant ($P \leq 0.01$). However, the interaction of plant species × extract concentration was not significant ($P \leq 0.01$). The extracts of *Artemisia sieberi* and *Salvia syriaca* considerably ($P \leq 0.01$) reduced the percentage and seed germination speed of purslane. Our results showed that inhibitory effect of *Artemisia sieberi* on purslane germination performance was stronger compared to *Salvia syriaca*. Compared to control treatment, Artemisia extract reduced seed germination and germination speed by 57 % and 54 % compared to significantly lower effect in Salvia with 44 % and 47 %, respectively (Table 1). Our results displayed that the increase in plant extract concentration led to stronger reduction in seed germination and germination speed. In terms of plant extract concentration level effect, the differences in germination and germination speed between the highest (20 %) and the lowest (control) extract concentrations were 73.5 % and 70 %, respectively (Table 1).

Purslane seedling growth parameters including leaf length, leaf number, leaf mass, leaf width, stem length and stem mass were greatly reduced by *Artemisia sieberi* and *Salvia syriaca* extracts. The negative effect of *Artemisia sieberi* on seedling growth of purslane was stronger compared to *Salvia syriaca* (Figure 1).

Table 1: Effect of plant species and concentration level of *Artemisia sieberi* Bess. and *Salvia syriaca* L. extracts on germination percentage and speed of purslane (*Portulaca oleraceae* L.) seed

Treatment		Germination percentage	Germination speed
Plant species	Control	61.25 a	6.43 a
	Artemisia sieberi Bess.	26.25 c	2.94 c
	Salvia syriaca L.	34.38 b	3.43 b
Plant extract Concentration (%)	0.0	61.25 a	6.43 a
	5.0	41.25 b	4.31 b
	10.0	34.38 c	3.5 bc
	15.0	29.38 d	3.0 c
	20.0	16.25 e	1.94 d

Different letters in each column for each trait indicates significant difference according to Duncan's multiple range test.



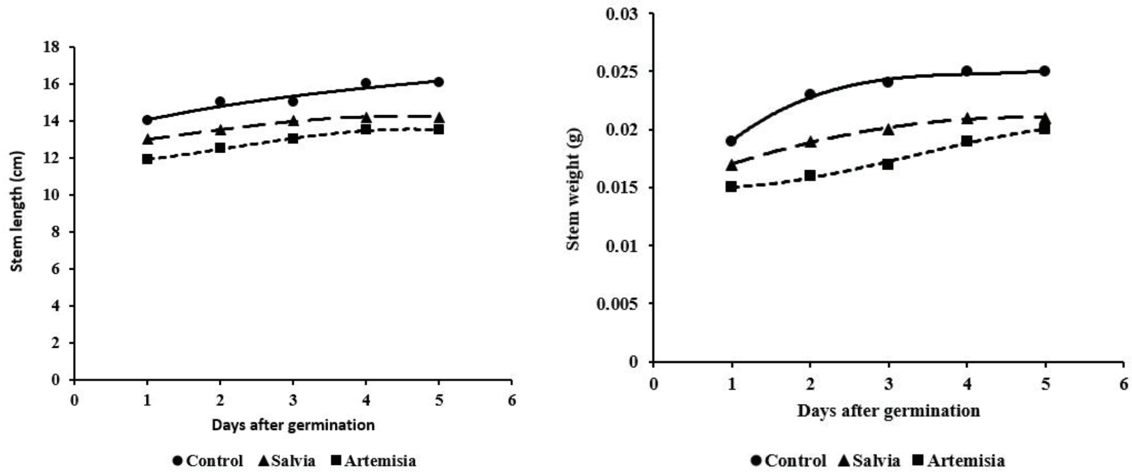


Figure 1: Effect of plant species on leaf and stem growth properties of purslane (*Portulaca oleraceae* L.) after seedling emergence**.**

The upsurge in extract concentration was accompanied by higher reduction in leaf and stem growth of purslane. The highest and the lowest leaf number, leaf width, leaf mass, leaf length and stem mass were observed in control and the concentration of 20 %, respectively (Figure 2).

The results of field experiment showed that the effect of mixing residue with soil was noteworthy ($P \leq 0.01$) on capsule number per plant, seed number per plant, seed number per unit area, seed production and shoot dry mass of purslane. However, seed number per capsule and 1000-seed mass were not significantly ($P \leq 0.01$) influenced by the mixture of plant residues and soil treatment. The highest seed production (kg ha^{-1}) was observed in control treatment. The purslane weed had the lowest seed production in the treatment of 4.0 g kg^{-1} mixture of residues with soil (Table 2).

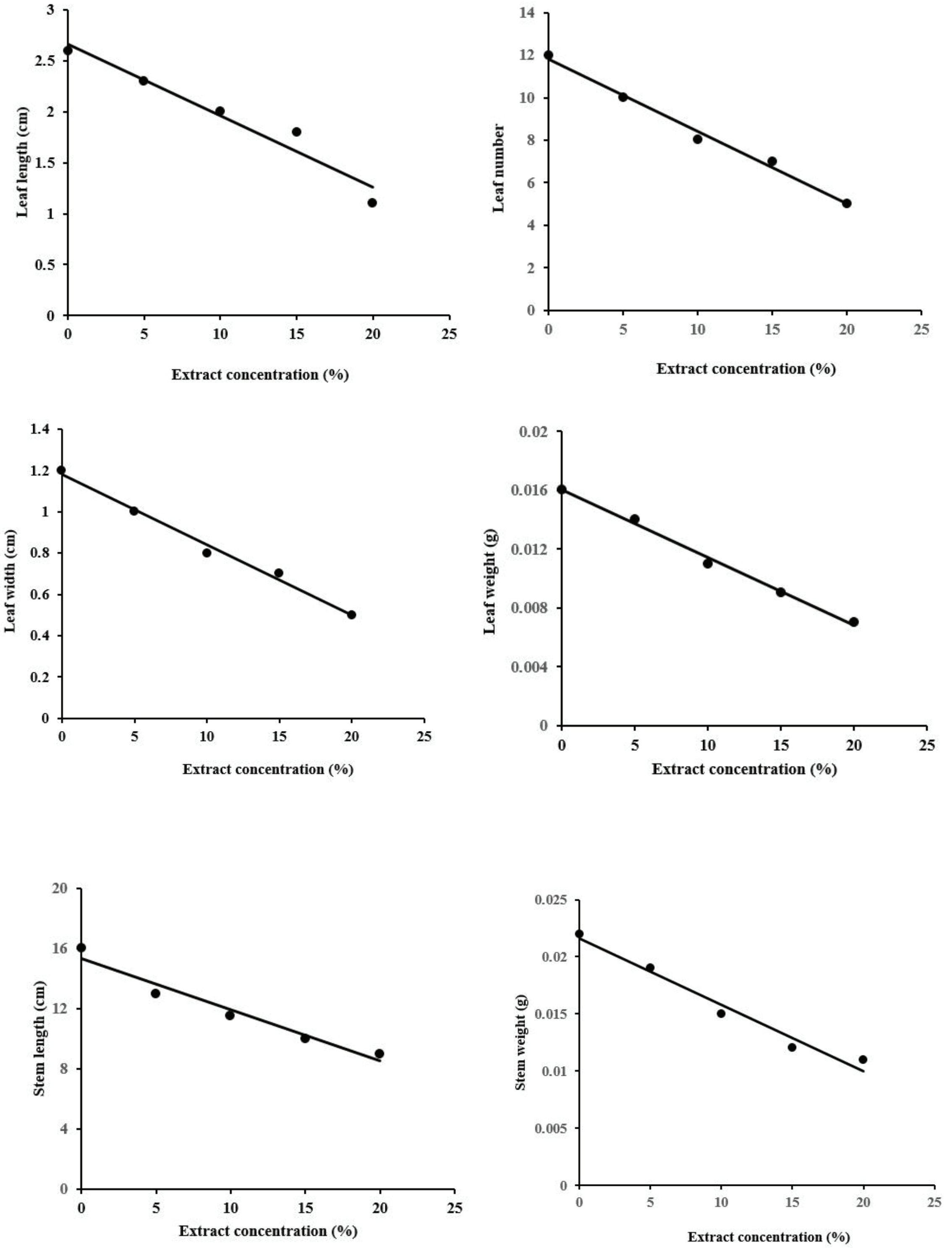


Figure 2: Effect of plant extract concentration (%) on leaf and stem growth properties of purslane (*Portulaca oleraceae* L.) after seedling emergence

Table 2: Effect of plant residues and soil mixture type on growth and seed production of purslane (*Portulaca oleraceae* L.) under field conditions

Treatment (mixture of plant residues and soil) (g kg^{-1})	Plant number/ unit area	Shoot dry mass (g)	Capsule number/ plant	Seed number/ capsule	Seed production (kg ha^{-1})
Control	20.0 a	38.3 a	9.7 a	211.3 a	12.1 a
1	13.0 b	26.8 b	9.1 ab	141.0 ab	6.0 b
2	9.5 bc	21.67 c	7.7 ab	172.8 ab	5.3 b
3	7.17 bc	16.83 d	6.2 bc	93.3 bc	2.4 bc
4	5.17 c	12.17 e	3.2 c	49.3 c	0.98 c

Numbers in different treatment mixtures indicate dry mass (g) of plant residues *(*Artemisia and salvia) per kg of fresh soil mass.
Different letters in each column for each trait indicates significant difference between them at $P \leq 0.01$ according to Duncan's multiple range test.

In the current experiment, the inhibitory effect of *Artemisia sieberi* was higher than that of *Salvia syriaca* which is compatible with the finding by Jafarpour et al. (2011) who reported that while seed germination of bean was not affected by low extracts of *Salvia syriaca*, all concentrations of *Artemisia sieberi* reduced germination and seedling growth of bean. Lydon et al. (1997) found that low concentration of *Artemisia sieberi* reduced seed germination and seedling growth of *Sinapis arvensis* L. weed, but had no effect on wheat, suggesting that *Artemisia sieberi* extract can be used for weeds control. The results of the present research revealed that *Artemisia sieberi* was more efficient in reducing purslane growth. However, the two crops have the potential for being applied for purslane weed control, where they not only reduced seed germination of purslane but also seed germination and seedling growth were delayed considerably. It has been emphasized that the delay in germination has negative impacts on seedling growth, where production of small seedlings reduces the competition ability of purslane (Chon et al., 2005).

Pinene, salween and tannins are allelopathic compounds found in *Salvia syriaca* extract (Kohli et al*.,* 2001) which denature mitochondrial structure, leading to inability of cells to use their storage materials (Mighati, 2003). This finally decreases purslane root and shoot growth. Inhibitory effect of *Salvia syriaca* on enzymatic activity, such as α-amylase, is another factor reducing seed germination (Ghomi & Tavili, 2011). There are some allelopathic compounds in *Artemisia sieberi* extract which inhibit cell division and elongation and, therefore, seed germination and seedling growth of purslane (Gholami et al., 2012). It has been reported that *Artemisia sieberi* has biological compound artemisinin which has toxic effects on plant seed germination (Lydon et al., 1997). The results of the research by Ghorbani et al., (2008) revealed that *Artemisia sieberi* extract decreased germination and seedling growth of *Avena fatua* L. and *Amaranthus retroflexus* L. which is in line with the findings of the present experiment.

When the share of Artemisia and salvia residues increased in plant soil mixture, purslane weed produced lower dry matter (Table 2). Medicinal plant residues, especially in the treatment of 4.0 g kg^{-1} mixture of residues with soil, led to a smaller number of emerged and established purslane plants, which is analogous with our laboratory findings in which germination percentage and speed of purslane seeds were negatively affected by Artemisia and salvia extracts (Table 1). Purslane showed 30 %, 43 %, 56 % and 69 % reduction in shoot dry mass in the 1.0, 2.0, 3.0 and 4.0 g kg^{-1} treatments, respectively. Similar results were reported by Erez and Fiden (2015) who found that germination of *Portulaca oleraceae* seeds was inhibited by extract of *Salvia macrochlamys* Boiss. & Kotschy.

Since the impact of residue mixing treatments was not substantial on 1000-seed mass, it can be concluded that reduction of seed production of purslane (kg ha^{-1}) resulted from the decrease in the number of seeds per unit area. On the other hand, seed number per capsule was not significantly affected by field experimental treatments, thus, lower seed number per unit area was induced by lower capsule number per unit area. In fact, allelopathic effects of *Artemisia sieberi* and *Salvia syriaca* reduced seed germination of purslane which induced lower plant per unit area and, finally, seed production. Seed production of 1.0, 2.0, 3.0 and 4.0 g kg^{-1} residue treatments was 35 %, 53 %, 64 % and 74 % lower compared to control treatment, indicating considerable decrease of purslane seed production affected by addition of artemisia and salvia plant residues in soil. Generally, *Artemisia sieberi* and *Salvia syriaca* residues can be successfully utilized for non-chemical control of purslane. However, the effects of allelo-chemicals of these two medicinal plants on the growth and yield of other crops need more investigation.

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Glomus intraradices (N.C. Schenck & G.S. Sm.) C. Walker & A. Schuessle enhances nutrients uptake, chlorophyll and essential oil contents and composition in *Anethum graveolens* L.

Weria WEISANY[1]

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi are plant-root symbionts whose application in agriculture has been proven its efficiency. However, their application in medicinal plants and their impact on accumulation of essential oils (EO) is still limited. In order to investigate the effect of AM fungi (*Glomus intraradices* N.C. Schenck & G.S. Sm.) C. Walker & A. Schuessle) on nutrients uptake, biomass production, yield components, chlorophyll content, and EO content and composition in dill (*Anethum graveolens* L.), a field experiment was conducted as randomized complete block design with three replications. This medicinal plant was grown under AM fungi colonization and non-colonization treatments. Plant inoculation by mycorrhiza increased aerial tissues P and Fe concentrations. However, K, Ca, and Zn concentrations were not affected by AM colonization. The plants inoculated with AM significantly increased plant biomass, chlorophyll content, and EO content by 363 g m^{-2}, 11.83 SPAD and 0.683 % in comparison with non-inoculated plants, respectively. Changes in EO composition were found in AM-colonized dill plants. The contents of myristicin, dill-ether and N-dihydrocarvone increased in EO obtained from AM-colonized plants, while AM colonization resulted in a lesser content of α-pinene, α-phellandrene, limonene, and β-phellandrene.

Key words: arbuscular mycorrhizal fungi; dill; essential oil; medicinal plants; nutrient uptake

IZVLEČEK

MIKORIZACIJA Z ARBUSKULARNO GLIVO *Glomus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schuessle POVEČUJE PRIVZEM HRANIL, KONCENTRACIJO KLOROFILA IN VSEBNOSTI ETERIČNIH OLJ PRI NAVADNEM KOPRU (*Anethum graveolens* L.)

Arbuskularne mikorizne glive (AM) so glivni simbionti večine kopenskih rastlin, tudi mnogih kmetijskih rastlin. Njihov pomen za uspevanje rastlin je potrjen, malo pa je znanega o njihovem vplivu na tvorbo eteričnih olj v zdravilnih rastlinah. Z namenom analize vplivov okužbe koreninskega sistema z AM na tvorbo sekundarnih metabolitov smo analizirali navadni koper (*Anethum graveolens* L.) z glivo *Glomus intraradices* N.C. Schenck & G.S. Sm.) C. Walker & A. Schuessle v poljskem poskusu organiziranem kot naključno zasnovani komplet s tremi ponovitvami. Analizirali smo absorpcijo hranil, proizvodnjo biomase, deleže pridelka, vsebnost klorofila in vsebnost eteričnih olj ter kemijsko sestavo nadzemnih delo rastline. Inokulacija rastlin z AM je povečala koncentracije P in Fe v nadzemnih tkivih, nismo pa ugotovili značilnih sprememb v koncentracijah K, Ca in Zn. Rastline, inokulirane z AM, so imele bistveno bujnejšo rast, večjo vsebnost klorofila in eteričnih olj v primerjavi z neinokuliranimi rastlinami. Pri inokuliranih rastlinah smo ugotovili tudi spremembe v sestavi nabora eteričnih olj, povečana je bila količina miristicina, koprovega etra in N-dihidrokarvona, zmanjšala pa se je količina α-pinena, α-felandrena, limonena, in β-felandrena.

Ključne besede: arbuskularna mikoriza; navadni koper; eterična olja; zdravilne rastline; privzem hranil

1 INTRODUCTION

Medicinal plants play major roles in human health services worldwide (Weisany et al., 2015), and herbal medicine is gaining importance at global level (Wondimu et al., 2007). Essential oils (EO) constituents of the medicinal and aromatic plants are most frequently used as a source of new bioactive molecules. The EOs

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are abundant in flowers, leaves, seeds, and are usually isolated via hydro-distillation, cold pressing methods (Edris, 2007). Their main active components are: carvone, carvacrol, eugenol, myristicin and apiole (Duke, 2001), although their mechanism of action is still poorly understood (Burt, 2004; Calo et al., 2015).

Dill (*Anethum graveolens* L.) is important essential oils producing plant. Myristicin and apiole in *A. graveolens* EO components are applied as a toxin and repellent to growing larvae and adults of *Tribolium castaneum* (Chaubey, 2007). Furthermore, the essential oils from its fruits and shoots are used in pharmacology as well as food and in soap industries.

It is well accepted that suitable use of irrigation and chemical fertilizers improve yield and quality of oil in aromatic plants (Singh and Randhawa, 1990; Tiwari and Banafar, 1995). However, for sustainable agriculture, traditional agriculture practices, including heavy fertilizer input are adverse owing to their long-lasting impact on the ground water quality (Yao et al., 2001). The alternative for sustainable plant production system to ensure increased productivity is to limit chemical input along with bio-inoculants so as to augment nutrient uptake by plants (Bethlenfalvay and Linderman, 1992).

Arbuscular mycorrhizal (AM) fungi can result in beneficial effects on soil and plant ecosystem such as improving soil structure (Rillig and Mummey, 2006; Bedini et al., 2009) and influencing plant nutrient uptake (Smith and Read, 2008; Clark and Zeto, 2000) and influencing major element cycles (for example, carbon, phosphorus and nitrogen) (Fitter et al., 2011). It is well accepted that AM fungi can improve the uptake of micronutrients and other mineral nutrients with low mobility including Fe (Clark and Zeto, 2000), Zn (Weisany et al., 2016a) and Mn (Weisany et al., 2016b). Utilizing management practices of AM fungi associated with promote effects of AM fungi on phosphorus uptake, growth, and grain yield of crops (Sohrabi et al., 2012b; Arihara and Karasawa, 2000; Karasawa et al., 2002). Previous studies have shown that different species and isolates of *Glomus* had various effects on mycorrhizal plants (Sohrabi et al., 2012a,b).

AM fungi promote the accumulation of effective ingredients of medicinal plants, which has become a hot area of research lately (Raei and Weisany, 2013). AM fungi application may provide a natural regulation mechanisms and ecological method to increase the accumulation of secondary metabolites in medicinal plants, because it contributes to a reduction of need to apply chemical fertilizers and development of sustainable agriculture (Pedone-Bonfim et al., 2013). AM fungi can affect the production of active ingredients in medicinal and aromatic plants (Karagiannidis et al., 2011), resulting from a better nutritional condition or by means of protecting the host from the pathogenic fungi (Volpin et al., 1994). Symbiosis between plants and AM fungi can increase the accumulation of several secondary metabolites in medicinal plants which plays important roles in treating human diseases (Weisany et al., 2015). The influence degree of different AM fungi varies among the medicinal plants. The scientific reports suggest the improved production of EO in coriander and dill inoculated by *Glomus fasciculatum* (Taxt.) Gerd.ec Trappe or *G. macrocarpum* Tul. & C. Tul. (Kapoor et al., 2002a, b), in mint inoculated by *G. fasciculatum* or a suite of AM fungi (Freitas et al., 2004), in oregano and dill inoculated by *Glomus mosseae* (T.H. Nicolson & Gerd.) Gerd. & Trappe 1974 (Khaosaad et al., 2006; Weisany et al., 2015), and in annual wormwood colonized by *G. faciculatum* (Kappoor et al., 2007; Chaudhary et al., 2008).

While AM colonization can increase the EO contents of medicinal plants, it is not clear whether the composition of the EO could be affected. Therefore, the overall aim of the present study is to investigate the fact that whether AM fungi can provide an effective and natural way of improving the growth, chlorophyll content, nutrient uptake, and EO content and composition in dill plants. More specifically, through testing two hypotheses we attempted to evaluate whether the composition of the secondary metabolites in dill are affected by AM fungi inoculation. The first hypothesis is that AM fungi enhance chlorophyll content and nutrient uptake in dill and this, in turn, increase the plant production. The second hypothesis is that symbiosis between plant and AM fungi boosts the accumulation of several secondary metabolites in dill.

2 MATERIALS AND METHODS

2.1 Experimental design

A field experiment was conducted in the Agriculture and Natural Resources Research Center of Kurdistan Province in 2014. Soil samples were taken from depths of 0–10 cm and 10–25 cm and mixed, using a soil auger. These samples were collected in spring from 8 points of experimental area. All soil samples were air dried at laboratory for 7 days and then crushed and sieved through a 2 mm sieve to determine the chemical composition (Rao, 1993). The texture of the soil was sandy clay loam. Different chemical and physical properties of soils are presented in Table 1. The experiment carried out as randomized complete block design with three replications. The medicinal plant studied in this research was dill (*Anethum graveolens* L.). It was applied as a colonized and non-colonized plant by arbuscular mycorrhizal fungi. The medicinal plant was managed according to the organic farming practices without using pesticides or fertilisers. Seeds were sown in plots (4 × 5 m), each with 8 rows. Three seeds were sown by hand on the eastern side of the ridges in each hole being at a 10-cm distance from another hole. After emergence, the seedlings were thinned and one plants was kept in each hole. Plots of non-colonized seeds were sown first in order to avoid AM cross contamination.

Table 1: Some physical and chemical properties of the soil of experimental area

Texture	Organic carbon %	pH (1:2.5)	K	P	Ca	Na	Zn	Mn	Fe	Cu
			(mg kg^{-1}soil)							
Sandy clay loam	1.14	7.12	131	12.2	1150.1	450.2	0.476	7.054	6.97	0.826

Thirty grams of soil inoculum (100 endomycorrhizal spore/10 g soil) along with 300 mg of chopped *G. intraradices* -colonized *Zea mays* L. roots were added to each plot at sowing time just below the seeds. The AM fungus (*G. intraradices*) was obtained from the culture collection of Tabriz University, Tabriz, Iran (Weisany et al., 2015).

2.2 Arbuscular mycorrhizal fungi colonization

Five plants from each plot were randomly collected at 95 days after AM fungi inoculation. The root samples were extracted by using a cylindrical corer (10 mm). The roots were washed, cut into about 1 cm long pieces and mixed thoroughly. The staining procedure was applied according to Phillips and Hayman (1970) with the modified parameters for the present study. The roots were cut into small pieces (1 cm) and placed in a beaker (10 % KOH) for 60 min in a water bath at 65 °C. The roots were then rinsed with tap water and acidified with 5 % lactic acid at room temperature for 12 h. Finally, they were stained by a solution containing 875 ml of lactic acid, 63 ml of glycerin, 63 ml of tap water, and 0.1 g of fuchsine acid for 30 min at 70 °C and were then de-stained by lactic acid for 15 min. Afterwards, root segments were mounted onto slides and examined at 100-400 magnification under a Nikon YS100 microscope. Beneath the glass slide an acetate film with 10 thin lines was adapted. At crossing points between roots and lines, each point that had an infection was recorded and the number of infections was expressed as percentage (Weisany et al., 2015). The percentage of AM root colonization was calculated by the following equation (McGonigle et al., 1990):

Root colonization (%) = (number of root segments colonized/number of root segments studied) × 100

2.3 Mineral nutrient analysis

The dry ash method was used (Jones and Case, 1990) to measure different elements in dill. In this way, the aerial tissues of plants were dried in an oven at 70°C. Five plants from the each plot were randomly harvested. Subsequently, 1 g of dry matter was transferred into ceramic vessels and was slowly subjected to 500 °C in the oven. The final product was a white ash. White ash was cooled in room temperature and then 20 ml 1N HCl was added to each sample, followed by the sand bath for 30 minutes. The samples were elutriated in a 100 ml volumetric balloon (Cottenie, 1980). Having provided plant extracts, the concentrations of calcium (Ca) and potassium (K) were measured via flame photometer (Model 410, Corning, Halstead, UK). Iron (Fe) and zinc (Zn) concentrations were measured by atomic absorption spectrometer (Shimadzu AA6600) (Jones 1972). Plant phosphorous was gauged through the yellow method, in which, vanadate-molybdate (Tandon et al., 1968) was employed as an indicator. P concentration was determined at 430 nm, using a spectrophotometer apparatus (Shimadzu, UV3100).

2.4 Plant growth measurements and chlorophyll content

Ten plants from each plot were randomly harvested after seed maturation. In the sampling plots, the randomly chosen plants from the each treatment were harvested along with complete roots, and the plant biomass (dry mass) was recorded (Weisany et al., 2015). Ten indiscriminately selected plants at full maturity stage in each plot were cut, and the plant height and yield components were defined.

Chlorophyll status of dill plants was evaluated in each plot by SPAD analysis (SPAD 502, Minolta Ltd. Osaka, Japan). SPAD measurements were performed at flowering stage, and the mean of three random SPAD measurements on the middle part of the leaf blade was recorded (Weisany et al., 2015).

2.5 Essential oil extraction

At the beginning of flowering, aerial tissues of dill were harvested from 1 m^2. The EOs were extracted by hydrodistillation in 500 ml of water, using a Clevenger apparatus for 2 h. The distillate was extracted using diethyl–ether as solvent (1/1, v/v) and drying the sample over anhydrous sodium sulphate. The organic layer was then concentrated at 35 °C by a Vigreux column and the EO was stored at 4 °C prior to analysis (Weisany et al., 2016b). The percentage of EO content was measured in volume/100 g dry mass basis.

2.6 Gas chromatography–mass spectrometry

Gas chromatography (GC) analysis was performed by means of a trace GC ultra-gas chromatograph coupled with a TSQ quantum tandem mass spectrometer upgraded to the XLS configuration. A DuraBrite IRIS ion source with pre-filter was installed so as to improve the performance of the spectrometer. The system was equipped with a triplus autosampler (Thermo Electron Corporation, Waltham, MA). The injection volume was 1 µl, post injection dwell time 4 sec, and tray temperature 7 °C. GC separation was done on a 30-m VF-WAXms capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 µm (Varian, Inc. USA). Temperature programming was as follows: 40 °C held for 4 min after injection and 6 °C min^{-1} up to 250 °C held for 5 min. Injection parameters were as follows: split injection, split ratio of 100:1, inlet temperature of 250 °C, carrier gas being helium 5.5, and constant flow: 1.2 ml min^{-1}. The mass spectrometry was used in scan mode in the range of 40-400 m/z with a scan time of 0.2 sec. The ionization mode was electron impact (EI), and the source temperature was kept at 250 °C (Perini et al., 2014).

2.7 Statistical Analysis

Analysis of variance was carried out, using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) (SAS Institute Inc. 1988). Means of the treatments were compared through orthogonal comparisons. The data showed normal distribution and no transformation has ben done.

3 RESULTS AND DISCUSSION

3.1 AM colonization

Arbuscular mycorrhiza colonization was observed in inoculated plants and root samples. The percentage of mycorrhizal root colonization was significantly greater in all the treatments of plant colonization with mycorrhiza in comparison with non-inoculated control plants (Table 2).

Table 2: Root AM colonization, growth and yield components, chlorophyll, mineral nutrient and essential oil contents of dill inoculated (+AM) and non-inoculated with arbuscular mycorrhiza (-AM)

Parameters	Dill		
	-AM	+AM	Pr > F
AM colonization (%)	0.00±0.00	80.6±6.568	0.0056**
Phosphorous (mg kg^{-1}DM)	13.78±0.016	14.29±0.025	0.0003**
Zinc (mg kg^{-1}DM)	0.449±0..009	0.458±0.001	1.0000ns
Iron (mg kg^{-1}DM)	1.131±0.338	1.946±0.499	0.0367*
Potassium (mg kg^{-1}DM)	255.45±33.80	320.05±21.33	0.3620ns
Calcium (mg kg^{-1}DM)	622.0±20.78	685.5±53.98	0.1959ns
Plant height (cm)	43.00±5.291	50.66±7.310	0.3995ns
Number of branches/plant	6.66±0.333	7.660±0.666	0.4226ns
Number of umbels/plant	6.67±0.333	7.33±0.881	0.4226ns
Number of umbelet/plant	94.66±3.179	109.33±5.456	0.3570ns
Root length (cm)	10.01±0.577	14.50±0.866	0.0701ns
Plant biomass (g m^{-2})	205.33±3.289	363.33±26.193	0.0204*
Chlorophyll (SPAD)	6.90±0.2645	11.83±0.2962	0.0121*
Total essential oil (%)	0.377±0.000	0.683±0.000	0.0251*

+AM, -AM: inoculated and non-inoculated with arbuscular mycorrhiza, respectively.
Results are the mean of three replications±SD. NS, * and **, non-significant and significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

3.2 Mineral nutrient uptake

Results of this study indicated that plant colonization with AM enhanced the aerial tissues P concentration of dill, compared with control plants (Table 2). So, the inoculated dill with *G. intraradices* had the most (14.29 mg kg^{-1}DM), but non-inoculated plants had the least (13.78 mg kg^{-1}DM) P concentrations (Table 2). Mycorrhization of target plant provides a better nutrient status by the wide extra-radical mycelium of AM that equips the plant roots with a more surface area for uptake of nutrients and water (Jefferies et al., 2003). AM fungal hyphae can transfer immobile P resources to the roots from a long distance (over centimeters) and therefore, play an even more important role in P uptake by their host plants than what was previously taken for granted (Richardson et al,. 2011, Weisany et al., 2016a).

The colonization of dill with *G. intraradices* increased Fe concentration to 1.946 mg kg^{-1}DM, whereas the non-inoculated plants had the least Fe concentration (1.131 mg kg^{-1}DM). However, there was no significant difference between inoculated and non- inoculated plants in terms of K, Ca and Zn concentration (Table 2). The increased utilization of soil volume is especially important in the uptake of less mobile nutrients such as P and Fe (Smith and Read, 2008). Increased uptake of Fe by mycorrhizal fungi may be in part due to production of siderophores that specifically chelate Fe (Shende and Rai, 2010). AM fungi moderate the ion balance in the plant that affecting the availability of mineral nutrients in plant tissues (Bermudez and Azcon, 1996).

3.3 Growth and yield components

Dill plant biomass was effected by AM colonization, so that the inoculated plants with *G. intraradices* had more (363.3 g m^{-2}) compared with non-inoculated plants (205.3 g m^{-2}) plant biomass. However, plant height, number of branches per plant, number of umbels per plant, number of umbelets per plant and root length of dill were not substantially affected by AM inoculation (Table 2).

Our previous studies have revealed that different species and isolates of *Glomus* increased plant height, total dry mass and root and aerial tissues dry mass of chickpea (Sohrabi et al., 2012a,b). The improvement of plant growth was also observed in coriander (*Coriandrum sativum* L.) by colonization of AM and application of phosphorus (Farahani et al., 2008). The results of the present study are consistent with these reports. The increase in growth can be attributed to improved P and Fe nutrition in these treatments (Table 2). In the current research, plant inoculation with AM augmented the growth of dill, supporting the observation that mycorrhizal plants obtain more nutrients. Furthermore, AM are able to share nutrients via an underground network of hyphal connections linking individuals within and between species (Simard et al., 2003).

The positive influence of P and Fe on growth and yield has been reported for many plants, including those with medicinal value (Naguib et al., 2007). The Fe element plays roles either as functional or structural co-factors or as the metal components of different enzymes (Marschner, 1995). Inoculation of plant by AM fungi results in higher growth and yield, since it offers an opportunity to optimize the rate of photosynthesis via improved uptake of P and Fe nutrients. Therefore, AM fungi provide balanced nutrition to the host plants, leading to increased growth and yield.

3.4 Chlorophyll content

Chlorophyll content in dill leaves was markedly influenced by inoculation with AM. In general, inoculated plants with *G. intraradices* had significantly more chlorophyll (11.83 SPAD) than the non-inoculated plants (6.90 SPAD) (Table 2). The association of AM fungi with the roots of dill plants influence Fe uptake (Table 2). The Fe plays an important role in various biochemical and physiological processes, such as chlorophyll synthesis, photosynthetic transport, respiration, nitrate reduction and N_2 fixation (Robinson and Postgate, 1980). The obtained AM-mediated higher chlorophyll content in dill leaves may be due to improved nutrient uptake by this plant, especially that of Fe. These findings are in agreement with those previously found by Mathur and Vyas (2000). They discovered that root colonization with AM increased chlorophyll synthesis. The contribution of AM fungi and the roots of dill plant influences Fe acquisition that has poor mobility rates. Fe is an integral component of the chlorophyll molecule (Taiz and Zeiger, 2004).

3.5 Essential oil yield and composition

Inoculation of plant with AM fungi considerably influenced EO yield. AM inoculation noticeably enhanced the total EO yield in dill aerial tissues (Table 2). The effect of AM fungi in increasing the production of EO has been reported in some of medicinal plant species (Khaosaad et al., 2006; Copetta et al., 2006; Chaudhary et al., 2008, Weisany et al., 2015). Karagiannidis et al., (2011) obtained similar results in their study on three AM fungi colonization that increased the nutrient concentration, plant growth and EO yield of oregano and mint plants. The boosted EO production is the outcome of enhanced shoot fresh mass (Subrahmanyam et al., 1992; Piccaglia et al., 1993). Kapoor et al. (2007) also noticed that plant inoculation with AM enhances the number of glandular trichomes of *Artemisia annua* L. and, as a consequence, increases artemisinin content in leaves. This bigger number of glands could be related to variation in the hormonal profile of plants due to enhanced amounts of auxins, cytokinins and gibberellins in plants inoculated with AM (Torelli et al., 2000). Additionally, inorganic P concentration can influence the biosynthesis of EO in the plants (Loomis and Corteau, 1972). In the present research, AM fungi improved the absorption of P in plants. P element may play a direct role in increasing the content of secondary metabolites (Abu-Zeyad et al., 1999). The same results were found by Kapoor et al. (2004) in their study on the accumulation of EO in fennel. The present findings concur with these conclusions, because a significant positive correlation between EO synthesis and shoot P concentrations was found for dill.

Photosynthesis of mycorrhizal plants can increase due to an increased plant chlorophyll content and by the drain of carbon, as a consequence of Calvin cycle activation and higher production of primary metabolites that act as precursors for secondary metabolism (Kaschuk et al., 2009). Gas chromatography (GC) analysis of EO composition showed that dill-apiole was the main component in all treatments and its amount varied among the treatments (Table 3, Fig 1). The second main component of the EO was carvone. The results indicated that inoculation of dill with AM increased myristicin, dill-ether and N-dihydrocarvone contents in plant (Table 3). These findings are similar with those of Karagiannidis et al. (2011). Variation in EO composition due to AM colonization (Table 3) maight be related to the nutrition of plants. The accumulation of flavonoids (Larose et al., 2002), phytoalexins (Yao et al., 2003), cyclohexanone derivatives and apocarotenoids (Fester et al., 2002; Vierheilig et al., 2000a,b), triterpenoids (Akiyama and Hayashi, 2002) and phenolic compounds (Devi and Reddy, 2002) in plants inoculated by AM fungi has been previously reported. AM fungi colonization of *Salvia officinalis* L. changes EO composition, and improves the relative amounts of 1,8-cineole, bornylacetate, α-thujones and β-thujones (Geneva et al., 2010). The mechanisms by which AM fungi changes the production of EO are not clear, but they are possibly associated with improved nutrition.

Table 3: Chemical composition (% of essential oil) of essential oils of dill shoots inoculated (+AM) and uninoculated with arbuscular mycorrhiza (-AM)

Compounds (Synonymous)	Dill			Confirmed by
	-AM	+AM	Pr > F	
α-pinene	0.15	0.11	0.2458ns	STD, MS
α-phellandrene	2.45	2.14	0.0011**	STD, MS
Limonene	4.22	3.18	0.0001**	STD, MS
β-phellandrene	0.47	0.39	0.0034**	RI, MS
Dill_ether (3,9-epoxy-1-p-menthene; anethofuran)	0.60	0.70	0.2879ns	RI, MS
N-dihydrocarvone (trans-dihydrocarvone)	4.80	5.99	0.0011**	STD, MS
Iso-dihydrocarvone (cis-dihydrocarvone)	10.07	11.66	0.0002**	STD, MS
Carvone	26.05	23.17	0.0001**	STD, MS
Neoiso-dihydrocarveol	0.41	0.43	0.3349ns	STD, MS
Iso-dihydrocarveol	0.73	0.69	0.2381ns	STD, MS
Trans-carveol	0.11	0.10	1.0000ns	STD, MS
Isopiperitenone	0.05	0.05	1.0000ns	RI, MS
Cis-carveol	0.08	0.07	0.2879ns	STD, MS
Thymol	0.02	0.09	0.0056**	STD, MS
Carvacrol	0.15	0.14	0.6560ns	STD, MS
Elemicin	0.24	0.24	1.0000ns	RI, MS
Myristicin	0.74	1.05	0.0006**	STD, MS
Dill_apiole	48.57	49.75	0.0001**	RI, MS

STD, MS = confirmed by injection of Standard and by Mass Spectra library; RI, MS = confirmed by n-alkanes Retention Index by Mass Spectra library;
+AM, -AM: inoculated and non-inoculated with arbuscular mycorrhiza, respectively.
NS, * and **, non-significant and significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. Five plants from each plot were randomly collected.

The results of the present research showed that inoculation of plant with AM decreased contents of α-pinene, α-phellandrene, limonene, and β-phellandrene in dill plant (Table 3). Most presumably these variations are due to changes in the synthesis pathways and the role of EO in plant physiology. AM colonization significantly increased activities of enzymes related to secondary metabolism. Among enzymes such as chalcone synthase and chalcone isomerase, the key enzymes in the synthesis of flavonoids, and phenylalanine ammonia-lyase (PAL) that catalyzing the deamination of phenylalanine (Ibrahim and Jaafar, 2011) provide precursors for the synthesis of secondary metabolites. The AM fungi also effects cytological changes in the host plant, such as an increase in the number of plastids and mitochondria, leading to the activation of the tricarboxylic acid cycle and the plastid biosynthetic pathways and the increase in the production of primary and secondary metabolites (Lohse et al., 2005, Strack and Fester, 2006).

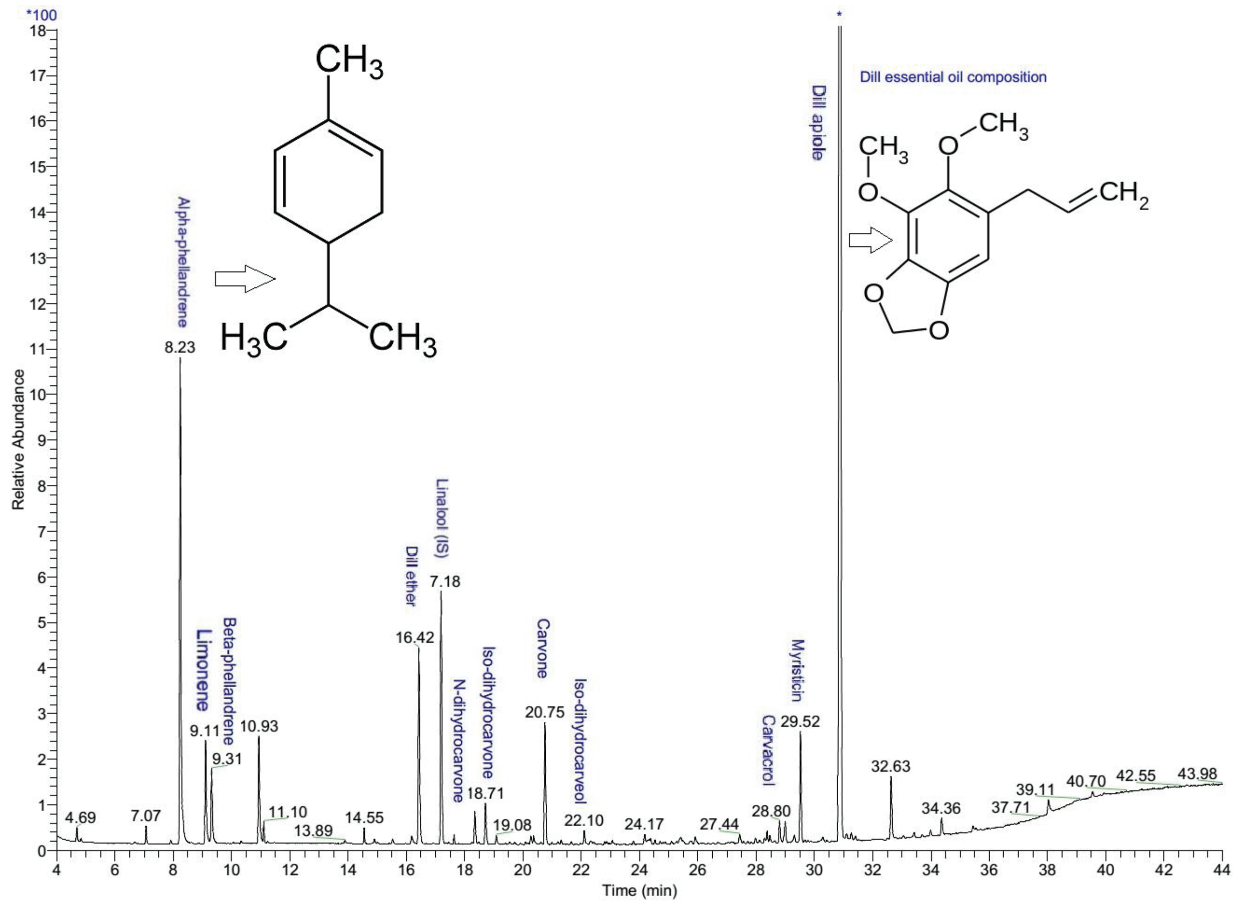

Figure 1: *Anethum graveolens* L. essential oil chromatogram carried out using a gas chromatograph mass spectrometry. Essential oils were obtained from inoculated with arbuscular mycorrhiza (*Glomus intraradices*) plant.

4 DISCUSSIONS

The study showed that inoculation of dill plant with AM improved mineral nutrition of plant in comparison with non- AM inoculated plants. Increases in growth and chlorophyll content were observed in AM colonized plants. When plant species are inoculated with AM fungi, it is concluded that yield benefits occur as a result of complementary use of resources by the plants. *A. graveolens* L. EO yield was enhanced in inoculated plants with AM fungi. Aerial tissues EO composition of dill was affected by colonization with AM. Our contribute to the expansion of effective organic and sustainable methods for the cultivation of medicinal plants. Further study in mycorrhizal technology is needed to develop the sustainability of the commercial cultivation of medicinal plants.

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Effects of seaweed extract on the growth, yield and quality of cherry tomato under different growth conditions

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ABSTRACT

An experiment was carried out to determine the effect of foliar application of seaweed extract (0.2 %) on the growth, yield and quality of cherry tomato under stress and non-stress conditions. The greenhouse experiment was set up in a randomized block design with four treatments in three replications. Treatments were as follows: V_1 - seedlings treated by seaweed extract and subjected to drought; V_2 - seedlings treated by seaweed extract and regularly watered; V_3 - non-treated seedlings subjected to drought; V_4 - non-treated seedlings regularly watered. Cherry tomato seedlings treated by seaweed extract had a lower content of proline and higher leaf water potential compared to non-treated seedlings under stress conditions, indicating that application of this fertilizer contributes to better adaptation of cherry tomato seedlings to stress. Treatment with seaweed extract also positively influenced the yield and quality of cherry tomato (total soluble solids, vitamin C, lycopene) under both standard and drought stress conditions as compared to untreated plants in same conditions. Positive effects of seaweed extract on growth and quality of cherry tomato are result of its specific composition, as well as ability of cherry tomato plants to utilize bioactive substances in seaweed extracts for its growth and development.

Key words: cherry tomato; seaweed extract; osmotic adjustment; photosynthesis; antioxidants; growth conditions

IZVLEČEK

UČINKI IZVLEČKOV MORSKIH ALG NA RAST, PRIDELEK IN KAKOVOST ČEŠNJEVEGA PARADIŽNIKA V RAZLIČNIH RASTNIH RAZMERAH

Izveden je bil poskus za določanje učinkov foliarnega gnojenja z izvlečkom morskih alg (0, 2 %) na rast, pridelek in kakovost češnjevega paradižnika v stresnih in nestresnih razmerah. V rastlinjaku je bil postavljen naključni bločni poskus s štirimi obravnavanji in tremi ponovitvami. Obravnavanja so bila: V_1 – tretma sadik z izvlečkom morskih alg in izpostavitev suši; V_2 - tretma sadik z izvlečkom morskih alg in redno zalivanje; V_3 – netretirane sadike so bile izpostavljene suši; V_4 – netretirane sadike so bile redno zalivane. Sadike češnjevca, ki so bile tretirane z izvlečkom morskih alg, so imele manjšo vsebnost prolina in večji vodni potencial listov v primerjavi z netretiranimi v stresnih razmerah, kar kaže, da je uporaba tega gnojila prispevala k boljši prilagoditvi sadik na stres. Foliarno gnojenje z izvlečekom morskih alg je tudi pozitivno vplivalo na pridelek in kakovost češnjevca (celokupno vsebnost topnih snovi, vitamina C, likopena) v kontroli in stresnih razmerah v primerjavi z netretiranimi rastlinami v enakih razmerah. Pozitivni učinki izvlečka morskih alg na rast in kakovost češnjevca so posledica njegove specifične sestave kot tudi sposobnosti tega paradižnika, da bioaktivne snovi iz izvlečkov morskih alg uporabi za rast in razvoj.

Ključne besede: češnjev paradižnik; izvleček morskih alg**;** osmotsko uravnavanje; fotosinteza; antioksidanti; rastne razmere

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1 INTRODUCTION

Water stress caused by drought induces morphology, biochemistry and physiology changes in plant, leading to considerable reductions in plant growth and productivity (Atkinson and Urwin, 2012; Li and Mattson, 2015). Besides, drought stress is able to promote reactive oxygen species (ROS) production which in turn leads to damage of all cellular components primarily proteins, lipids and nucleic acids (Ali and Anjum, 2016; Ali et al., 2016).

Plants possess a number of defense mechanisms to cope with stress and some of the more important are osmotic adjustment and efficient antioxidant systems. The osmotic adjustment is indicated by the accumulation of proline, glycine betaine and other metabolites the structural capabilities to maintain homeostasis and improve plant functioning under drought stress (Hayat et al., 2012). The ability of plants to improve their defense mechanism against stress also depends on the possibility of plant to produce secondary metabolites with strong antioxidant activity, among them phenolic compounds (Sanchez-Rodriguez et al., 2011).

Tomatoes are very sensitive to drought (Nuruddin et al., 2003). There are currently several approaches that potentially reduce the impact of drought stress on vegetable cultivation, such as development of drought stress tolerant cultivars, adopting agronomic practices, efficient irrigation systems and use organic fertilizers that can contribute to mitigate drought stress (Tilman et al., 2002; Mikiciuk and Dobromilska, 2014). Application of seaweed extract also might contribute to the strengthening of the plant defense system against stress since the seaweed extracts are very rich in bioactive compounds, including betaine, proline, and aromatic amino acids (Arioli et al., 2015). Currently, many types of seaweed extracts can be purchased for commercial agriculture, especially for vegetable cultivation (Craigie, 2011). Bio-algeen S-92 (Shulze & Hermsen GmbH, Germany) is an organic fertilizer for foliar supplemental feeding, derived from seaweed *Ascophyllum nodosum* (L.) Le Jol. According to the product specification Bio-algeen S-92 contains 96 % water, 0.02 % N, 0.006 % P, 0.096 % K, 0.31 % Ca, 6.3 mg l^{-1} Fe, 1 mg l^{-1} Zn, 0.6 mg l^{-1} Mn, vitamins (B_1, B_3, B_6, B_9 and vitamin E), a certain amount of essential amino acids: alanine, glycine, tryptophan, histidine, proline, glutamine and other active natural substances such as organic acids and microelements that provide lots of benefits for plant growth and development (Dobromilska et al., 2008). As far as we know, the possible application of this preparation in greenhouse production of cherry tomato has not been tested so far, especially under drought stress conditions.

Cherry tomato was selected as the subject of this study, particularly because this species is commonly affected by a lack of moisture, and also because this vegetable an important part of a healthy diet.

2 MATERIALS AND METHODS

2.1 Field experiment

The study was carried out in 2015 under controlled conditions, in the greenhouse of public communal company ’Park’ in Sarajevo. In the experiment air temperature in greenhouse was maintained at 23 to 25 °C during day and 20 to 22 °C at night. Relative humidity (RH) was maintained between 60 % and 70 %, with combined venting to reduce RH, and with high-pressure fogging to increase RH. During warm days shade cloth over the top of a greenhouse was used to reduce solar radiation entry.

The first part of the study involved transplanting of cherry tomato seedlings into individual pots (20 cm diameter × 13 cm height), containing substrate Florahum-SP (8 April 2015). Cherry tomato seedlings used in the experiment were produced at a certified nursery located near the greenhouse and showed no significant difference in terms of size and appearance. The substrate used in this study represented a mixture of white and black peat enriched with nutritional supplements. The main chemical characteristics of substrate were as follows: pH 5.5 - 6.5, EC 1.2 - 1.8 mS cm^{-1}, content of N 140 - 180 mg l^{-1}, content of P_2O_5 160 - 300 mg l^{-1}, and content of K_2O 180 - 400 mg l^{-1}.

The second part of the study related to setting up an experiment in which the cherry tomato seedlings were treated by 0.2 % solution of Bio-algeen S92, extract from *Ascophyllum nodosum* (ANE). The experimental trial was set up in a randomized block design with four treatments in three replications. Each of these treatments was present with sixty seedlings. The treatments were as follows:

V_1 - double foliar treatment with ANE (100 ml per plant) before exposure to drought stress,
V_2 - double foliar treatment with ANE (100 ml per plant) and regularly watered (non-stressed),

V_3 - without ANE treatment before exposure to drought stress,
V_4 - without ANE treatment and regularly watered (non-stressed).
ANE application was carried out manually, using small bottles with sprayers. The first ANE application was done immediately after the transplanting of seedlings (8 April 2015), and the second fifteen days later. Five days after the second treatment, the cherry tomato seedlings (V_1 and V_3) were exposed to drought stress conditions (non-watering), while the other cherry tomato seedlings (V_2 and V_4) were not exposed to drought stress, that is, they were regularly watered.

Exposure of cherry tomato seedlings to drought stress conditions lasted until the moment in which first visually observable effects of drought appeared on the seedlings as wilting leaves. This moment was also represented the beginning of the third part of study, which included measurement of selected physiological parameters for evaluating of drought tolerance in cherry tomato seedlings: leaf water potential, leaf area, photosynthetic pigments, content of proline, total phenolic and flavonoid content, and total antioxidant capacity. Leaf water potential was estimated by the dye method (Knipling, 1967), content of proline was measured by acid-ninhydrin method (Bates et al., 1973), photosynthetic pigments were extracted with 80 % acetone (Wettstein, 1957) and the total amount of pigments were determined with equations recommended by Lichtenthaler and Wellburn (1983), leaf area was measured by millimeter graph paper method (Pandey and Singh, 2011), total phenolic content was estimated using Folin Ciocalteu method (Ough and Amerine, 1988), total flavonoids according to Aluminium chloride colorimetric assay (Zhishen et al., 1999), and the ferric reducing/antioxidant power (FRAP) assay was used to determine total antioxidant capacity (Benzie and Strain, 1996).

The next part of study involved the cultivation of cherry tomato under standard growth conditions in all variants until the time of technological maturity of fruits, in order to test how the exposure of seedlings to drought stress and application of ANE affect the yield and quality of fruit. Fruit nutritional quality was analyzed by detecting the following parameters: total soluble solids, titratable acidity, lycopene, vitamin C, total phenolic and flavonoid content, content of rutin and naringenin, and total antioxidant capacity of cherry tomato fruits. Estimation of total soluble solids was performed by digital refractometer according to the International standard method (ISO 2173, 2003), titratable acidity was estimated by titration with NaOH according to AOAC Official method No. 942.15 (AOAC, 2000) and vitamin C by titration with 2,6-dichlorophenolindophenol according to AOAC Official method No. 967.21 (AOAC, 2006).

2.2 Estimation of proline

Estimation of proline was carried out as follows: 1 g of fresh leaf samples was homogenized in 3 % (w/v) aqueous 5-sulfosalicylic acid and the homogenate was filtered through a glass-fiber filter to a plastic test tube. 2 ml of filtrate was mixed with 2 ml of ninhydrin reagent and 2 ml of glacial acetic acid in a test tube and boiled for 1 hour at 100 ^{0}C (ninhydrin reagent was prepared as follows: 2.5 g of ninhydrin was dissolved in a mixture of 60 ml glacial acetic acid and 40 ml 6 mol l^{-1} phosphoric acid). After termination of reaction in ice bath, the reaction mixture was extracted with 4 ml of toluene, and mixed vigorously with a vortex mixer for 15 - 20 sec. The reddish layer of mixture was transferred to cuvette and absorbance read at 520 nm with a UV/Vis spectrophotometer (Thermo Scientific, Madison, USA) using toluene as blank. The proline concentration was determined from a standard curve (0 - 5 µg ml^{-1}) and then the values were recalculated on fresh mass (µg g^{-1} FM).

2.3 Estimation of photosynthetic pigments

Extraction of pigments was made from 200 mg of fresh leaves in acetone (80 %) and absorbance of extract was read spectrophotometrically at 662 nm, 645 nm, and 470 nm. The total amounts of pigments were determined with equations recommended by Lichtenthaler and Wellburn (1983) as follows:

Chlorophyll $a = 11.75\ A_{662} - 2.350\ A_{645}$

Chlorophyll $b = 18.61\ A_{645} - 3.960\ A_{662}$

Carotenoids = $1000\ A_{470} - 2.270$ Chl a - 81.4 Chl $b/227$

The results were expressed as mg of pigment per g of fresh mass (mg g^{-1} FM).

2.4 Extraction of the plant material

Extraction of phenolic compounds from dry leaves and fruits of cherry tomato was performed in reaction flasks using a 30 % aqueous solution of ethanol. The flasks were boiled at 60 ^{0}C for 1 hour using a reflux condenser. Extracts thus obtained were used for the estimation of the total content of phenolic and flavonoids, and total antioxidant capacity.

2.5 Estimation of total phenolic content

The total phenolic content of the extract was determined as follows: 0.25 ml of extract, 15 ml of distilled water, and 1.25 ml of Folin-Ciocalteu's reagent (diluted by distilled water in the ratio 1:2) was mixed into 25 ml flask. The mixture was incubated at room temperature for 15 min and then 3.75 ml saturated sodium carbonate solution was added. Flask was filled to the mark with 30 % ethanol and heated in water bath at 50 ºC, for 30 min. After cooling to room temperature absorbance was measured at 765 nm. The total phenolic content was calculated using a standard curve with gallic acid (0 - 500 mg l^{-1}), and results were expressed as mg of gallic acid equivalent per g dry mass (mg eq. GA g^{-1} DM).

2.6 Estimation of total flavonoids content

The total flavonoid content of the extract was determined by the aluminium chloride colorimetric assay as follows: 1 ml of extract was added to 10 ml volumetric flask containing 4 ml of distilled water and 0.3 ml 5 % $NaNO_2$. After 5 min. 0.3 ml 10 % $AlCl_3$ was added, and the mixture was incubated at room temperature for 6 min. Then 2 ml of 1 mol l^{-1} NaOH was added and the flask was made up to 10 ml with distilled water. The flask was incubated at room temperature for 15 min, and absorbance was read at 510 nm. The total flavonoid content was calculated using a standard curve with catechin (0 - 100 mg l^{-1}) end results were expressed as mg of catechin equivalent per g of dry mass (mg eq. C g^{-1} DM).

2.7 Estimation of total antioxidant capacity

The total antioxidant capacity of the extract was determined by ferric reducing antioxidant power (FRAP) assay as follows: 240 µl of distilled water, 80 µl of extract, and 2080 µl of FRAP reagent (reagent was obtained by mixing 0.3 mol l^{-1} acetate buffer (pH = 3.6), 10 mmol l^{-1} TPTZ (2,4,6-tripyridyl-s-triazine) and 20 mmol l^{-1} $FeCl_3$ x 6 H_2O in ratio 10 : 1 : 1) were added into a 10 ml Erlenmeyer flask and heated in water bath at 37 ºC, for 5 min and the absorbance was measured at 595 nm. The values of total antioxidant capacity were calculated using a standard curve with $FeSO_4$ x 7 H_2O (0 - 2000 µmol l^{-1}) and results were expressed as µmol Fe^{2+} per g of dry mass of extract (µmol Fe^{2+} g^{-1} DM).

2.8 Estimation of lycopene

Lycopene content was determined according to method of Davis et al. (2003) as follows: Approximately 0.3 to 0.6 g of the homogenized samples of cherry tomato fruits were weighed in Erlenmeyer flasks and 5 ml of 0.05 % (w/v) butylated hydroxytoluene (BHT) in acetone, 5 ml of ethanol and 10 ml of hexane were added. Samples were extracted on an orbital shaker for 15 min on ice. After shaking, 3 ml of deionized water were added to each flask and the samples were shaken for an additional 5 min on ice. Samples were then left at room temperature for 5 min to allow the separation of both phases. The absorbance of the upper layer was measured in a 1-cm-path-length quartz cuvette at 503 nm blanked with hexane, and results were expressed as mg lycopene per g of fresh mass (µg g^{-1} FM).

2.9 Individual flavonoid compounds extraction and analysis

The extraction of samples (5 g) was made in 10 ml of extracted solution (methanol + 3 % formic acid + 1 % m/v 2,6-di-tert-butyl-4-methyilphenol/BHT) according to Escarpa and Gonzales (2000).

Individual flavonoid compounds (naringenin and rutin) were analyzed by using Thermo Scientific Finnigan Surveyor HPLC-DAD system, controlled by a ChromQuest 4.0 chromatography workstation software system (Thermo Scientific, San Jose, CA, USA). Separation of flavonoid compounds was achieved by using Pursuit XRs 3 C-18 column (4.6 × 150 mm, 5 µm; Agilent Technologies, Santa Clara, CA, USA) operated at 25 ºC. The mobile phase consisted of the following linear gradient: 97 % acetonitrile + 3 % redistilled water + 0.1 % formic acid (A) and 97 % redistilled water + 3 % acetonitrile + 0.1 % formic acid (B). Sample injection volume was 20 µL and the flow rate was 0.6 ml min^{-1}. The sample was eluted in accordance with method described by Marks et al. (2007). Detection of flavonoid compounds was performed with a diode array detector (DAD) at 280 and 350 nm. Naringenin and rutin were identified on the basis of their retention times and addition of external standards and quantification was made according to concentrations of corresponding external standard and expressed as mg per 100 g of fresh mass (mg 100 g^{-1} FM).

2.10 Statistic data processing

All experimental measurements were carried out in triplicate and the results were expressed as mean ± standard deviation. The data obtained were processed by application of standard statistic methods of variance analysis (ANOVA) using Microsoft Excel 2013 software program, and the significant differences between the variants were determined using Least Significant Differences at 0.05 level of probability ($LSD_{0.05}$).

3 RESULTS AND DISCUSSION

3.1 Leaf water potential

Exposure of cherry tomato seedlings to drought stress caused a decrease in leaf water potential (Ψ) as compared with control (non-stressed seedlings), regardless of ANE treatment, and as expected, with the progression of the stress, plant water potential decreased. However, the reduction of Ψ being less pronounced for stressed plant treated by ANE (Figure 1), suggesting that this treatment helps the maintain homeostasis in plant cells under stress conditions.

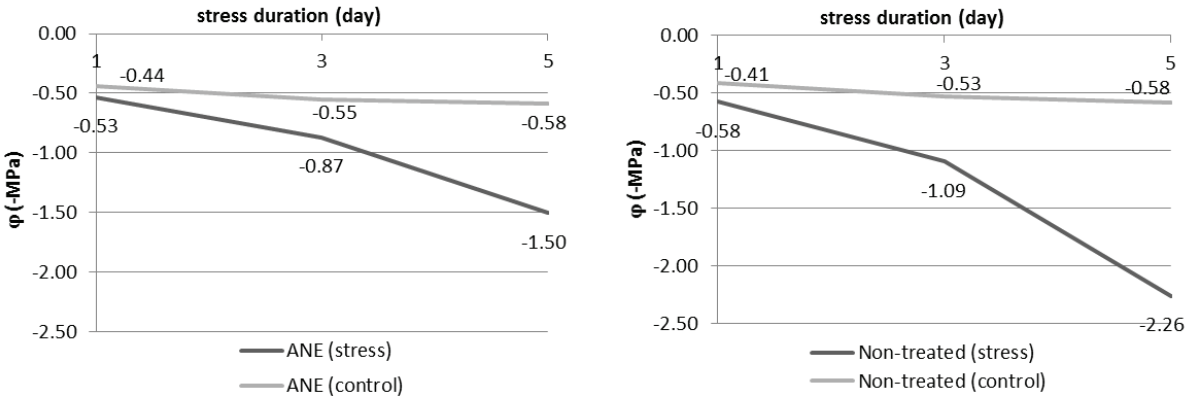

Figure 1: Leaf water potential (-MPa) depending on treatment with ANE and exposure to water stress

Numerous studies have also found that ANE application contributes to better osmotic adjustment of plants to stress (Khan et al., 2009; Ha et al., 2014). Karabudak et al. (2014) reported that substances such as glycine betaine and sterols present in ANE act as a buffer against major osmotic changes in plant cells and thus reduces negative effects of stress on plants. In addition, ANE contain many other osmolytes including amino acids: proline, valine, isoleucine and aspartic acid, vitamins and microelements and numerous other active natural substances that improve the stress tolerance of agricultural crops (Spann and Little, 2011).

3.2 Proline content

The results of the proline estimation indicated an increase of the proline level in leaves of all cherry tomato seedlings exposed to drought stress conditions. Furthermore, in experiment variant where cherry tomato seedlings were treated by ANE, the increase of proline in leaves during drought stress was much lower compared with non-treated seedlings grown under same conditions (Figure 2).

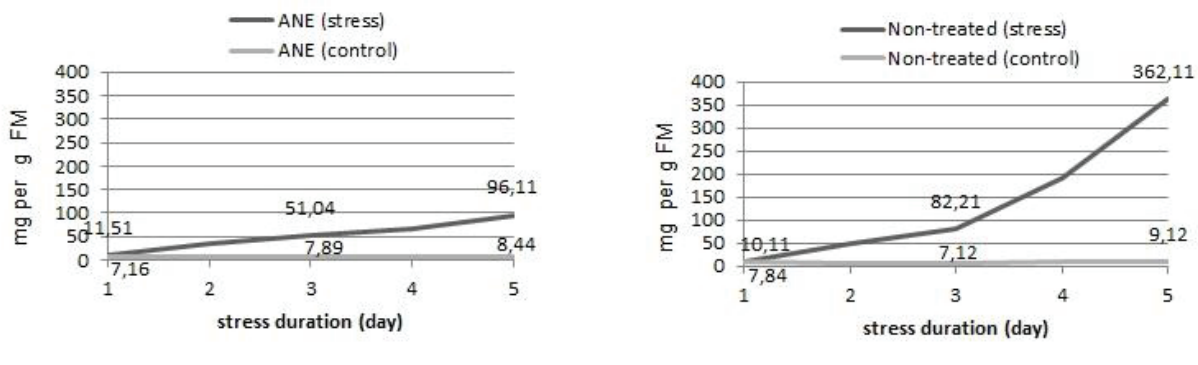

Figure 3: Proline content (μg g^{-1} FM) depending on the ANE treatment and exposure to water stress

Since the faster increase of proline content in leaves indicates plant stress, the results of the present study suggest that application of ANE contributes to a better osmotic adjustment of cherry tomato seedlings to stress conditions. Extracts from seaweed have also been reported to reduce drought stress in cultivation of vegetable, ornamental crops and grasses (Zhang and Ervin, 2004; Neily et al., 2010).

3.3 Photosynthetic pigments and leaf area

The results of the analysis of photosynthetic pigments showed that the content of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids in leaves of cherry tomato seedlings were decreased under drought stress conditions, regardless of ANE treatment (Table 1). This decrease was statistically significant for pigment Chl *a*, while for pigment Chl *b* and carotenoids were not. Numerous studies have also found that plant reduces the content of pigments in leaves under stress conditions (Ghorbanli et al., 2013; Yuan et al., 2016). Jaleel et al. (2009) reported that reducing the photosynthetic pigments content in leaves may be the result of the impairment in pigment biosynthesis or destruction of pigments due to disturbing in uptake of nutrients under drought stress conditions. Anjum et al. (2011) found that drought causes not only the reduction of the photosynthetic pigments content but it also leads to the destructive changes in the chloroplast, resulting in decreased photosynthetic capacity of plant.

The research results also indicate that ANE application contributes to higher synthesis of photosynthetic pigments in cherry tomato leaves and thus improving plant survival under subsequent stress. González et al. (2013) reported that the efficiency of seaweed extracts application to increase the content of pigments has been mainly attributed to large number of natural nitrogenous compounds present in seaweed which are important for the synthesis of chlorophyll pigments.

Table 1: Photosynthetic pigments content and leaf area of cherry tomato seedlings

Treatment	Chl *a* (mg g^{-1} FM)	Chl *b* (mg g^{-1} FM)	Carotenoids (mg g^{-1} FM)	Leaf area (cm^2)
V_1 ANE (stress)	1.36 ± 0.12^{bc}	0.46 ± 0.03	0.45 ± 0.01	16.06 ± 3.01^{bc}
V_2 ANE (non-stress)	1.51 ± 0.07^{a}	0.51 ± 0.10	0.53 ± 0.11	19.86 ± 5.88^{a}
V_3 Non-treated (stress)	1.13 ± 0.11^{d}	0.45 ± 0.05	0.46 ± 0.05	13.21 ± 2.95^{d}
V_4 Non-treated (non-stress)	1.38 ± 0.04^{b}	0.47 ± 0,05	0.47 ± 0.05	17.84 ± 3.54^{b}
$LSD_{0.05}$	0.095	-	-	2.186

Values expressed as main ± standard deviation.
Different letters in each column represent significant difference among variants at 0.05 level of probability

As shown in Table 1, leaf area in stressed cherry tomato seedlings were lower than in treatments where the seedlings were regularly watered. Many studies have shown the similar results about the effect of drought stress on leaf area (Jureková et al., 2011; Aldana et al., 2014). Galmés et al. (2013) reported that reducing the leaf area of plant under stress conditions is primarily result of reduction of cell enlargement and limited cell division due to lack of water.

The present data also showed that in stressful conditions, cherry tomato seedlings treated by ANE had a higher leaf area compared to untreated plants. This data indicates that the treated plants were less stressed, supporting the hypothesis that the application of ANE postpones and thus reduces negative effects of drought on cherry tomato seedlings.

3.4 Total phenolic, total flavonoid content, and antioxidant capacity of leaf extracts

A secondary effect of drought stress on plants is the increased of reactive oxigen species (ROS) such as superoxide radicals (O^{2-}), hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), and other oxidant substances (Appel and Hirt, 2004). In order to achieve balance between the production and scavenging of ROS, plants activate their defense systems, which include enzymatic and non-enzymatic systems. Plants have ability to synthesize a wide range of antioxidants that can contribute the strengthening of the defense system of the plant, and some of these substances are phenolic compounds. The protection activity of phenolic compounds is achieved mainly due to their redox potential, which allowed them to act as scavenger of free radicals (Atmani et al., 2009).

The results of this study showed that the total antioxidant capacity (FRAP), total phenolic (TPC) and total flavonoid content (TFC) were significantly higher in leaves of cherry tomato seedlings exposed to drought stress than in non-stressed seedlings (Table 2), suggesting that plant initiates the intensive synthesis of phenolic compounds as a response to drought stress, and this hypothesis has been confirmed by many scientists (Basu et al., 2010; Cramer et al., 2011).

Table 2: Total phenolic (TPC), total flavonoid content (TFC) and antioxidant capacity (FRAP) in leaves of cherry tomato seedlings

Treatment	FRAP (μmol Fe^{2+} g^{-1} DM)	TPC (mg g^{-1} DM)	TFC (mg g^{-1} DM)
V_1 ANE (stress)	140.03 ± 5.78^{a}	8.06 ± 0,44^{a}	4.08 ± 0.23^{a}
V_2 ANE (non-stress)	123.68 ± 3.29^{c}	7.13 ± 0,22^{b}	3.74 ± 0.19^{bc}
V_3 Non-treated (stress)	133.93 ± 5.63^{b}	7.07 ± 0,06^{bc}	3.86 ± 0.09^{b}
V_4 Non-treated (non-stress)	102.87 ± 1.68^{d}	5.99 ± 0,22^{d}	2.88 ± 0.09^{d}
$LSD_{0.05}$	5.70	0.427	0.127

Values expressed as main ± standard deviation.
Different letters in each column represent significant difference among variants at 0.05 level of probability

As shown in Table 2 cherry tomato seedlings treated by ANE have a higher content of phenolic and among them flavonoids in leaves of cherry tomato seedlings compared to non-treated seedlings, both in standard as well as stressful growth conditions. These effects can also be attributed to the specific chemical composition of ANE. It's well known that seaweed extracts contains amino acid phenylalanine, tyrosine and tryptophan which serve as precursor for the synthesis of a wide range of phenolic compounds, so it can be assumed that application of ANE promote the synthesis of phenolic compounds in cherry tomato plants.

3.5 Yield and quality parameters of cherry tomato fruits

In order to test how the exposure of cherry tomato seedlings to drought and application of ANE impact on the yield and quality parameters of cherry tomato fruits, the analysis of yield and quality parameters of fruit were carried out, and the results are shown in Table 3 and 4.

Table 3: Yield, total soluble solids (TSS), titratable acidity (TA) and vitamin C content of cherry tomato fruits

Treatment	Yield (kg per plant)	TSS (Brix)	TA (%)	Vitamin C (mg 100 g^{-1} FM)
V_1 ANE (stress)	1.77 ± 0.2^{bc}	6.59 ± 0.13^{ab}	0.64 ± 0.01^{b}	13.77 ± 1.34
V_2 ANE (non-stress)	2.41 ± 0.25^{a}	6.54 ± 0.09^{abc}	0.61 ± 0.02^{c}	13.33 ± 0.67
V_3 Non-treated (stress)	1.07 ± 0.8^{d}	6.66 ± 0.13^{a}	0.66 ± 0.02^{a}	13.66 ± 0.66
V_4 Non-treated (non-stress)	2.07 ± 0.41^{ab}	6.34 ± 0.12^{d}	0.62 ± 0.01^{c}	13.22 ± 1
$LSD_{0.05}$	0.395	0.126	0.019	-

Values expressed as main ± standard deviation.
Different letters in each column represent significant difference among variants at 0.05 level of probability

Table 4: Lycopene, total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (FRAP) of cherry tomato fruits

Variant	Lycopene (μg g^{-1} FM)	TPC (mg g^{-1} DM)	TFC (mg g^{-1} DM)	FRAP (μmol Fe^{2+} g^{-1} DM)
V_1 ANE (stress)	90.66 ± 2.69^{a}	10.56 ± 0.81^{a}	5.39 ± 0.49^{a}	188.35 ± 8.37^{ab}
V_2 ANE (non-stress)	88.25 ± 2.97^{ab}	9.48 ± 0.56^{c}	4.71 ± 0.44^{c}	155.9 ± 13.41^{c}
V_3 Non-treated (stress)	88.25 ± 2.12^{ab}	10.44 ± 1.06^{ab}	5.31 ± 0.34^{ab}	194.26 ± 10.82^{a}
V_4 Non-treated (non-stress)	86.17 ± 3.11^{b}	8.55 ± 0.37^{d}	4.26 ± 0.12^{d}	141.43 ± 4.90^{d}
$LSD_{0.05}$	2.47	0.617	0.346	12.91

Values expressed as main ± standard deviation.
Different letters in each column represent significant difference among variants at 0.05 level of probability

As shown in Table 3, total soluble solids, titratable acidity and vitamin C of fruits were higher in plants exposed to stress as compared to non-stressed plants, regardless of ANE treatment, but for vitamin C, that increase was not statistically justified. The total antioxidant capacity and the content of secondary metabolites such as lycopene, phenolic and flavonoids were also significantly higher in fruits of cherry tomato exposed to drought stress (Table 4). Besides, analyses of individual flavonoid compound indicate that the dominant flavonoid in fruits of cherry tomato naringenin and rutin, also accumulates more in cherry tomato fruits as response to drought stress (Table 5).

Table 5: Naringenin and rutin content of cherry tomato fruits

Treatment	Naringenin (mg 100 g^{-1} FM)	Rutin (mg 100 g^{-1} FM)
V_1 ANE (stress)	3.87 ± 0.36^{a}	6.62 ± 0.19^{b}
V_2 ANE (non-stress)	3.01 ± 0.21^{bc}	5.64 ± 0.08^{c}
V_3 Non-treated (stress)	3.29 ± 1.03^{b}	7.55 ± 0.07^{a}
V_4 Non-treated (non-stress)	2.89 ± 0.14^{c}	5.71 ± 0.63^{c}
$LSD_{0.05}$	0.306	0.217

Values expressed as main ± standard deviation.
Different letters in each column represent significant difference among variants at 0.05 level of probability

These results indicate that the contents of antioxidants in plant are closely related to the growth conditions and that their content in plant increases if the plant is exposed to controlled drought stress conditions. Many studies have shown the similar effect of drought stress on the content of antioxidant substances and generally secondary metabolites in tomato fruits (Atkinson et al., 2011; Giannakoula and Ilias, 2013). There is therefore no doubt that higher production of secondary metabolites is one of basic response of plant to controlled drought stress and conclusions of many studies support this hypothesis (Murshed et al., 2013; Okunlola et al., 2015). These observations are very interesting in terms of improving cherry tomato quality since that vegetable containing phytochemicals with high antioxidant power are drawing increased interest from consumers (Kubota et al., 2006).

The negative impact of drought stress on cherry tomato fruits in the present study was related to the yield, that was significantly lower in stressed plants, what was expected since the lack of water causes losses in tissue water content which reduce turgor pressure in cell, thereby inhibiting enlargement and division of cell, causing of reduce of yield. However, in experiment where cherry tomato seedlings were treated by ANE before exposure to stress, the yield was significantly higher compared to non-treated plants exposed to stress, indicating that this fertilizer reduces negative effects of drought on yield.

Furthermore, treatment of cherry tomato seedlings with ANE, has significantly contributed to increase the content of phenolic and flavonoids in fruits of cherry tomato under standard (non-stress) growth conditions, confirming that this fertilizer stimulate the synthesis of phenolic compounds, and thereby strengthen antioxidant defense mechanism of the plant. This fertilizer was also positively influenced by some quality parameters of cherry tomato (total soluble solids, content of ascorbic acid and lycopene) under standard growth conditions (non-stress) as compared to untreated plants.

4 CONCLUSIONS

ANE application in cherry tomato cultivation contributes to better adaptation of seedlings to drought conditions. The application of ANE also positively influenced by the yield and quality of cherry tomato under both standard and drought stress conditions as compared to untreated plants in same conditions. Positive effects of application of ANE are result of its specific composition, as well as ability of cherry tomato plants to utilize bioactive substances in seaweed extracts for its growth and development. The results of this study also indicate that the controlled exposure of cherry tomato plants to drought stress improves fruit quality, increasing nutritional components but decreasing yield.

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Effect of gold mining on total factor productivity of farmers: Evidence from Ghana

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ABSTRACT

Gold mining comes with several benefits to developing countries, manifested mainly in the form of employment and revenue, but simultaneously impacts negatively on the immediate environment. It affects the economic structure including agriculture and its productivity. Hence, this study investigated the effect of gold mining on total factor productivity of farmers in Ghana using 110 cocoa farmers from Asutifi North and Asutifi South districts of the Brong Ahafo Region, categorised into mining and non-mining areas respectively. About 83 % of the farmers in the mining areas were affected by gold mining through channels such as land disputes, relocation of farm/residence, high cost of labour, illegal small-scale mining and dust settlement on crops. Also, about 64 % of cocoa farmers in the mining areas lost their farm lands (between 0.4 and 3.64 ha as a result of gold mining. The Tornqvist Total Factor Productivity (TFP) indices for cocoa farmers in the non-mining areas (mean TFP of 1.404) were also statistically higher than those in the mining areas (mean TFP of 0.371). The study concluded that gold mining activities adversely affect productivity of farmers in the catchment areas. The study recommends, among others, that a policy of land-for-land should be in place and effectively implemented to ensure that mining companies in order to enhance and ensure continuity of livelihoods must fully replace lands lost through mining activities.

Key words: gold mining; total factor productivity; cocoa; Tornqvist; Ghana

IZVLEČEK

VPLIV ZLATOKOPOV NA CELOKUPNI DEJAVNIK PRODUKTIVNOSTI KMETOV: PRIMERI IZ GANE

Zlatokopi prinašajo v dežele v razvoju številne koristi, ki se kažejo v obliki zaposlitev in prihodku, a imajo hkrati negativne učinke na neposredno okolje. Vplivajo na gospodarstvo, vključno s kmetijstvom in njegovo produktivnostjo. V raziskavi je bil na osnovi ankete med 110 pridelovalci kakava na območjih Asutifi North in Asutifi South, regije Brong Ahafo preučevan vpliv zlatokopov na skupno produktivnost kmetov v Gani, ki so bili razdeljeni na območja z in brez rudarjenja. Okrog 83 % kmetov na območjih z rudarjenjem je bilo prizadetih zaradi te aktivnosti in sicer zaradi prepirov za zemljišča, premestitev kmetij/bivališč, velikih stroškov dela, ilegalnega malopovršinskega rudarjenja in usedanja prahu na posevke. Okrog 64 % pridelovalcev kakava je na območjih z rudarjenjem izgubilo svoja kmetijska zemljišča (od 0,4 do 3,64 ha kot posledica zlatokopov). Indeksi Tornqvistove skupne faktorske produktivnosti (TFP) pridelovalcev kakava so bili na območjih brez rudarjenja statistično značilno večji (poprečje TFP = 1,404) kot na območjih z rudarjenjem (poprečje TFP = 0,371). V raziskavi je bilo ugotovljeno, da zlatokopi negativno vplivajo na produktivnost kmetov na preučevanem območju. Na osnovi raziskave lahko priporočamo med drugim, da je učinkovita uporaba doktrine menjave zemljišča za zemljišče primerna, da zagotovi, da se ob delovanju rudarskih družb spodbuja in zagotavlja kontinuiteta kmetijstva preko popolne nadomestitve zemljišč, izgubljenih zaradi rudarjenja.

Ključne besede: zlatokopi; skupna faktorska produktivnost; kakav; Tornqvist, Ghana

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1 INTRODUCTION

Ghana's agriculture is vastly dominated by smallholder famers; many commodities including cocoa, maize, cassava and yam produced predominantly on small farms. According to Chamberlin (2007), more than 70 % of Ghanaian farms are 3 hectares (ha) or smaller in size and cocoa and maize represent the two most cultivated crops in Ghana by smallholder farmers (Millennium Development Authority (MiDA) 2010; Asuming-Brempong et al., 2007).

Cocoa takes a remarkable position in Ghana's economy since it has long played an important role in Ghana's economic development and remains an important source of rural work and national income. It also remains the country's most important agricultural export crop (Asuming-Brempong et al., 2007; International Cocoa Organization (ICCO) 2010; Boadi-Kusi et al., 2016). Ghana is currently the world's second major producer of cocoa beans, after Cote d'Ivoire with 21 % share of world cocoa production. Cocoa provides the second largest source of export earnings after gold, representing about 19 % of Ghana's total export earnings in 2015 (Ashitey, 2012; ISSER, 2016).

Mining has also been an important component of developing country economies. The grandness of the mining sector, particularly gold mining in the economy of Ghana has increased considerably since the 1980's (Akabzaa, 2009). The Ghana Chamber of Mines report in 2008 indicated that, mining activities generated around 45 % of total export revenue, 12 % of government's fiscal revenue and attracted almost half of Foreign Direct Investment (FDI). Gold exports revenue in 2015 represented 41 % of the total exports of Ghana, followed by cocoa beans, which account for 19 % (Observatory of Economic Complexity, 2016). This mining expansion has been attributed to the structural reforms in the 1980s that encouraged foreign investment in large-scale mines, especially in gold mining (Ghana Chamber of Mines, 2008; Akabzaa, 2009). Ghana has a long tradition of gold mining with an estimated 2,488 metric tons (80 million ounces) of gold produced between the periods of 1493 to 1997. It is the second largest gold producer in Africa, after South Africa, the third-largest African producer of aluminium metal and manganese ore and a significant producer of bauxite and diamond (Coakley, 1999). Mining, specifically gold mining, has contributed immensely to the economy of Ghana through employment generation, attracting foreign direct investment, contributing to export earnings and Gross Domestic Product (GDP).

Gold mining has effects on economic, social, environmental, agricultural and food security of the communities in which the mining takes place (Amankwah & Anim-Sackey, 2003). A lot of studies have established linkages between mining and agriculture with the effects of gold mining either beneficial or detrimental to the affected population or communities. Despite these linkages, the impact of mining on agriculture has not been extensively studied in Ghana especially at the micro level and available results are mixed. According to Mining Facts (2012) (a Resource for Canadian Mining Information), agriculture is growing in some areas as a result of mining and declining in others, depending on local circumstances. According to Aragon and Rud (2013) and Van der Ploeg (2011), most modern mines in the developing world are located in rural areas, where agriculture is noted to be the main source of livelihood and thereby having both direct and indirect effects on them. Gold mining and agriculture are linked directly through the dependence on same or similar inputs (land, water resources and labour). The competition between gold mining and agriculture for key inputs (such as land and labour) and environmental pollution from mining creates potential negative spillovers to farmers (Aragon & Rud, 2013). They are also indirectly linked where mining firms have improved infrastructure in a way that supports agricultural development. A study by Cartier and Bürge (2011) found that mining has the potential to kick-start local economic development, such as agriculture and service oriented industries and also concluded that small-scale agriculture and mining are not livelihood alternatives, but are instead livelihood complements and therefore have the potential to contribute to more sustainable rural livelihoods.

In Ghana, gold mining coincidentally takes place in rural communities/areas where lands earmarked for gold mining are arable lands that farmers cultivate or have reserved for future use. Gold mining therefore reduces farmers' access to their farmlands and degrades the environment where farm lands are located (Aragon & Rud, 2013) and these factors have the potential to affect the productivity of farmers.

There have been concerns over the low productivity and environmental impacts on cocoa production which makes the long-term sustainability of the sector uncertain (Gockowski, 2007). Average annual cocoa yield in Ghana is about 400 kg ha^{-1} in recent years and this is among the lowest in the world compared to countries such as Cote d'Ivoire (800 kg ha^{-1}) and Malaysia (1880 kg ha^{-1}). The low productivity has been attributed to environmental conditions (climatic and atmospheric) and other factors such as hybrid seed type, input variables and cultural practices (Kolavalli & Vigneri, 2011; Tom-Dery et al., 2012). Gockowski (2007) showed that cocoa production has focused on land expansion and intensive use of labour rather than on land productivity. Thus output has increased mainly due to increase in area cultivated and partly due to increase in yield. The arable land and labour used for this expansion is also competed for by mining companies (Aragón & Rud, 2013). Some farmers as a result have portions of their farmlands and others their whole farmlands taken over by mining companies. Talule and Naik (2014) indicated that farmers experienced dust settlement on plantations after gold mining was started in the state of Goa, India which impeded crop growth. These conditions (competition for land and labour, pollution from gold mining) have the potential to reduce crop productivity. There is therefore growing concerns with regard to the real benefits of gold mining to the ordinary Ghanaian farmer in the gold mining communities as it affects their welfare and productivity (Akabzaa, 2009). Though, gold mining and agriculture have all contributed immensely to the economy of Ghana in general, whether local farmers benefit in any way from gold mining activities within the catchment communities is not well established. Instead, environmental regulators and opponents of the mining industry have focused mostly on other aspects such as risk of environmental degradation, health hazards, and social impacts. What is lacking in the policy debate, however, is the crowding out mechanisms such as loss of land and agricultural output through gold mining. Does gold mining in Ghana reduces farmers' productivities in gold mining areas? This study seeks to determine the factors through which gold mining affects the total factor productivity of farmers in mining and non-mining areas of Ghana.

2 MATERIALS AND METHODS

2.1 Total factor productivity

The two most widely adopted methods employed in agricultural productivity estimations are the superlative index approach and the quantity-only based index approach (Bjurek, 1996; Førsund, 1997). The advantage of using the superlative index method is more apparent when it comes to the issue of aggregation consistency: the superlative index method is robust to various levels of disaggregation while the quantity-only index is not (Sheng et al., 2014). A number of different types of economic indices using the superlative index approach exist. Each type of index offers an approximate scalar measure of a multidimensional change over time in prices, quantities or productivity. The different indices approximate these inter-temporal changes in different ways according to their theoretical properties. Differences in indices can be viewed as differences in their abilities to provide approximations to the inter-temporal changes in prices, quantities or productivity.

Four economic index numbers are commonly applied in estimating economic index: Laspeyres, Paasche, Fisher Ideal, and Törnqvist. These indices produce different methods of approximation (reflected in the formulae of their aggregator functions) with correspondingly different properties. The Laspeyres and Paasche indices have traditionally been widely applied, but the Tornqvist and Fisher Ideal are increasingly used. The Laspeyres, Paasche and Fisher output quantity indices can be defined as follows, using the quantity aggregates given in Equation (1) – (3) respectively

$$Q_{st}^{L} = \sum_{i=0}^{N} p_{is} q_{it} \Big/ \sum_{i=0}^{N} p_{is} q_{is} \tag{1}$$

$$Q_{st}^{P} = \sum_{i=0}^{N} p_{it} q_{it} \Big/ \sum_{i=0}^{N} p_{it} q_{is} \tag{2}$$

$$Q_{st}^{F} = \sqrt{Q_{st}^{L} \times Q_{st}^{P}} \tag{3}$$

Where $q_{it} = [\, q_{it}, \ldots\ldots q_{Nt}\,]$ and $p_{it} = [\, p_{it}, \ldots\ldots p_{Nt}\,]$ are output and output price vectors respectively; *t* and *s* denote time or period or firms; *i* = [1,..... *N*] are different outputs. Thus, p_{it} is the price of i-th good in t-th period or firm and q_{it} is the quantity of i-th good in t-th period or firm. The input indices, Laspeyres ($Q_{st}^{L^*}$), Paasche ($Q_{st}^{P^*}$), and Fisher ($Q_{st}^{F^*}$) are obtained in a similar fashion and the ratio of output index to the corresponding input index gives the Total Factor

Productivity (TFP) index. Therefore, in general terms, *TFP is* expressed as $TFP^a = Q^a / Q^{a^*}$ where a = [P, L, F] representing Paasche, Laspeyres and Fisher.

The Tornqvist (or Translog) index is an alternative index, which is the weighted average of growth rates of microeconomic data. For the output quantity index, this is expressed as follows:

$$Q_{st}^{T} = \prod_{i=1}^{N} \left[\frac{q_{it}}{q_{is}} \right]^{\frac{\omega_{is}+\omega_{it}}{2}} \tag{4}$$

$$\ln Q_{st}^{T} = \sum_{i=1}^{N} \left[\frac{\omega_{is}+\omega_{it}}{2} \right] \left(\ln q_{it} - \ln q_{is} \right) \tag{5}$$

$$\ln Q_{st}^{T} = \frac{1}{2} \sum_{i=1}^{N} \left[\left(P_{is} q_{is} / \sum_{i=1}^{N} P_{is} q_{is} \right) + \left(P_{it} q_{it} / \sum_{i=1}^{N} P_{it} q_{it} \right) \right] \ln \frac{q_{it}}{q_{is}} \tag{6}$$

The ratio of $ln\, Q_{st}^{T}$ to its input counterpart ($ln\, Q_{st}^{T^*}$) provides the Tornqvist TFP index. Fisher is geometric average and hence may also be a good approximation of TFP. However, Tornqvist uses share weights often expressed in log-change form for calculation. Tornqvist is thus a geometric weighted average, while Laspeyres and Paasche are arithmetic and harmonic averages, respectively.

$$TFP^{T}\, Index_{st} = \frac{Output^{T}\ index_{st}}{Input^{T}\ index_{st}} \tag{7}$$

$$\ln TFP^{T}\, Index_{st} = \ln Output^{T}\ index_{st} - \ln Input^{T}\ index_{st} \tag{8}$$

$$\ln TFP_{st}^{T} = \frac{1}{2} \sum_{i=1}^{N} \left(\omega_{is} + \omega_{it} \right) \left(\ln q_{it} - \ln q_{is} \right) - \frac{1}{2} \sum_{j=1}^{K} \left(\delta_{js} + \delta_{jt} \right) \left(\ln x_{jt} - \ln x_{js} \right) \tag{9}$$

N is number of outputs and K is the number of inputs, q is output quantity, x is input quantity, ω denotes output revenue share and δ denotes input cost share. This approach (equation 9) of estimation is also known as the Hicks-Moorsteen Approach and defines productivity index simply as the ratio of output and input index numbers (Diewert, 1992).

According to Diewert (1976), there are two methods used to assess the suitability of an index formula and they are; economic theory or functional approach (exact and superlative index number) and axiomatic or Test approach (index numbers that satisfy a number of desirable properties). The Tornqvist and Fisher indices provide more accurate approximations to changes than the Laspeyres or Paasche index because intermediate substitution possibilities are incorporated. According to the index number theory, Tornqvist and Fisher Ideal indices are a group of index numbers whose underlying formula, as shown in equation 3 and equation 9, provides a second order differential approximation to any unknown production function (Diewert, 1976) and these indices can be interpreted as a production function shift (Technical change) if we assume technical efficiency, allocative efficiency and constant return to scale. This second order flexibility makes the Fisher and Tornqvist indices 'superlative' indices (Mishra & Pujari, 2008). Diewert (1976) demonstrated that the Tornqvist index is an exact index for (*i.e.* is consistent with) a "translog" structure of production whiles fisher is exact for quadratic. But the Laspeyres and Paasche employs simplistic linear production function. The merits of the translog production function include the fact that it places fewer restrictions on input (and output) relationships than other functions (Dean et al., 1996).

The Tornqvist index satisfy almost all the basic and commonly used axioms (positivity, proportionality, continuity, units invariance, time-reversal, mean value, factor). However, the axiom of circularity (transitivity) and factor reversal test are not satisfied by the Tornqvist index but the factor reversal test it is not considered very serious and important (Diewert, 1992; Mishra & Pujari, 2008). The non-transitive indices are transformed into transitive ones by applying the Elteto-Koves-Szulc (EKS) transformation. The transitive property is very important for a proper comparison between various time periods or among various cross-sections (Diewert, 1992; Mishra and Pujari, 2008). EKS method constructs geometric mean of all indirect comparisons via the N firms in the sample. EKS adjustment is a minimum mean squared deviation from original index. It is expressed as

$$\mathrm{I}_{st}^{Transitive} = \prod_{r=1}^{N} \left[\mathrm{I}_{\mathrm{sr}} \times \mathrm{I}_{\mathrm{rt}} \right]^{\frac{1}{N}} \tag{10}$$

$$\ln TFP_{st}^{Transitive} = \left[\frac{1}{2}\sum_{i=1}^{M}\left(\omega_{it}+\overline{\omega_i}\right)\left(\ln q_{it}-\overline{\ln q_i}\right) - \frac{1}{2}\sum_{i=1}^{M}\left(\omega_{is}+\overline{\omega_i}\right)\left(\ln q_{is}-\overline{\ln q_i}\right)\right] - \left[\frac{1}{2}\sum_{j=1}^{K}\left(\delta_{jt}+\overline{\delta_j}\right)\left(\ln x_{jt}-\overline{\ln x_j}\right) - \frac{1}{2}\sum_{j=1}^{K}\left(\delta_{js}+\overline{\delta_j}\right)\left(\ln x_{js}-\overline{\ln x_j}\right)\right] \tag{11}$$

where

$$\omega_{is} = p_{is}q_{is} \Big/ \sum_{i=1}^{M} p_{is}q_{is} \qquad \delta_{js} = p_{js}x_{js} \Big/ \sum_{j=1}^{K} p_{js}x_{js}$$

q_i denotes output, x_j denotes inputs and the p_i and p_j are the output price and input cost respectively. The bars refer to sample means. The transitive Tornqvist can be calculated directly using equation 11. In productivity studies, the Fisher index has been used less frequently than the Törnqvist. However, the Tornqvist index method has been preferred by many researchers in the area of productivity measurement and analysis because of the desirable properties outlined above (Dean et al, 1996; Ali & Iqbal, 2004). Tornqvist Total Factor Productivity approach therefore was used to estimate the TFP index of various respondents for this study.

The Tornqvist TFP has been used by several researches after its development but mostly at macro levels with few at the micro level (Mishra & Pujari, 2008). Kumar and Mruthyunjaya (1992) analysed the TFP growth of wheat in India. They used the Divisia-Tornqvist index to compare the total output, total input, TFP and input price indices for wheat grown in the major states of India, based on micro-level data. Coelli (1996) investigated productivity growth in agriculture in Western Australia using Tornqvist indices using three output groups (crops, sheep products and other) and five input groups (livestock, materials and services, labour, capital and land) from 1953/4 to 1987/8. The total factor productivity was observed to grow at an average annual rate of 2.7 %. Rosegrant and Evenson (1992) assessed the sources of TFP growth in the crops sector in India, and compared the same with Pakistan and Bangladesh. They used the Tornqvist index to analyse TFP for 271 districts in India from 1956 to 1987 and the study covered five major and fourteen minor crops. They concluded that the main sources of productivity growth have been public research and extension and private research. Sidhu and Byerlee (1992) analysed technical change and wheat productivity in Punjab, in the post-Green Revolution period and found that the use of inputs such as fertilizers and herbicides increased from the 1970s to the 1980s but the use of labour-saving technologies such as tractors increased rapidly which was also synonymous with the TFP changes. Kumar and Rosegrant (1994) assessed TFP growth in 15 states of India and examined the sources of productivity growth. They used the Divisia Tornqvist index for computing the total output, total input and TFP indices for rice, using farm-level data from 1971 to 1988. They found TFP and growth in crop inputs to have contributed roughly 3.5 per cent per year to rice production growth and have enabled India to increase rice production per capita in the presence of high population growth rates and limited land resources within the period.

2.2 Study area

The Brong Ahafo Region, as shown in Figure 1 is the second largest region in Ghana with a land area of 39,557 km^2 and 27 administrative districts/municipalities. It covers 16.6 % of the country's total land area. The region has an estimated population of 2,310,983 (2010 census) and located within longitude 00 15' E-30 W and Latitude 80 45' N-70 30' S in the west central part of Ghana which is in the transition zone of Ghana. The transition zone stretches across the centre of the country from East to West, where soils are deep, friable, and well drained, and there is less dense forest cover. It has a bi-modal rainfall with average annual rainfall and temperature of 1,300 mm and 27 ^{0}C respectively. The productive soil and bimodal rainfall season permit all year round cocoa production.

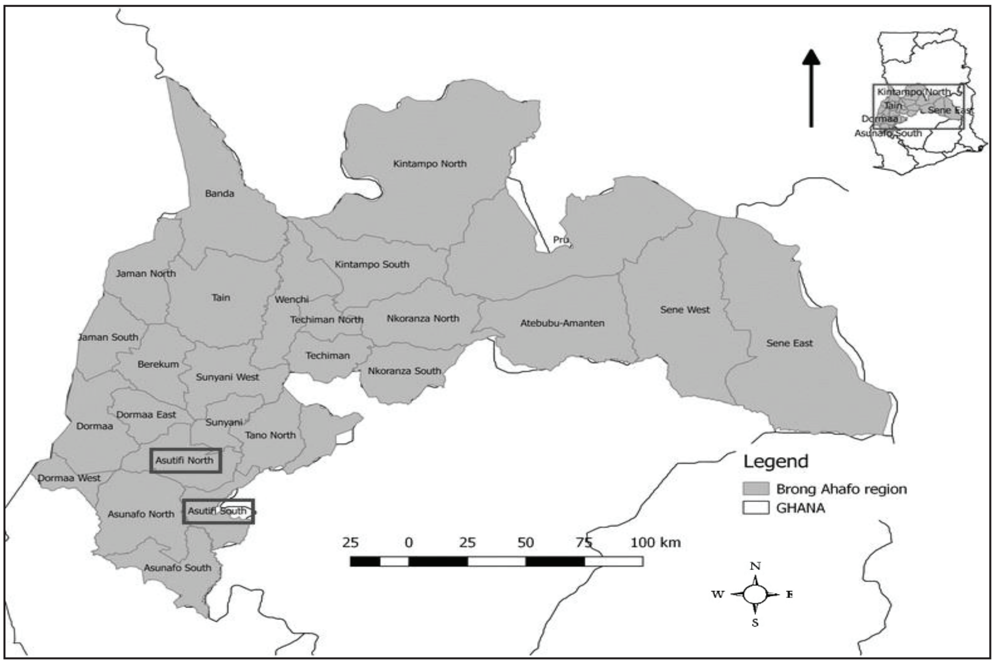

Figure 1: Map of Brong Ahafo Region of Ghana
Source: Geography Department, University of Ghana

With an arable land area of 23,734 km^2 (60 % of land area) and land under cultivation being 9,746 km^2 (41 % of arable land area), opportunities exist to expand cultivated land and improve productivity (MOFA, 2006; MOFA, 2013). Agriculture plays a very important role in the region's economy as it engages 61 % of the population. The various farming systems/methods practiced by the farmers in the region include; shifting cultivation, continuous cropping, mixed cropping, mono cropping, inter cropping, land rotation and bush fallows. Some of the major crops cultivated include yam, cassava, maize, cocoyam, rice, potato, pepper, plantain, garden eggs, okra, watermelon, ground nut, cowpea, and other tree crops such as cocoa, cashew and mango. Some non-traditional farming activities practiced by the farmers include grass cutter rearing and bee-keeping. Gold mining is also one of the economic activities in the region with Newmont Gold Ghana Limited (NGGL) being the largest gold mining company situated in the Asutifi North and South Districts (Ghana Statistical Service 2010; Ghanadistrict.com 2014).

Figure 2: The map of Africa showing Ghana
Source: https://www.pinterest.com/pin/157414949459332821/

2.3 Data collection

A pretested structured questionnaire was used to collect data on output, input and relevant socioeconomic variables of cocoa farmers from January to February 2015 and covered both the major and minor seasons of 2013/2014 cropping year. A multi-stage sampling technique was employed in this household survey. Purposively, Asutifi North and South districts were chosen because its land area falls within the forest agro-ecological zones of the Brong Ahafo region where cocoa production is concentrated and all the communities located close to the operational areas of Newmont Gold Ghana Limited (NGGL) involved in cocoa production. Respondents were farmers in both the mining and non-mining areas. The farmers in mining areas were farmers who have their farms around the operational area of NGGL. The farmers in the non-mining communities were farmers with their farms located at least 10 kilometres from the operational area of NGGL such that they do not experience any direct impact or effect of mining operations, such as hauling through or around their farms and dust from mining operations settling on their crops. Finally, a simple random sampling of cocoa farmers from each community was employed, resulting in 110 cocoa farmers. Table 1 shows the distribution of respondents by communities (69 from the mining areas and 41 from the non-mining areas).

Table 1: Communities and number of cocoa farmers sampled

District	Community/Town	Mining	Non-mining	Total
Asutifi North	Kenyasi	40	0	40
	Ntotoroso	29	0	29
	Obengkrom	0	19	19
	Sub-Total	69	19	88
Asutifi South	Amanfrom	0	5	5
	Achirensua	0	11	11
	Nkasiem	0	6	6
	Sub-Total	0	22	22
	Total	***69***	***41***	***110***

Source: field survey, 2015

TFPIP 1.0 software developed by Coelli (1997) was employed to estimate the transitive TFP indices. The variables used in the estimation include the output and output prices as well as input and input cost of cocoa produced in 2013/14 production year. Cocoa output (2013/14 production year) was measured in kilograms and output price is measured in Ghana Cedis per kilogram. Labour is captured based on the total man-days employed by the i-th farm during the production year. One man-day for labour is calculated as one adult male working for one day (8 hours); one female working for one day (8 hours) equals 0.75 man days. Seedling is the quantity of seedling used by the i-th farmer for the production year, measured in number for cocoa seedlings and price per seedling is measured in Ghana Cedis. Total quantity of weedicide, fungicide and insecticide used by the i-th farmer measured in litres. The price per litre is measured in Ghana Cedis.

2.4 Productivity differences among farmers in mining and non-mining areas

The assumption underlying the differences in cocoa productivity is that productivity should be the same for farmers in the mining and non-mining areas in the absence of gold mining since they are in the same agro-ecological (transitional) zone, experience similar environmental and climatic conditions and encounter the same input market and cocoa output market arrangements and challenges. To determine whether there is productivity difference, the study adopts and performs a number of *z*-tests (of equality of means) to analyse whether farmers in the non-mining communities are more productive than those in mining communities. The mean values of the Tornqvist TFP, inputs and output indices are estimated and their mean differences are statistically examined. In the determination of the differences in the values of the means in the two areas, the z-test used for the analysis is given as:

$$Zcal = \frac{\bar{y}_2 - \bar{y}_1}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}} \qquad (12)$$

where $\bar{y}_1$ and $\bar{y}_2$ are the mean TFP index of the mining and non-mining areas respectively, s_1 and s_2 are the standard deviations of the two samples, n_1 and n_2 are the sizes of the two samples.

2.5 Statement of hypothesis

$H_0: \bar{y}_1 = \bar{y}_2$ there is no significant difference between the mean TFP index of farmers in mining and non-mining areas

$H_1: \bar{y}_1 < \bar{y}_2$ the mean TFP index of farmers in mining area is significantly less than the mean TFP of farmers in non-mining area

This hypothesis is repeated for the output and input indices. The decision rule is that if z_{cal} is greater than (in absolute terms) the z_{crit}, then we reject the null hypothesis (H_0) in favour of the alternate hypothesis (H_1).

3 RESULTS AND DISCUSSION

3.1 Socio-demographic characterisation of respondents

Males represent the majority (68 %) of the respondents which affirms the dominance of males in cocoa production, mostly because of the laborious and cost intensive nature of cocoa farming which discourages most females from investing into cocoa production. Also, in Ghana, land is mostly owned and controlled by the male head of the household which also gives them an advantage. From Table 2, the age of cocoa farmers ranges between 20-85 years with a mean age of 50 years. The majority of cocoa farmers (51) fall between 41 to 50 years, representing 46 % of the respondents. One can infer from these results that most cocoa farmers in the study area are in their economically active (15-60 years)[1] ages and this implies that quality of labour is good which may positively affect their productivity. Diverse age groups cultivate cocoa therefore improvement in cocoa productivity will positively affect livelihoods. The majority (41 %) of the respondents have completed middle school or junior high educational level. However, 28 % of the farmers had no formal education at all. In general, about 72 % of the farmers had access to some level of formal education. The educational level of farmers is known to affect farming activities. The majority (67 %) of the respondents have a household size between 5 and 9 and one household (1 % of the respondents) has a household size of 15 people. The mean household size is seven (7). A greater percentage (40 %) of the farmers in the study

[1]Ghana Statistical Service (GSS) (2012) definition for economically active age

area had farm sizes less than 2.02 ha. This suggests that the majority of the farmers are peasant and small-scale farmers. However, as shown in Table 2, very few cocoa farmers cultivated between 6.47 – 8.09 ha (8 %) and above 8.09 ha (3 %).

Table 2: Socio-demographic characteristics of the respondents

Socioeconomic variables	Item	Cocoa Farmers Mining area	Percent	Non-mining area	Percent	Total	Percent
Sex	Female	23	20.91	12	10.91	35	31.82
	Male	46	41.82	29	26.36	75	68.18
Age (years)	20-30	5	4.55	1	0.91	6	5.50
	31-40	10	9.09	12	10.91	35	31.80
	41-50	22	20.00	8	7.27	51	46.40
	Above 50	32	29.09	20	18.18	18	16.40
	(Minimum = 25 Mean = 49.8 Maximum = 85)						
Education	No Schooling	18	16.36	13	11.82	31	28.18
	Primary	7	6.36	7	6.36	14	12.73
	JHS/MSLC	28	25.45	17	15.45	45	40.91
	SHS/O/A Level	14	12.73	3	2.73	17	15.45
	Technical/Vocational	1	0.91	1	0.91	2	1.82
	Tertiary	1	0.91	0	0.00	1	0.91
Household size	1-4	7	6.36	7	6.36	14	12.73
	5-9	48	43.64	26	23.64	74	67.27
	10-14	14	12.73	7	6.36	21	19.10
	Above 14	0	0.00	1	0.91	1	0.90
	(Minimum = 1 Mean = 7 Maximum = 15)						
Land size (ha)	<5	26	23.64	18	16.36	44	40.00
	6-10	23	20.91	11	10.00	34	30.91
	4.45 – 6.07	13	11.82	7	6.36	20	18.18
	6.47 – 8.09	4	3.64	5	4.55	9	8.18
	Above 8.09	3		0	0.00	3	2.73
	(Minimum = 0.5 Mean = 7.2 Maximum = 55)						

Source: field survey 2015

3.2 Perceived effects of gold mining on crop production

The majority (83 %) of cocoa farmers in mining areas (69 farmers) indicated that gold mining has affected their crop production. According to them, the channels through which they have been affected included high cost of farmland, high cost of labour, and relocation of farm/residence, illegal small-scale gold mining, land disputes, and settlement of dust on their crops. As shown in Figure 3, Relocation of farm/residence (32 %) and high cost of farmlands (26 %) were the major channels through which gold mining has affected cocoa farmers. This confirms a study by Taphee et al. (2015) on the economic efficiency of cocoa production which concluded that high cost of production per hectare was a major problem to cocoa farmers in Ondo State, Nigeria. The same reasons were given by Schueler et al. (2011) on the study of the impacts of surface gold mining on land use systems in Western Ghana where farmers described their livelihood situation after relocation as worse, due to the loss of their traditional farmlands and inadequate compensation schemes from mining companies. Another 4 % and 9 % of cocoa farmers in the mining areas mentioned illegal small-scale mining and settlement of dust on their crops respectively as impacting negatively on cocoa productivity. Dust settlement on cocoa leaves inhibit the growth as well as injuring the plants and thereby reducing the productivity.

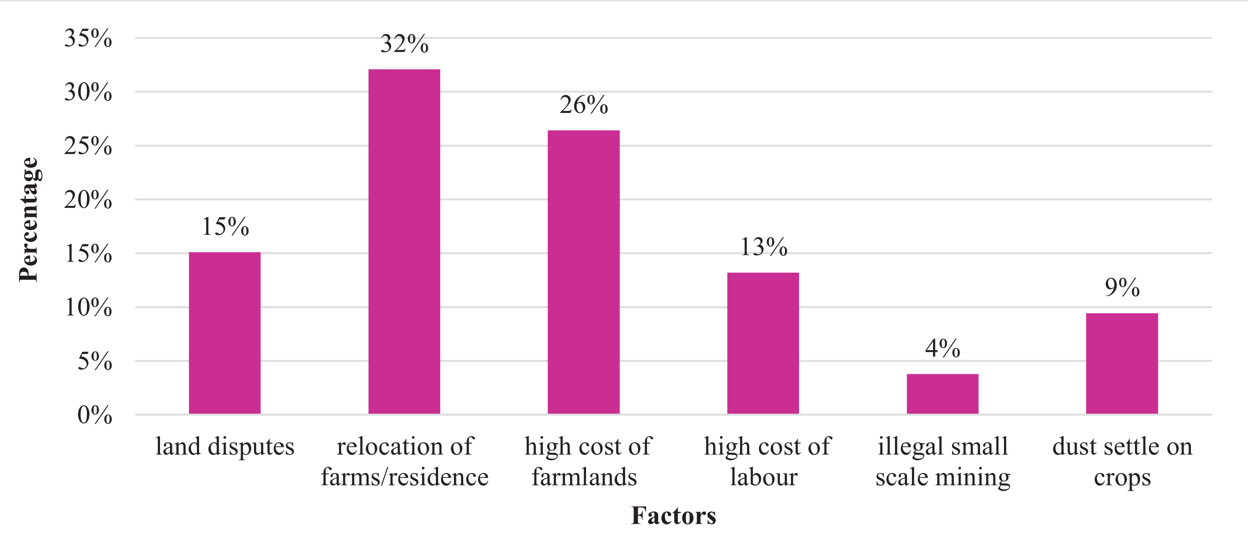

Figure 3: Factors affecting cocoa farming and productivity
Source: field survey 2015

Figure 4 shows the average reduction in cocoa farm sizes because of the commencement of gold mining operations in the mining areas. About 36 % of cocoa farmers in the mining areas indicated that their farm sizes have not reduced as a result of the gold mining. However, the rest of the farmers (64 %) in the mining areas mentioned various reductions in farm acreages. About 52 % indicated their farmlands reduced by 0.4 to 1.21 ha. The study by Mumuni et al. (2012) found similar results where an estimated 9,575 individual crop farmers in the Asutifi North and South districts lost 7,500 hectares of farmlands, an average of 0.8 ha per farmer which were annexed by Newmont Gold Ghana Limited for gold exploration.

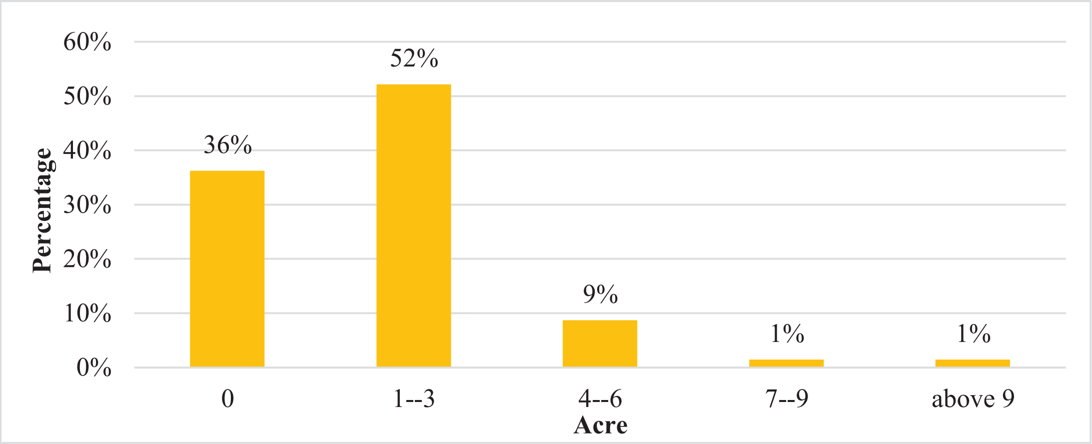

Figure 4: Cocoa farm size lost/reduced through gold mining
Source: field survey 2015

3.3 Total factor productivity (TFP) in mining and non-mining areas

Table 3 presents the summary statistics of the estimated input, output and TFP indices. In general, the study finds that, farmers have higher averages of indices in non-mining areas as compared to the mining areas.

Table 3: Summary statistics of Tornqvist total factor productivity indices

Observation	Area *Mining*	*Non-Mining*	Total
Mean TFP Index	0.371	1.404	0.756
Standard Deviation	0.463	1.512	1.106
Mean Output Index	0.430	1.193	0.714
Standard Deviation	0.641	1.610	1.160
Mean Input Index	1.755	1.770	1.760
Standard Deviation	3.068	2.900	2.993

Source: field survey 2015

Table 4 shows the summary of the compared means. The mean difference between the input indices for farmers in the two categories was not statistically different and thus the null hypothesis is not rejected. The reasons may likely be that both farmer groups have access to same input types and prices from same markets and also utilise similar input amounts. For the output and TFP index, the mean differences were statistically significant at 1 % significance level. The Output and Tornqvist TFP indices were higher in non-mining areas than in mining areas (see Table 3). Since there is not any difference between the inputs index between farmers in mining and non-mining areas, the difference in the output and TFP could likely be attributed to the fact that gold mining has significantly contributed to lower cocoa productivities of farms in the mining areas mainly through dust settlement on cocoa trees that impede the growth of cocoa trees and thereby reducing the productivity of cocoa farms in the mining areas. To a lesser effect, the lower use of inputs could also contribute to lower productivities.

Table 4: Mean comparison (t-test) of output, input and TFP indices for mining and non-mining areas

Index	Cocoa TFP *T-statistics*	*Significance*
Input Index	-0.0256	0.9796
Output Index	-2.8990	0.0056
TFP Index	-4.2564	0.0001

Source: field survey 2015

4 CONCLUSIONS

The study estimated the TFP difference among cocoa farmers in gold mining and non-mining areas using micro-level data from the Asutifi North and South Districts of the Brong Ahafo Region. Based on the findings from the study, it is concluded that gold mining in the study area has a negative effect on productivity of farmers located in mining areas. The adverse impacts are mainly dust settlement on cocoa trees from mining activities, which impedes cocoa growth and thereby reducing the productivity. To lesser extent, the use of relatively less productive inputs contributes to lower TFP for this group of farmers. Cocoa farmers also perceived land disputes, relocation of farms/residence, high cost of farmlands, high cost of labour and illegal small-scale mining as factors contributing to low productivities.

The uniqueness of this study is rooted in the application of Total Factor Productivity (TFP) and not the effect of one single input (i.e., partial factor productivity) on cocoa productivity. Often, qualitative approaches are adopted to highlight the effect of mining on crop production and productivity. Using a quantitative approach, this study has identified and attributed low cocoa productivity in mining areas to mining activities.

The findings of the study are important to inform policy on how to eliminate or reduce the existing negative effect of gold mining on cocoa productivity of rural farmers. A policy of land-for-land should be in place and effectively implemented to ensure that lands lost through mining activities (whether currently in use or lying fallow) must fully be replaced by mining companies to enhance and ensure continuity of livelihoods. In the absence of this, areas devoted to cocoa production will dwindle, labour may shift from cocoa production and productivity may fall (reducing government revenue, household income and livelihood). Secondly, if farmers loose crop lands (tree crop and food crop lands) adequate crop compensations that reflect current economic realities must be paid by mining companies to farmers. Government through the Land Valuation Board (LVB) must review crop compensation rates to reflect economic realities and must also factor in the sustainability of cocoa trees (projected income flows of the economic life of cocoa trees) when rural livelihoods are at stake. When farms are to be relocated, mining activities need not interfere with crop production activities. The findings of the study also suggest that mining companies should adequately compensate for crops. The farmers in Asutifi were compensated based on the Mining and Minerals Law, 1986 (PNDCL 153). The existing policies and laws relating to mining should incorporate the education of farmers and mining companies on the effect of mining activities on crop productivity.

Thus, key lessons from the study are that: mining activities impact negatively on cocoa productivity and rural livelihoods in spite of its contribution to government revenue. Farmers in gold mining catchment communities perceive mining activities as inimical to their food security situation and livelihoods through the loss of croplands and inadequate crop compensation.

There is a high level of confidence in the study's empirical findings: use of primary data collected from statistically representative cocoa farmers and the use of basic and robust quantitative approach to determine results. In other words, the approaches adopted in the study have provided enough data and information to make informed decisions on the phenomenon under study.

The study employed the use of primary cross-sectional data and therefore recommends that, subsequent research should consider the use of a time series or panel data for the analyses and also to determine TFP growth rates. Moreover, future studies could quantify, in dollar terms, the losses in cocoa productivity and livelihoods resulting from mining activities in catchment communities and compare with cocoa revenues generated from such areas.

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Determinants of Savings among Smallholder Farmers in Sokoto South Local Government Area, Sokoto State, Nigeria

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ABSTRACT

Low savings in the economy could lead to ineffective mobilization of funds for domestic investment. This could be part of the reason why Nigeria depends heavily on external borrowing for its developmental and investment projects. There is little or no documented evidence from available literatures of savings culture of farmers in the area. The dearth of such conclusive evidence has left gap which this study tried to fulfill by investigating the intervening variables. Purposive sampling was used to select four and two wards from Sabongari and Gagi wards. The choice of the selection was based on the preponderance of smallholder farmers in these locations. Two (2) villages were randomly selected from each ward. A random selection of twenty (20) farmers from each village gave a total of 120 respondents for the study. Data were elicited through a structured questionnaire. Data were analyzed using descriptive statistics and multiple regression technique. On the whole, age, farm income, non-farm income, interest rate and the distance were significant in determining the amount of saving by smallholder farmers in the study area. Thus, these factors have to be considered in designing strategies aimed at improving the savings of smallholder farmers.

Key words: savings; smallholder farmers; determinant

IZVLEČEK

DETERMINANTE VARČEVANJA MED MALIMI KMETI V OKROŽJU SOKOTO SOUTH, SOKOTO STATE, NIGERIA

Majhni prihranki v gospodarstvu lahko vodijo v neučinkovito črpanje sredstev za domača vlaganja. To bi lahko bil vzrok, da je Nigerija tako močno odvisna od zunanjega zadolževanja pri razvojnih in investicijskih projektih. V dostopni literaturi je na voljo zelo malo ali skoraj nič podatkov o kulturi varčevanja med kmeti na tem območju. Pomankanje teh pomembnih informacij je bila vrzel, ki jo skuša ta raziskava zapolniti s preučevanjem vplivnih spremenljivk. Izbrano je bilo selektivno vzorčenje za izbor štirih vasi in dveh območjih v provincah Sabongari in Gagi. Odločitev za izbor je temeljila na večinskem stanju malih kmetov na območjih. Na vsakem izmed območij sta bili naključno izbrani po dve vasi. Naključen izbor 20 kmetov iz vsake vasi je dal celokupno 120 respondentov v raziskavi. Podatki so bili pridobljeni z vprašalnikom in analizirani z opisno statistiko in multiplo regresijo. V celoti so starost, prihodek kmetije, prihodek iz nekmetijske dejavnosti, obrestna mera in razdalja do banke značilno določali velikost prihrankov malih kmetov na tem območju. Te dejavnike bi torej morali upoštevati pri oblikovanju strategij za povečanje prihrankov malih kmetov.

Ključne besede: prihranki; mali kmeti; determinante

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1 INTRODUCTION

In most developing countries, sustained growth in agriculture is often the keystone of overall socioeconomic growth and development (Dethier & Effenberger, 2011). These agro-based economies have rural farmers as stakeholders in the path to agricultural development. In Nigeria, for instance, smallholder farmers account for about 90 % of food production (Adams & Vogel, 1990; CBN, 2004). The importance of agriculture in developing country, especially rural households, cannot be overemphasized – provides employment for 60 percent of the labor force: accounting for 25 percent of GDP and provides livelihood for 86 percent of rural households (Dethier & Effenberger, 2011).

Due to the dominance of agricultural activities by rural farmers, improving rural food productivity and production is central to agricultural growth and development. However, smallholder farmers are confronted by peculiar circumstances and government policies and programmes which have limited their capacities for increased production. In many African countries, including Nigeria, lack of access to credit or its inadequacies is most frequently mentioned as a leading constraint to increased agricultural production. Generally, lack of access to credit by rural farmers is attributed to the fact that not only that most of the rural farmers rarely attain formal education, but also lack collateral, which virtually locks them out of the conventional banking system (Awotide et al., 2015). Worse still, credit obtained from informal financial institutions is not always enough for a meaningful increase in their agricultural production (Ike, 2009).

In view of the credit situation in rural sector, and the peculiar constraints surrounding credit availability to rural farmers, a sustainable way for capital formation for investment is via savings mobilization from rural farmers themselves to increase the amount of loanable funds in rural banks as well as to increase the extent to which they accumulate capital for farming (Vonderlack & Schreiner, 2001; CBN, 2004). Savings is a common word used by individuals on daily basis. It is a means of accumulating assets that perform specific function for the saver (Ike & Idoge, 2006). It is also the setting aside of some items for future use or what will be considered as deferred expenditure (Amu & Amu, 2012). Savings plays a foundational role in economic development as it is a key to capital formation which is necessary for investment. There is proportional relationship between savings and investment as the size distribution of savings reveals a potential for further investment. If investment remains localized in accordance to size of savings generated in a specific area, there are likelihoods for reinvestment in areas wherein higher savings is recorded compared to those of meager savings (Odoemenem et al., 2013). Economic theory posits that high level of savings is translated to higher investment, with financial institutions playing the intermediation role of mobilizing and allocating financial resources from savers to investors. Thus, at the national level, savings are crucially important because they allow for investment which in turn creates jobs and enhances production. This leads to an increase in income, which permits additional savings and investments.

Most smallholder farmers utilize their savings for both agricultural and non-agricultural purposes (Odoemenem et al., 2013). Agricultural investments are in the purchase of agrochemicals - fertilizers and pesticides, hired labour, machineries and acquisition of land for farming. Investment in non-agricultural activities are mainly centered on education, trade expansion and other cultural obligations.

As stated earlier, inadequate savings, income and access to capital for investment is one of the basic challenges confronting agricultural development in Nigeria. Despite this challenge, there have been limited attempts to address the saving cultures in rural Nigeria (Odoemenem et al., 2013). Although studies have been conducted in Nigeria with the aim of determining savings and capital formation in the southern Nigeria, but due to the cultural difference between the rural dwellers in north of Niger River, it is necessary to understand the saving culture in Northern Nigeria. In the light of the foregoing, this study seeks to (i) describe socioeconomic characteristics of smallholder farmers in the study area; (ii) determine factors influencing savings among smallholder farmers in the study area; and (iii) identify the major reasons why smallholder farmers save in the study area.

2 MATERIALS AND METHODS

2.1 Study area

Sokoto south local government area is located between latitudes 12^0 20'N and 13^0 58'N and longitudes 4^0 8'E and 6 ^{0} 54'E. It is bounded by Kware local government to the east, Wamakko local government in the west, Sokoto north local government in the north and Dange-Shuni local government in the south. The average rainfall is about 750 mm, the mean temperature is 34.9 ^{0}C. It has an area of 41 km^2 and a population of 194,914 amongst which there are 102,270 males and 92, 644 females (NPC, 2006). Sokoto south local government is blessed with vast arable land and vegetation across its domain used for agricultural cultivation. The land is generally fertile for agricultural activities. Crops cultivated in the area include maize, guinea corn, millet, cassava, and potatoes in large quantities. There is also availability of large amount of livestock in the area. The livestock include cattle, sheep, goats, and poultry. Vast proportions of the people in the local government area are traders and are also involved in farming and other economic activities. Apart from the arable crops, economic activities like dyeing of cloths and tanning are also practiced.

2.2 Sampling procedure

Purposive sampling was used to select four (4) and two (2) wards from Sabongari and Gagi wards respectively based on the preponderance of small holder farmers in these locations. This gave a total of 6 wards. From each of the wards, two villages each were randomly selected. From each village, the lists of respondents were compiled. Random sampling technique was used to select 20 farmers from each village. This gave a total of 120 respondents.

2.3 Data Collection

The study used primary data for its analysis. Data were elicited in 2013 by the use of pre-tested and structured questionnaire administered in an interview schedule to the smallholder farmers. The data elicited include the socioeconomic characteristics of the farmers: age, sex, income (farm and off-farm), monthly savings and distance from financial institutions, among other information.

2.4 Analytical technique

To achieve the objectives of the study, set of statistical tools were employed in analyzing the data obtained. Descriptive statistical tools such as percentages and frequency were used to analyze the socioeconomic characteristics of the farmers and to identify the reasons for savings. Multiple regression technique was employed in the analysis of factors influencing savings by using the socioeconomic variables as proxies. The regression model was implicitly specified as:

$$Y = F(X_1, X_2, X_3, X_4, X_5, X_6 + X_7 + X_8 + X_9 + X_{10} + e) \quad (1)$$

Where Y = volume of savings accumulated in a year
X_1 = Age (years), X_2 = Farm size (hectares), X_3 = Household size (Number of persons), X_4 = Farm income (N), X_5 = Non-farm income (N), X_6 = Experience from saving programme (Years),
X_7 = Interest rate (%) X_8 = Household level of education (Years), X_9 = Membership of organized group (Dummy) X_{10} = Distance to the nearest formal financial institution (Km), e = Error term

The model was explicitly specified as:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_5X_5 + \beta_6X_6 + \beta_7X_7 + \beta_8X_8 + \beta_9X_9 + \beta_{10}X_{10} + e \quad (2)$$

Where: β_0=Constant term; β_1 – β_{10}=Regression Coefficients. Other variables are as previously defined.

3 RESULTS AND DISCUSSION

3.1 Socioeconomic characteristics of smallholder farmers in the study area

The socioeconomic characteristics of the farmers were elicited and thus presented in Table 1.

Table 1: Summary of the descriptive statistics of the smallholder farmers

Variables	Frequency	Percentage	Mean
Age (years)			
20-35	22	18.3	
36-41	38	31.7	43.0
42-57	43	35.8	
>57	17	14.2	
Household size (numbers)			
<3	5	4.2	
3-6	54	45.0	6.6
7-10	46	38.3	
>10	15	12.5	
Famers education (level)			
Primary education	19	15.8	
Secondary education	28	23.3	
Tertiary education	35	29.2	
Islamic education	38	31.6	
Farm size (hectares)			
<1	45	37.5	
1-3	37	30.8	2.7
4-6	26	21.7	
>6	12	10.0	
Household income (Naira/annum)			
<20,000	4	3.3	
20,000-50,000	10	8.3	71,758.33
51,000-81,000	27	22.5	
>81,000	79	65.8	
Mode of savings			
Formal financial institution	70	58.3	
Informal financial institution	50	41.7	
Monthly savings(Naira)			
1-1000	46	38.3	
1001-2000	50	41.7	1217.07
> 2000	24	20.0	
Distance to financial institution(km)			
<1	15	12.5	
1-5	41	34.2	5.8
6-10	43	35.8	
>10	21	17.5	

Source: Field Survey, 2013

The mean age of the smallholder farmers was observed to be 43 years. The implication is that the smallholder farmers are middle aged and still agriculturally active. This however could have effect on agricultural production with the mean reflecting the age group that can withstand the rigours, and stress involved in agricultural production (Onyenucheya & Ukoha, 2007). The mean household composition of the farmers was 7 persons with the majority (52.5 %) of them with secondary and tertiary level of education. As anticipated, higher education tends to enhance the ease of adopting innovation and hence increased farm income (Ezeh, 2007). On the distribution of farm size in hectares, it was observed that the average farm size was 2.7 hectares. Farmers with less than 1 ha constituted about 37.5 % of the respondents and only 30.8 % had 1-3 ha, an indication that the farmers are indeed operating on a small scale with a mean annual household income of N 71,758.33. It can be seen that the average monthly saving (N 1217.07) is 20.4 % of the average monthly income (N5979) of the farmers. The distance of the farmers to financial institution at average, was about 5.8 km.

3.2 Factors influencing saving among farmers in the study area

Multiple regression estimates on the determinant of saving in the study is presented in Table 2.

Table 2: Regression estimates for the determinants of savings among smallholder farmers

Variable	Coefficients	t values
Constant	7.124***	11.024
Age (Years)	0.039***	3.845
Farm Size (Hectares)	0.027	0.904
Family Size (No)	0.031	1.042
Farm Income (N)	0.165***	2.703
Non-farm income (N)	0.512**	2.058
Experience from saving programme (Years)	0.016	0.531
Interest rate (%)	0.120***	2.656
Household level of Education (Years)	0.182*	1.855
Membership of organized group (Dummy)	-0.261	-1.15
Distance	-0.295***	-2.66
R square value	0.643	
Adjusted R square value	0.414	
F Statistics	25.97***	

Source: Field Survey, 2013.
Note: *** significant at 1 %, ** Significant at 5 %, * Significant at 10 %

The estimated determinant function is shown in Table 2. It shows a R^2 value of 0.643. This is an indication that 64.3 % variability of farmers' saving is attributed to the specified explanatory variables in the model. This shows that the specified explanatory variables were important determinants of saving among respondents. The F statistics confirms the suitability of the overall regression equation and it is however significant at 1 % probability level.

The coefficient of age is positive and in conformity with *apriori* expectation of the amount of savings accumulated. This positive relationship implies that older farmers save more. This is in agreement with the work of Attanasio & Szekely (2000) who found that savings capacity is enhanced as age increases. Old people tend to be more frugal and thrifty. This may be due to the facts that middle aged people are required to save more, owing to the financial obligations for their immediate families.

The farm income is expected to bear a positive relationship with the volume of savings. The result of this study shows that volume of savings was sensitive to farm income of the farmers as this was found to be significant at 1 %. This implies that the higher the farm income, the more likely a farmer would save or accumulate capital to meet investment demands. This corroborates the findings of Adeyemo & Bamire (2005).

Non- farm income turnout is one of the major determinants of savings in the area. Non-farm income is statistically significant at 5 %. This implies that the earning from non-farm activities is likely to influence their level of saving. If they are intensively involved in these non-farm activities like petty trading, transportation business, non-agricultural processing, sewing etc., they are likely to save more capital than those who are not involved.

Interest on amount saved has a positive and significant coefficient. This corroborates theoretical expectation. This implies that smallholder farmers will continue to save even with a marginal interest. This may be affirmed to the distribution of society's surplus according to members' patronage (in terms of savings, purchase and loan) at the end of the accounting year and could serve as a way in motivating members to keep saving at higher rate.

The relationship between the level of savings and distance to financial institutions is negative. Its coefficient is statistically significant. This implies that farmers' proximity to financial institutions is one of the major determinants of level of saving because the nearer farmers are to financial institutions, the more the likelihood of saving some part of their income.

3.3 Major reasons why smallholder farmers save in the study area

The various reasons for savings as attested to by the farmers through multiple responses to items are presented in Table 3.

Table 3: Reasons for Saving

Reasons	Frequency*	Percentage
Precautionary motive	63	52.5
Capital formation	115	95.8
Sense of independence	31	25.9

Source: Field Survey, 2013, * Multiple response

Capital accumulation (capital formation) was observed by 95.8 % as the reason why they save. This will in turn help increase their level of agricultural production while over 52.50 % of the respondents save as against precautionary motive aimed at tackling unforeseen circumstance that might evolve during the production period and as well ensuring the proper education of their children. To marry more wives so as to add to the family labour input might be a good reason for the response on sense of independence by the farmers.

4 CONCLUSION

The study examined the determinants of savings among smallholder farmers in Sokoto south local government area of Sokoto State, Nigeria. Ten parameters were fitted into the regression model and only five were found to be significant determinant of saving. It shows that these socioeconomic variables have impact on the farmer's level of savings. It can also be concluded that these farmers save in order to accumulate capital which might enhance their production. As such farm level, policies which remove agricultural production constraints will increase the farmer's income and promote accumulation of financial capital in form of savings among farmers. Also the determinants of savings as observed should be considered in designing strategies aimed at improving the savings of smallholder farmers.

Given the significance of the income (both farm and non-farm) to the improvement of savings among farmers, appropriate financial instruments such as medium and long term loans should be provided by financial and other credit institutions to farmers in order to boost their income level. Only then, the savings being accumulated in the rural economy can be transformed into productive investment that will enhance or uplift their present standard of living.

Finally, to improve savings among smallholder farmers in Nigeria, technologies – mobile banking - that bring financial institutions close to smallholder farmers should be encouraged among the farmers via their relevant farm associations. This will in turn improve their saving cultures as this strongly relates to distance from financial institution.

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Humic acid and boron treatment to mitigate salt stress on the melon plant

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ABSTRACT

Salinity is one of the main abiotic stress factors which limit the growth and productivity of plants, however, the nutritional status of plants is the first brick in the resistance wall against stresses. Therefore, a factorial experiment was undertaken to investigate effects of soil applied humic acid (0, 7, 14, 21 $l.ha^{-1}$) and boron foliar spraying (0, 50, 100 ppm) and their interaction on growth and yield of melon plant under saline conditions. The results suggested that treatments, soil application of humic acid and boron foliar spray, successfully mitigated the deleterious effects of salt stress and influenced growth and yield of melon plant. Humic acid at 21 $l.ha^{-1}$ or boron spray at 50 ppm exhibited an improvement in growth and yield of melon, in terms of plant length, plant fresh and dry mass, chlorophyll (SPAD), fruit mass, total yield, and also leaf nutrient content (N and K) and total soluble solids (TSS) of fruits, while reduced the sodium content of leaves. The combined treatment of humic acid at 21 $l.ha^{-1}$ and boron spraying at 50 ppm was found to be more effective for the melon plant to improving growth performance and the crop yield by 21 % as compared with the control group under saline conditions.

Key words: melon; salt stress; humic acid; boron foliar spray

IZVLEČEK

OBRAVNAVANJE S HUMINSKO KISLINO IN BOROM ZA OBLAŽITEV SOLNEGA STRESA PRI MELONI

Slanostni stres je eden izmed najpomembnejših abiotskih stresnih dejavnikov, ki omejuje rast in produktivnost rastlin in prehranjenost rastline je prvi člen v odpornosti proti temu stresu. V ta name je bil izveden faktorski poskus za preučevanje učinkov talnega dodajanja huminske kisline (0, 7, 14, 21 $l.ha^{-1}$) in foliarnega škropljenja z borom (0, 50, 100 ppm) ter njune interkacije na rast in pridelek melon v razmerah slanostnega stresa. Na osnovi rezultatov priporočamo talno uporabo huminske kisline in foliarno škropljenje z borom za uspešno odpravo škodljivih učinkov solnega stresa na rast in pridelek melon. Huminska kislina (21 $l\ ha^{-1}$) ali škropljenje z borom (50 ppm) sta izboljšala rast in pridelek melon preko naslednjih dejavnikov: dolžine rastlin, njihove sveže in suhe mase, vsebnosti klorofila (SPAD), mase plodov, pridelka, vsebnosti hranil v listih (N in K) in celokupne vsebnosti topnih snovi v plodovih (TSS), ob hkratnem zmanjšanju vsebnost natrija v listih. Kombinirano obravnavanje s huminsko kislino (21 $l.ha^{-1}$) in škropljenje z borom (50 ppm) se je izkazalo za bolj učinkovito. Pridelek melon je bil v razmerah slanosti v primerjavi s kontrolo za 21 % večji.

Ključne besede: melona; solni stres; huminska kislina; foliarno škropljenje z borom

1 INTRODUCTION

Salinity of both soil and water in the arid and semi-arid regions is one of the major concerns facing the development in the agricultural sector in these regions, higher salt concentrations and excess exchangeable sodium in the soil has led to the deterioration of soil structure and make plants suffer more, the harmful effects of salinity on plants occur due to its adverse effects on osmotic potential, photosynthesis and protein synthesis (Ouni et al., 2014). Salinity negatively affects soil fertility, inhibit plant growth and reduce the productivity via to the excess uptake of some toxic ions, and lack of availability of water and essential nutrients (Khaled, 2011). Romero et al. 1997, Kaya et al. 2007 observed a significant decrease in the plant growth, fresh mass, fruit yield, contents of Ca, K and N, and chlorophyll, as well as the rates of photosynthesis and transpiration, while the Na uptake has increased in the melon plant under salt stress conditions. The high salt concentration in the soil solution decreases the water potential, reduces the osmotic potential of plant cells,

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and slow down the division and elongation of plant cells (Kusvuran et al. 2007). Humic acid is an organic matter found naturally in the soil, provide several benefits to the soil with regard to structure improvement, retain moisture and microbial activity, also act as a chelating agent, which prevent fixation, leaching, and oxidation of elements and thus promote the ion exchange capacity and nutrient availability in the soil (Ouni et al., 2014), humic acid is one of the organic-mineral fertilizers that increases the soil fertility (Kahraman, 2017), has positive effects at the different stages of plant growth, from seed germination and seedling growth to the development of shoots and roots (Adani et al.,1998), acts like the plant growth regulators, stimulates growth, development and yield (Karakurt et al., 2009), enhances enzyme activity and the permeability of cell membranes (Asri et al.,2015). A good conditioner for the saline soil which reduces the uptake of toxic elements e.g. sodium ions and increases the plant's ability to resist the deleterious effects of salt stress (Aydin et al., 2012). Boron is one of the essential micronutrient elements, boron in the proper concentration has several physiological roles within the plant including the function and integrity of cell walls and membranes, sugar transport, differentiation, and root elongation, as well as the metabolism of protein, carbohydrate, nucleic acid, and indole acetic acid (Pinho et al., 2010), plays a vital role in the division and elongation of plant cells as well as in the osmotic adjustment, metabolism of phenols and oxidative stress (Naeem et al., 2017). It is necessary for the flowering and fruiting processes, from the germination of pollen and growth of pollen tube to growth and development of fruit (Krudnak et al., 2013), enhances the nutrient status, marketable yield and shelf life of fruits (Davis et al., 2003). Boron foliar spray mitigates the deleterious effects of salt stress and enhances the growth performance and productivity (Hellal et al., 2015). However, the availability of boron element decreases when the soil pH is higher than 7 in the saline-alkaline soil (Brady, 1990), boron deficiency has an unfavorable impact on the physiological processes of plants, thus diminishing growth and yield of plants (Pinho et al., 2010).

Melon (*Cucumis melo* ‘Galia’) is one of the popular vegetables in Egypt, belonging to the Cucurbitaceae family. Melon is classified as a moderately sensitive crop to salinity (Olave and Guzman, 2004). Salinity is the most important problem facing the cultivation of melon plant in the dry and semi-dry regions (Kusvuran et al. 2007). Yasar et al 2006 reported that salt stress causes plant growth inhibition as well as metabolic disturbances, consequently losses of yield and quality. Our experiment was designed to investigate the influence of soil application of humic acid and boron foliar spray on melon plants grown under saline conditions and to determine their roles in reducing the damage of salt stress on plants.

2 MATERIALS AND METHODS

The experiment was conducted at the experimental farm of Desert Research Center at Ras Sudr, Sinai, Egypt in 2015 and 2016. Soil samples were taken from the experimental site and analyzed according to the methods described by Klute (1986) and Page et al. (1982), detailed soil description is provided in Table 1.

Table1: Chemical properties of the experimental soil

Depth (cm)	pH	E.C (dS/m)	Saturation soluble extract ($mg.100\ g^{-1}$)							Available nutrients ($mg\ kg^{-1}$)			
			Cations			Anions				N	P	K	Fe
			Ca^{++}	Mg^{++}	Na^{+}	CO_3^{-}	HCO_3^{-}	Cl^{-}	$SO_4^{=}$				
0-30	7.7	8.65	24.5	5.2	57.2	0.0	6.0	61.5	26.2	26.0	5.1	51.5	4.2
30-60	7.9	7.35	16.8	3.8	42.5	0.0	3.5	49.0	23.5	18.5	3.4	35.3	3.4

Based on the soil analysis the soil pH is 7.7, indicating a decrease in the availability of boron under these conditions (Brady, 1990). The experiment was arranged in a split plot design with three replications, the main plot factor was humic acid levels (0, 7, 14, 21 $l.ha^{-1}$) and the subplot factor was boron foliar application levels (0, 50, 100 ppm) after consideration of previous studies. Humic acid treatment was carried out in the soil using a potassium humate compound 83 % obtained from Union company for agricultural development. Boron applications were made using boric acid (B 17 %) with a back-sprayer, while the humic acid treatments were performed through the drip irrigation system. In the fourth week of March, the 30 days old seedlings of melon (*Cucumis melo* 'Galia') were transplanted into the soil at a spacing of 50 cm within rows, where each plot contained 5 rows, 4 m in length. Standard agricultural practices were followed for fertilization, irrigation, and pesticides for all experimental plots as per the recommendations of the Egyptian Ministry of Agriculture, ,except for the application of humic acid and boron spraying treatments which were conducted three times with a 15-day interval, starting from two weeks after transplanting. Five plants per treatment were harvested after 60 days after transplanting, and plant length, fresh mass and chlorophyll reading (SPAD) were recorded. Plant samples were washed with distilled water and dried at 70 ^{o}C for 72 h, and dry mass of plants was determined. For chemical analysis, plant leaf samples were dried, ground and digested with concentrated sulfuric acid, and then nitrogen content was determined according to the Kjeldahl method (Jackson, 1973), and flame photometer was used for the potassium, calcium and sodium analyses, while phosphorus was determined by spectrophotometric method (Irri, 1976). Yield measurements were also taken, such as fruit mass, fruit soluble solids (TSS) using a hand-held refractometer and total yield. Data for this study were analyzed statistically using the MSTAT software (MSTAT-C, 1991), and significant differences between means were obtained using Duncan's multiple range test at a significance level of P < 0.05 as described by Snedecor and Cochran (1982).

3 RESULTS AND DISCUSSIONS

3.1 Vegetative growth

Soil-applied humic acid has a significant impact on growth and development of melon plants under saline conditions as shown in Fig.1. Increasing concentrations of humic acid caused a gradual increase in the vegetative growth parameters in terms of plant length, leaf chlorophyll reading (SPAD), and fresh and dry mass as compared with the control group. Maximum values of growth parameters were recorded at 21 l.ha^{-1} of humic acid.

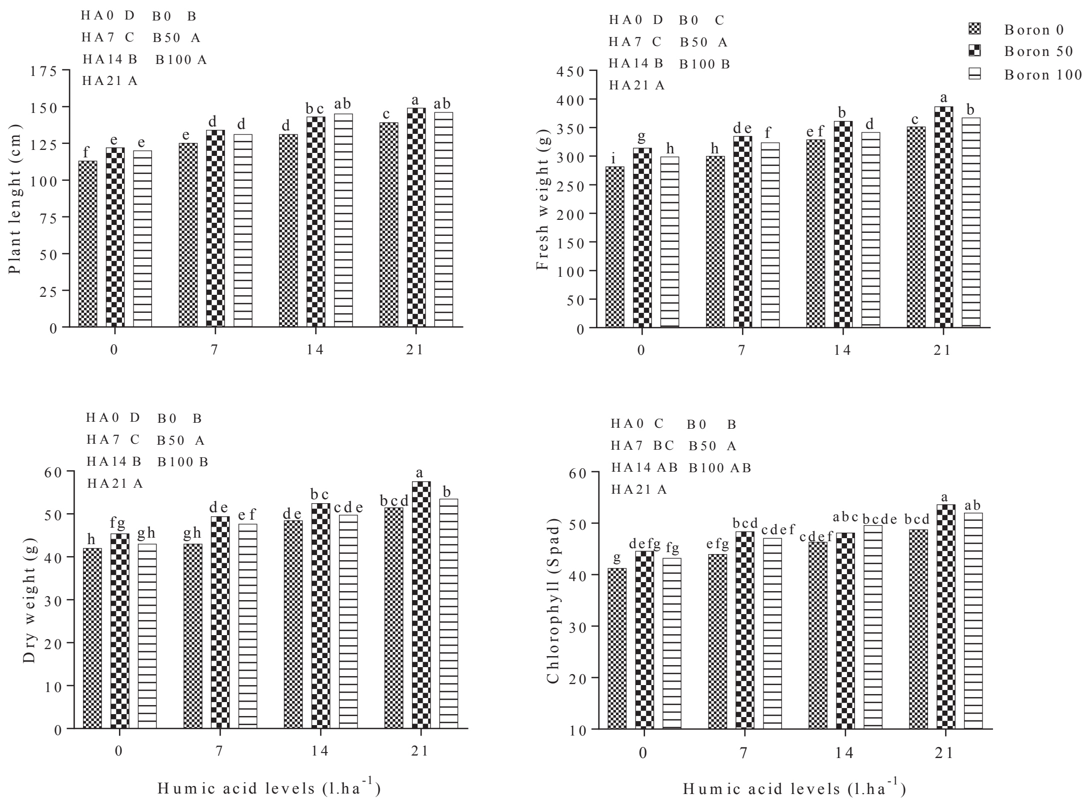

Figure 1: Main effects and interactions for humic acid and boron on plant length, plant fresh and dry mass and chlorophyll content of melon plants grown under saline conditions

The promotion of vegetative growth by humic acid application has also been reported in many studies, Adani et al. (1998) on tomato, Karakurt et al. (2009) on pepper, Aydin et al. (2012) on bean under salt stress, and Mirdad (2016) on lettuce.Ahmad et al. (2016) found that the humic acid application at 20 kg ha^{-1} improved growth of canola plant, while 70 kg ha^{-1} was the most appropriate for cowpea growth (Kahraman, 2017). Boron spray improves vegetative growth of melon plant, plant growth parameters (plant length, leaf chlorophyll reading, plant fresh and dry mass) were significantly increased by the boron application (Fig.1). The highest values of growth parameters were recorded with boron spray 50 ppm. These results are in agreement with Davis et al. (2003) on tomato, Baranwal et al. (2017) on guava and Hellal et al. (2015) on faba bean under salt stress. The interaction between humic acid and boron had a synergistic impact on the vegetative growth under salt stress conditions, 21 $l.ha^{-1}$ of humic acid plus 50 ppm boron spray was the superior treatment for the melon plant.

3.2 Chemical components

The data in Figs. 2 and 3 indicate that chemical components of the melon plant were significantly affected by humic acid treatment. The content of potassium and nitrogen in leaves and TSS of fruits were increased, whereas calcium and sodium contents in leaves were decreased with increasing levels of humic acid. 21 $l.ha^{-1}$ of humic acid led to the highest values of N, K, and TSS contents and the lowest values of Ca and Na. However, under salt stress conditions the potassium absorption is markedly reduced due to the competitive effect with sodium, where plants absorb Na ions more than necessity, resulting in malnutrition status in plants grown under these conditions (Kusvuran et al. 2007). These results are in consonance with the finding of Khaled (2011) for N, K, and Na in corn, Asri et al. (2015) for N, K, and TSS in tomato, Ahmad et al. (2016) for N and K in canola, Mirdad (2016) for N, K and TSS in lettuce, Kahraman (2017) for K and Ca in cowpea, as well as, humic acid increases the contents of N and K, and decreases sodium content of bean plant under saline conditions (Aydin et al., 2012). The positive effect of humic acid on plants may be due to improving chemical, physical and biological properties of the soil, elements chelation and pH adjustment (Karakurt et al., 2009), thus the low soil pH may affect plant growth indirectly, at the low soil pH the availability of many elements increases while the other nutrients such as calcium were limited (Yan et al. 1992). Boron foliar application led to significant increases in the contents of N, K, and Ca in plant leaf, and fruit TSS, while the sodium content in leaves was decreased as compared with the control group, however, the increase and decrease in the chemical parameters were highly statistically significant in plants sprayed with 50 ppm of boron. The effect of boron spray on the content of chemical parameters in plants has also been observed by Davis et al. (2003) for N, K and Ca in tomato, Hellal et al. (2015) for K, Ca and Na in faba bean and Baranwal et al. (2017) for TSS in guava fruits. A cooperative interaction between the humic acid 21 $l.ha^{-1}$ and boron spray 50 ppm was obtained, which increased N, K, and TSS values and decreased Na and Ca values, whereas the highest values of Na and Ca were recorded with the control treatment.

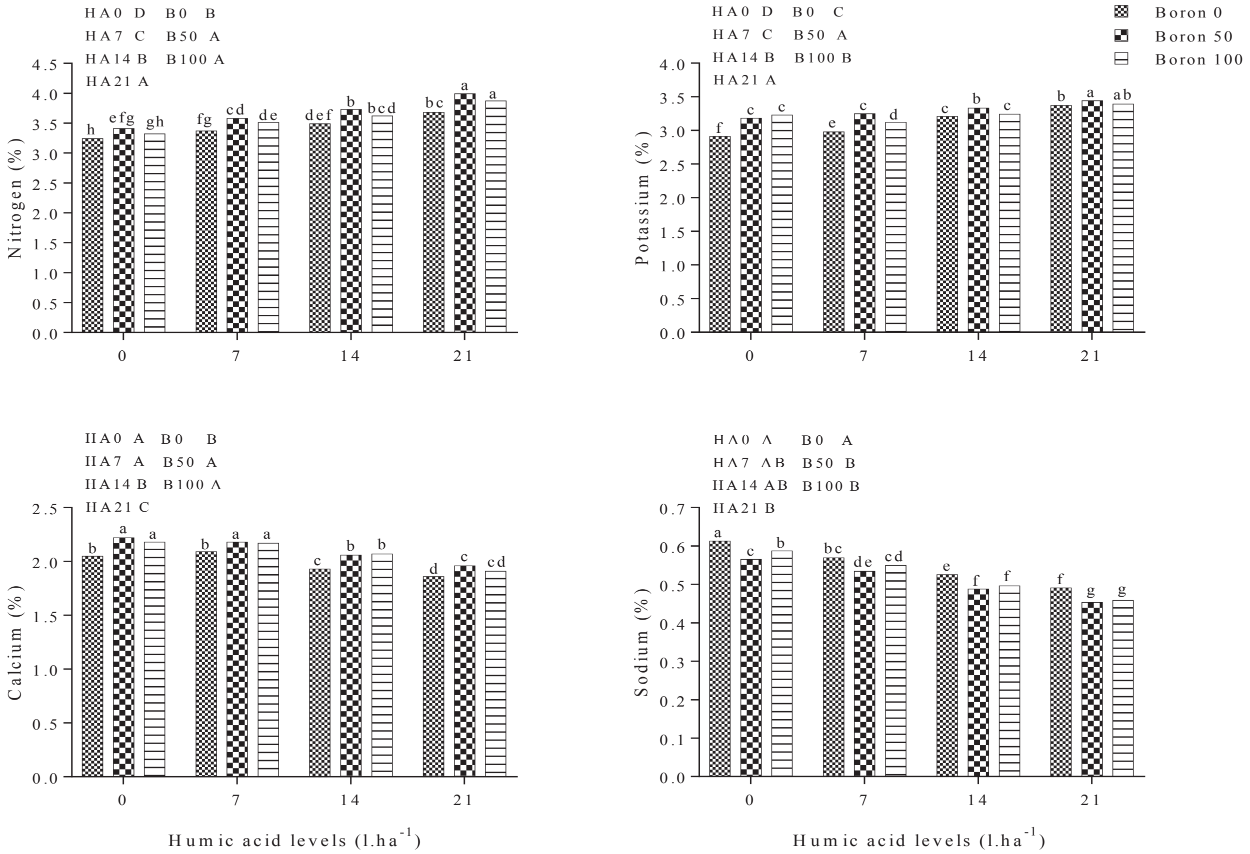

Figure 2: Main effects and interactions for humic acid and boron on nitrogen, potassium, calcium and sodium contents of melon plants grown under saline conditions

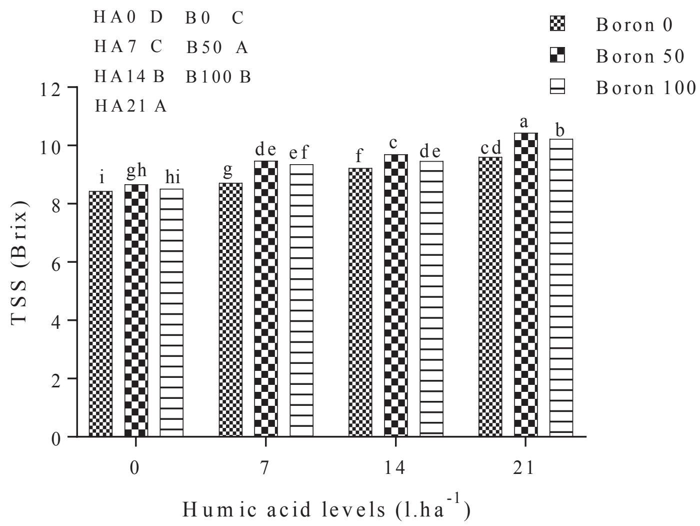

Figure 3: Main effects and interactions for humic acid and boron on TSS content of melon fruits under saline conditions

3.3 Yield

A significant increase in fruit mass and total yield of the melon plant was obtained with increasing levels of humic acid under saline conditions as shown in Fig. 4. Fruit mass and total yield were correlated positively and significantly with the soil application of humic acid, the increase was more pronounced in the plants which received 21 $l.ha^{-1}$ of humic acid. There are many studies that indicate the stimulatory influence of humic acid on crop productivity Karakurt et al. (2009) in pepper, Asri et al. (2015) in tomato, Ahmad et al. (2016) in canola and Kahraman (2017) in cowpea. Fruit mass and total yield of the melon plant were significantly increased with boron foliar spray (Fig. 4). The maximum values of fruit mass and total yield were obtained by the application of boron spray 50 ppm. The promotion effect of boron application on the plant productivity has also been detected by many studies, Davis et al. (2003) in tomato, Baranwal et al. (2017) in guava ,also by Hellal et al. (2015) in faba bean under saline conditions. The interaction between humic acid and boron showed a positive effect on the yield of melon under saline conditions, the highest values for fruit mass and total yield were recorded in plants received 21 $l.ha^{-1}$ of humic acid plus 50 ppm of boron spray.

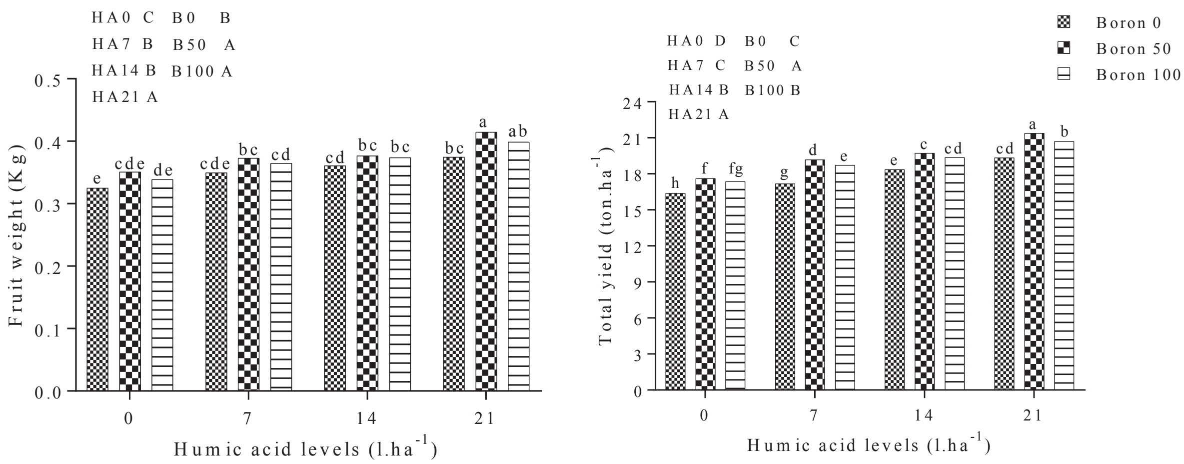

Figure 4: Main effects and interactions for humic acid and boron on fruit mass and total yield of melon plants grown under saline conditions

Humic acid increases the permeability of the cell membrane and transportation of nutrients (Aydin et al., 2012), as well as increases the root elongation and growth of lateral roots and root hairs and decreases the electrical conductivity of soil (Ahmad et al., 2016). Boron application stimulates the physiological processes within plants under salinity conditions such as osmotic adjustment, photosynthetic rate, antioxidant enzymes activity (superoxide dismutase, catalase, peroxidase and ascorbate peroxidase), and total protein and nucleoprotein synthesis (Naeem et al., 2017), also affect flowering process, it increases viability and germination of pollen and promotes the fruit composition (Kroenke et al., 2013). These effects together could mitigate the adverse impact of salt stress on plant and increase nutrient availability in the soil and ability of plant roots to uptake the nutrients, thus the contents of potassium and nitrogen in plant leaves has increased, hence, improved the vegetative growth performance and increased crop yield of melon plant under saline conditions.

4 CONCLUSIONS

1- The deleterious effect of salt stress on melon plant has been alleviated by the treatments with humic acid and boron.
2- Vegetative growth and chemical contents of melon plants improved under saline conditions using humic acid and boron treatments.
3- The fruit mass, fruit TSS and total yield were increased in the plants receiving humic acid or boron.
4- Plants grown under saline conditions received a double benefit through the treatment with humic acid and boron. The best results were obtained by the combination of soil-applied humic acid at 21 $l.ha^{-1}$ and boron spray at 50 ppm.

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Potential benefits and toxicity of nanoselenium and nitric oxide in peppermint

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ABSTRACT

Taking account of nano-compounds and biofortification, this research was conducted to evaluate peppermint (*Mentha x piperita* L.) responses to nano-selenium (nSe; 0, 2, and 20 mg l^{-1}) and/or nitric oxide (NO; 0 and 8 mg l^{-1}). Significant increases in leaf length, and area, and shoot fresh mass were enhanced by the low level of nSe and/or NO, contrasted with the high dose. The inhibitory effects of the high dose of nSe on the growth-related characteristics were significantly mitigated by NO. The adverse impact of nSe20 on chlorophyll concentration was alleviated by NO. The individual and combined treatments of nSe2 led to the significant inductions in the activities of nitrate reductase and peroxidase, whereas nSe20 inhibited. The proline contents in the nSe and/or NO-treated plants were higher than in the control. The nSe and/or NO provoked stimulation in activities of phenylalanine ammonia lyase enzyme. The foliar applications of nSe and/or NO triggered the accumulations of soluble phenols. Interestingly, the toxicity of nSe at the high dose led to the severe cell destruction in the cortex layer of the basal stem, which was partially alleviated by NO. The simultaneous applications of these supplements may consider as an alternative strategy for fortifying and improving plant protection, regarding sustainable agriculture.

Key words: biofortification; elicitor; metal-based nanoparticle; *Mentha x piperita*; nitrate reductase; proline; selenium

IZVLEČEK

POTENCIALNE KORISTI IN STRUPENOST NANOSELENA IN DUŠIKOVEGA OKSIDA PRI POPROVI METI

Raziskava je bila izvedena za ovrednostenje odziva poprove mete (*Mentha x piperita* L.) na nano selen (nSe; 0, 2, in 20 mg l^{-1}) in/ali dušikov oksid (NO; 0 in 8 mg l^{-1}). Značilno povečanje dolžine in površine listov in sveže mase poganjkov je bilo vzpodbujeno z majhnimi količinami nSe in/ali NO, nasprotno od učinkov velikih količin. Zaviralni učinki velikih koncentracij nSe na z rastjo povezane parametre so bili značilno zmanjšani z dodatkom NO. Negativni vpliv nSe20 na koncentracijo klorofila je bil ublažen z NO. Posamično in kombinirano obravnavanje z nSe2 je vodilo k značilnem povečanju v aktivnosti nitrat reduktaze in peroksidaze, medtem ko jo je tretiranje z nSe20 zavrlo. Vsebnost prolina je bila v z nSe in/ali NO obravnavanih rastlinah večja kot v kontroli. Obravnavanje z nSe in/ali NO je povečalo aktivnost fenilalanin ammonium liaze. Foliarno dodajanje nSe in/ali NO je vzpodbudilo kopičenje topnih fenolov. Toksičnost nSe je pri velikih dozah vodila k izrazitim razgradnjam celic v plasteh primarne skorje bazalnega dela stebla, kar je bilo delno oblaženo z dodatki NO. Hkratno uporabo teh dveh dodatkov lahko imamo kot alternativno strategijo za okrepitev in izboljšanje zaščite rastlin pri trajnostnem kmetovanju.

Ključne besede: biofortifikacija; elicitor; nano delci kovin; *Mentha x piperita*; nitrat reduktaza; prolin; selen

1 INTRODUCTION

Selenium (Se) is characterized as an essential trace mineral nutrient for many microorganisms, animals, and humans but it is not classified as an essential element for plants, despite its beneficial effects on the growth and physiology at low doses (Kaur and Nayyar, 2015). Awareness and concern about the Se significance to the human health issues are worldwide growing (Liu et al., 2017). In soil condition, selenite and selenate are the main sources of Se and may be efficiently uptake by plants. These mentioned forms of Se are mainly utilized to fortify different plants in the field production (Liu et al., 2017). Contrasted with many other living organisms, Se is not considered as an essential nutrient for plants. Otherwise, it seems to be a benefit for many plants, especially hyper-accumulators (El-Ramady et al., 2016). Thus, the functional significance of Se may lead to

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enhance plant growth, modify cellular physiology, and/or improve plant resistance to abiotic and biotic stress conditions (Ardebili et al., 2014; Ardebili et al., 2015; El-Ramady et al., 2016).

Nitric oxide (NO) is known as a vital reactive nitrogen specie, a bioactive multi-task signaling molecule, and an elicitor of an array of signaling pathways by which regulates various aspect of plant development, physiology and defense responses (Gupta et al., 2011; Mur et al., 2013; Zhao et al., 2016). It is implicated in the control process of meristem cells (Sanz et al., 2014). There is evidence that NO may change auxin sensitivity and affects meristem cell activity (Sanz et al., 2014). NO, mainly via nitrosylation, can affect the activity of various proteins and play the role as a Ca^{2+}-mobilizing intracellular messenger (Courtois et al., 2008). The application of sodium nitroprusside (SNP) as an exogenous NO donor is a most widely effective method to supplement plants with NO.

As nanotechnology science and technology is rapidly developing, the concerns about its benefits and toxicity on the environment are growing (Asgari-Targhi et al., 2018). Nano-forms possess unique physicochemical characteristics by which triggering especial responses different from their bulk type (Asgari-Targhi et al., 2018). Plants are considered as a key component for nanoparticle transport and bio-accumulation into the food chains (Tripathi et al., 2017). Therefore, various studies in different plant species are needed to elucidate the potential benefits and phytotoxicity of the nanoparticles, especially trace elements.

Some evidence provides data on NO-mediated alleviation toxicity signs of heavy metals, while little is known about NO-mediated mitigation of heavy metal stress and nanoparticle toxicity in plants (Tripathi et al., 2017). The Se nano-particles (nSe) with different characteristics of shape and size may be synthesized from Se salts especially selenates and selenite using reducing agents (Husen and Siddiqi, 2014). Moreover, there is evidence that the endogenous NO status at different developmental stages was altered by selenite treatment in *Arabidopsis* plants, mainly in a concentration and time-dependent manners (Lehotai et al., 2011).

On the basis of our knowledge, there is no research on the application of nSe on the plant growth, anatomy, and physiology. Also, as human body may intake Se from plants (White, 2015), finding an effective eco-friendly procedure for bio-fortifying, improving the defense-related system, and triggering secondary metabolism of medicinal plants, as well as introducing its toxicity range and signs are of critical importance. However, there is no report on the simultaneous application of NO and nSe.

Peppermint (*Mentha x piperita* L.) is an important economic, medicinal, and aromatic plant. Its leaves (as a spice) and vital secondary metabolites with the various critical antimicrobial, antioxidant and cytotoxic activities are widely utilized in the food and pharmaceutical-related industries (Çoban & Baydar, 2016). It is well known that growth, physiology, and secondary metabolism in the medicinal plants is strongly affected by the different physicochemical and environmental factors. It is obvious that various researches are required to elucidate the plant responses to these factors and clarify the exact involved mechanisms. These findings may be helpful to improve our knowledge and agro-technologies, thereby developing new alternative strategies to improve plant growth, yield, and protection regarding sustainable agriculture and environmental issues. Therefore, regarding the above-highlighted significance of Se, nanoparticle, NO, and peppermint the main objectives of the current research were to clarify the key physiological and anatomical mechanisms involved in the peppermint responses to the foliar applications of nSe and/or NO at different doses, for the first time.

2 MATERIALS AND METHODS

2.1 Materials and treatments

The applied nSe was purchased from the reliable company (NanoSany Corporation, Iranian Nanomaterials Pioneers Company; Mashhad City, Khorasan Province, Iran). Its characteristics were as follows: CAS#: 7446-08-4; high purity: 99.95 %, APS: 10-45 nm; morphology: near spherical; true density: 3.89 g cm^{-3}. The purchased nSe product was the stock solution of 1000 mg l^{-1} containing 0.1 % polyvinylpyrrolidone (PVP) as stabilizer and thus, there is no need to a sonication protocol. Also, TEM image of nSe is represented in Figure 1, showing the morphology and size of the nanoparticle. The supplied nSe was the stock solution of 1000 mg l^{-1}, containing 0.1 % polyvinylpyrrolidone (PVP) as a stabilizer and thus, there is no need to sonication protocol. SNP was used as a source of NO. The peppermint (*Mentha x piperita* L.) seedlings of eight leaves were grown under natural conditions (mean temperature: 20 ˚C, RH: 32 %) treated with three different levels of nSe (0, 2, and 20 mg l^{-1}) and/or two levels of NO (0 and 8 mg l^{-1}). The foliar supplementations with nSe and/or NO were done fifteen times with 48 h intervals. It was equal to 0, 0.05, and

0.5 mg nSe spray^{-1} plant^{-1}. Two weeks after the last treatment, the treated plants were harvested for the further studies on the plant growth, anatomy, and physiology. The treatment groups were called as follows: C-control; NO- nitric oxide of 8 mg l^{-1}; nSe2- nSe of 2 mg l^{-1}; nSe20- nSe of 20 mg l^{-1}; NO + nSe2- nitric oxide of 8 mg l^{-1} and nSe of 2 mg l^{-1}; NO + nSe20- nitric oxide and nSe of 20 mg l^{-1}.

2.2 Growth characteristics and photosynthetic pigments

Various growth-related characteristics, including total fresh mass of seedlings, leaf area, and leaf length were measured. The photosynthetic pigments were extracted by 80 % (v/v) acetone as a solvent and determined according to the formula presented by Arnon (1949).

2.3 Enzyme extraction

M phosphate buffer (pH of 7.5), containing 0.5 mM Na_2-EDTA and 0.5 mM ascorbate was applied as an enzyme extraction buffer. Then, the prepared homogenates were centrifuged at four °C and the supernatants were used as an enzyme extract.

2.4 Nitrate reductase activity

The activity of nitrate reductase was measured according to the previously presented method by Sym (1984).

2.5 Peroxidase activity

Peroxidase activity was determined based on the method described by Hemeda and Klein (1990). Peroxidase activity was expressed in unit E g^{-1}fresh mass.

2.6 Measurement of phenylalanine ammonia lyase (PAL) activity

PAL activity was determined according to the method represented by Beaudoin-Eagan and Thrope (1985). The enzyme activity was calculated based on the amount of cinnamate production using the standard curve of cinnamic acid. The enzyme activity was expressed in micromole cinnamate per minute per gram fresh mass (µmole Cin. min^{-1}g^{-1}fm).

2.7 Estimation of proline content

Proline was extracted by sulfa salicylic acid (3 % w/v) and measured based on the method represented by Bates et al. (1973). The proline contents were calculated, based on the proline standard curve and expressed in microgram per gram fresh mass (µg g^{-1}f m).

2.8 Determination of total soluble phenols

The soluble phenols were extracted using ethanol solvent of 80 % (v/v) and holding in boiling water bath for 20 min. Folin Ciocalteu method was applied. Content of total soluble phenols were calculated based on the standard curve of tannic acid as a standard and finally, expressed in milligrams per gram leaf fresh mass (mg g^{-1}f m).

2.9 Histological procedure

Ethanol: glycerol (80: 20) was applied as fixator solution. Cross sections of basal stems were prepared. Handmade cross sections of the basal stems, were stained by carmine and methylene blue, observed with the light microscope and photographed.

2.10 Statistical Analysis

Data of three independent replicates was subjected to statistical analyses by SPSS software. Significant mean differences between the groups were determined according to Duncan's multiple range test at the level of $P \leq 0.05$.

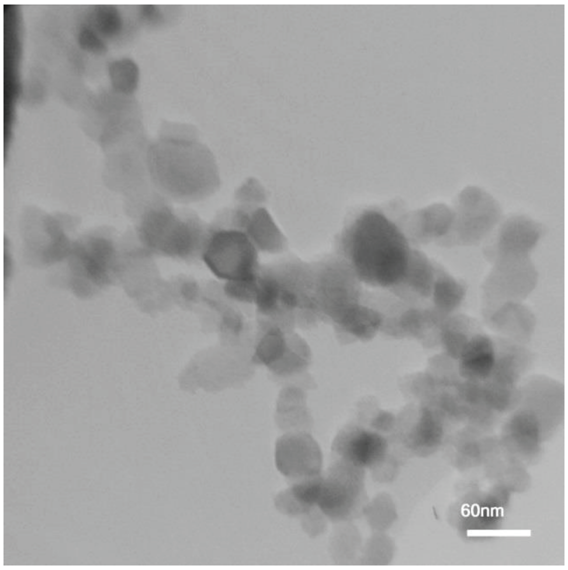

Figure 1: Transmission electron microscope (TEM) image of the spherical nSe.

3 RESULTS AND DISCUSSION

3.1 Growth, biomass, and photosynthetic pigments

It is interesting to note that leaf length and area were affected by the independent factors of the current research (nSe and NO). The significant rise in the leaf length parameter was enhanced by 7 %, 16 %, and 12 %, respectively for the NO, nSe of 2 mg l^{-1}, and NO + nSe 2 treatments, relative to the untreated control plants (Figure 2A). However, the nSe of 20 mg l^{-1} treatment decreased the leaf length about 31 % and 20.8 % for the nSe of 20 mg l^{-1} and NO + nSe 20 treatment groups (Figure 2A). Similarly, the leaf area in the NO, nSe of 2 mg l^{-1} and NO + nSe 2 treatment groups were found to be significantly ($P \leq 0.05$) higher than the control about 15 %, 38 %, and 30 %, respectively, whereas the significant reductions in this trait were recorded in the nSe of 20 mg l^{-1} (40 %) and NO + nSe 20 (24.7 %) (Figure 2B). The recorded changes in the leaf characteristics may result from the nSe and/or NO-induced alterations in the differentiation process of the leaf meristems. NO plays the critical roles in regulating the meristem cell (Sanz et al., 2014). NO is known as a vital reactive nitrogen specie and elicitor of an array of signaling pathways by which regulates the various aspect of plant development and stress responses (Sanz et al., 2014). Moreover, there is evidence that Se may alter the endogenous NO status in plants, mainly in a concentration-dependent manner (Lehotai et al., 2011). Also, the findings clearly indicated that NO alleviated the toxicity sign of the highest applied dose of nSe (20 mg l^{-1}), probably via triggering the specific signaling pathways and activating the defense-related system. Shoot fresh masses were significantly ($P \leq 0.05$) increased by 26 %, 30 %, and 42 %, respectively for NO, nSe of 2 mg l^{-1}, and NO + nSe 2 treatment groups, over the control (Figure 2C). In contrast to the nSe of 2 mg l^{-1}, the nSe of 20 mg l^{-1} treatment significantly reduced the shoot fresh mass by 33 %, relative to the control (Figure 2C). However, this growth-inhibiting impact of the nSe of 20 mg l^{-1} was mitigated by NO and the reduction percentage reached to 21.5 % (Figure 2C). In comparison with the control, the applications of the nSe of 2 mg l^{-1} and/or NO treatments, especially the latter factor, significantly ($P \leq 0.05$) improved the chlorophyll a (Chla) contents about 24 %, whereas nSe of 20 mg l^{-1} adversely affected this characteristic about 24.8 % and 15.7 % for the nSe of 20 mg l^{-1} and NO + nSe 20 groups, respectively (Figure 2D). Interestingly, the adverse impact of nSe of 20 mg l^{-1} on the Chlb content was mitigated by the applied level of NO, 8 mg l^{-1}, (Figure 2E). Similarly, the applications of the nSe of 2 mg l^{-1} and/or NO treatments, especially the latter, was significantly ($P \leq 0.05$) effective to increase total chlorophyll contents relative to the untreated control (Figure 2F). The nSe of 20 mg l^{-1} adversely influenced the total chlorophyll contents about 20 %, which was partially relieved by the NO agent and declined to 9.5% in the NO + nSe20-treated plants (Figure 2F).

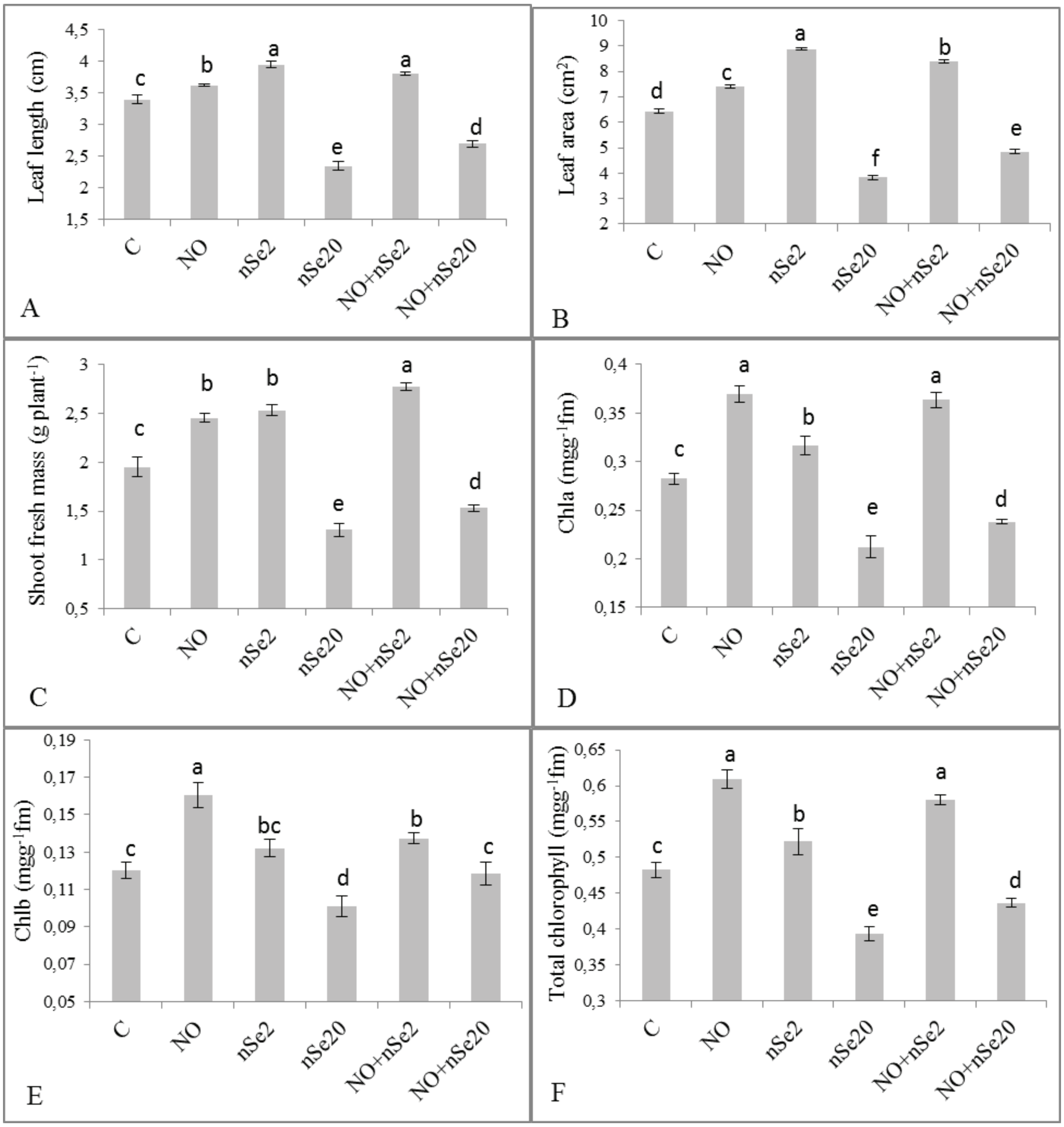

Figure 2: The recorded alterations in different growth-related characteristics and photosynthetic pigments resulted from the foliar applications of peppermint plants with the different concentrations of nSe and/or NO. C-control; NO- nitric oxide of 8 mg l^{-1}; nSe 2- nSe of 2 mg l^{-1}; nSe 20- nSe of 20 mg l^{-1}; NO + nSe 2- NO of 8 mg l^{-1} and nSe of 2 mg l^{-1}; NO + nSe 20- NO of 8 mg l^{-1} and nSe of 20 mg l^{-1}.

The modifications in the photosynthetic pigments found in the nSe and/or NO-treated plants could be caused by the possible changes in the nutrition process, redistribution of minerals, and/or the status of antioxidant system. Se-provoked alterations in the different photosynthesis-related characteristics have been reported in the various plant species, like potato (Germ et al., 2007) and basil (Ardebili et al., 2015). Se toxicity have been attributed to its similarity to sulfur (S), thereby producing nonfunctional proteins and enzymes (Pilon-Smits and Quinn, 2010; Lindblom et al., 2013). The cyto-protective role of NO in the photosynthesis has been attributed to modifying the antioxidant system, stomatal conductance, and interplay with Ca^{2+} signals (Courtois et al., 2008; Velikova et al., 2008). There is evidence that NO may improve the plant responses to stress conditions, mainly via enhancing sulfur assimilation, regulating the synthesis of reduced glutathione (GSH), and modulating the activity of antioxidant enzymes (Fatma et al., 2016). Also, it has been reported that Se provokes changes in the phytohormones, especially three vital key elicitors of defense signaling-related pathways (salicylic acid, jasmonic acid, and ethylene) (Tamaoki et al., 2008). It is interesting to note that NO may change the metabolism of auxin (Sanz et al., 2014). A specific and significant cross-talk occurs between NO and other signaling pathways organized by auxin, cytokinin, salicylic acid, jasmonic acid, ethylene, and active oxygen species (Wang et al., 2013). Thus, the above described valuable evidence highlights the significant possible potency of the nSe or NO treatments to change the growth, differentiation, physiology, and/or phytohormonal balances (a critical factor contributes to the relation between the source and sink tissues). Moreover, NO-mediated signaling may lead to the modification in assimilation-related processes, thereby dissipating the excess energy and reducing the photo-inhibition process and improving the cellular protection.

3.2 Nitrate reductase and proline

The significant inductions in the activities of leaf nitrate reductase were recorded by 80 %, 42 %, and 32 % respectively for the nSe2, NO + nSe 2, and NO + nSe 20 treatment groups, compared with the control (Figure 3A). It is interesting to note that; the nSe of 20 mg l^{-1} significantly inhibited the activity of the nitrate reductase enzyme by 31 % (Figure 3A). Taking account of the nitrate reductase (a critical enzyme involved in the nitrogen assimilation), the recorded dramatic decrease in the activity of this enzyme in the nSe 20 group, contrasted with the nSe 2 clearly reflected that nSe in a dose and application method dependent manner may lead to the improvement or disturbance in the nitrogen assimilation process. The findings showed that NO mitigated the toxicity signs of the nSe of 20 mg l^{-1}. The induction in the nitrate reductase activity found in the NO + nSe 20 treatment group may be responsible for mitigating the toxicity sign of the high dose of nSe. Therefore, the nitrate reductase activity may regard as a key mechanism and index contributing to the potential benefits and toxicity of nSe. In agreement with our results, Se fortification stimulated the nitrogen assimilation process, via improving the activities of the nitrate reductase, in potato (Munshi and Mindy, 1992), and wheat (Hajiboland and Sadeghzade, 2014). Also, it has been reported that the Se fortification altered contents of the leaf nitrate in spinach (Golubkina et al., 2017). Although, there is evidence that Se different salts in a dose and source dependent-ways prevented the nitrate uptake and activity of the nitrate reductase in barley (*Hordeum vulgare* L.) which was partially alleviated by the sulfate (Aslam et al., 1990). The proline contents in the nSe and/or NO-treated plants were found to be significantly higher than the control, among which the highest amounts were recorded in the NO + nSe 20 (43.7 %) and NO + nSe 2 (39 %) groups (Figure 3B). The dramatic increase in the proline content as a multifunctional cytoprotective agent may result from the nSe and/or NO applications. The Se-mediated modification in the amino acid metabolism has been introduced as a critical mechanism by which it may enhance plant resistance to stress condition (Ježek et al., 2011; Ardebili et al., 2015). Moreover, the modification in the nitrogen assimilation and metabolism may be considered as an alternative way to dissipate the excess energy, thereby reducing photo-inhibition process and improving cellular protection.

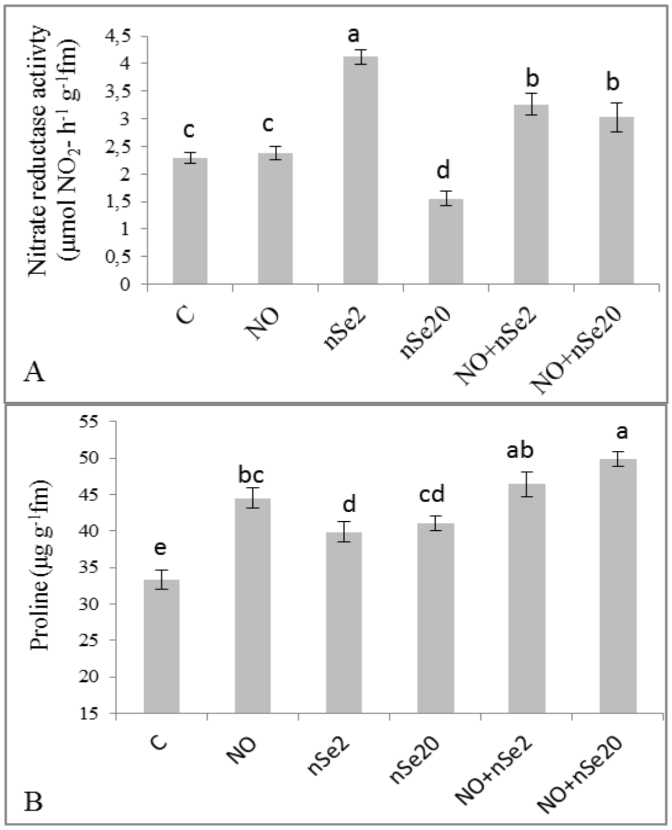

Figure 3: The recorded changes in the activity of nitrate reductase enzyme and proline contents caused by the different concentrations of nSe and/or nitric oxide. C-control; NO- nitric oxide of 8 mg l^{-1}; nSe 2- nSe of 2 mg l^{-1}; nSe 20- nSe of 20 mg l^{-1}; NO + nSe 2- NO of 8 mg l^{-1} and nSe of 2 mg l^{-1}; NO + nSe 20- NO of 8 mg l^{-1} and nSe of 20 mg l^{-1}

3.3 Peroxidase, PAL, and soluble phenols

In comparison to the control, the peroxidase activities were induced by 57 %, 35 %, 68 %, and 50 % respectively for the NO, nSe 2, NO + nSe 2, and NO + nSe 20 treatment groups, while this parameter was adversely influenced about 33 %, for the individual treatment of the nSe of 20 mg l^{-1} (Figure 4A). The alleviated toxicity sign of nSe of 20 mg l^{-1} by NO may be partially attributed to the dramatic increases in the peroxidase activity, a crucial antioxidant enzyme. In plants, Se- induced antioxidant system has been regarded as a main mechanism contribute to its protective roles against various abiotic stress conditions (Feng et al., 2013; Ardebili et al., 2014; Ardebili et al., 2015).

The foliar supplementations with nSe and/or NO led to the improvement in the activities of PAL enzyme by 68 %, 28 %, 45 %, 47 %, and 70 % respectively for the NO, nSe 2, nSe 20, NO + nSe 2, and NO + nSe 20 groups, over the untreated control samples (Figure 4B). In addition, except for the NO group (5 %) the other applied treatments had significantly higher amounts of the soluble phenols by 12 %, 26 %, 20 %, and 20.5 %, over the control respectively (Figure 4C). Considering the observed inducing effects of the PAL activity (a key enzyme of phenylpropanoid metabolism) and the soluble phenols by the applied supplements, it seems that these compounds have a considerable potency to affect secondary metabolism, thereby changing the quality and quantity of the secondary metabolites in plants, especially towards medicinal crops. As mentioned above an increase in the salicylic acid, a major phenylpropanoid involved in a plant acclimatization response to various stress factors, has been observed in the Se-treated *Arabidopsis* (Tamaoki et al., 2008). Also, rise in the phenylalanine content has been reported in the Se-fertilized plant (Munshi et al., 1990; Ježek et al., 2011). The production of the phenylpropanoids, mainly derived from phenylalanine and tyrosine, is triggered as one of the crucial defense mechanisms under a variety of biotic and abiotic stress agents. Therefore, it seems that the possible enhances in the salicylic acid contents may be responsible for the recorded results.

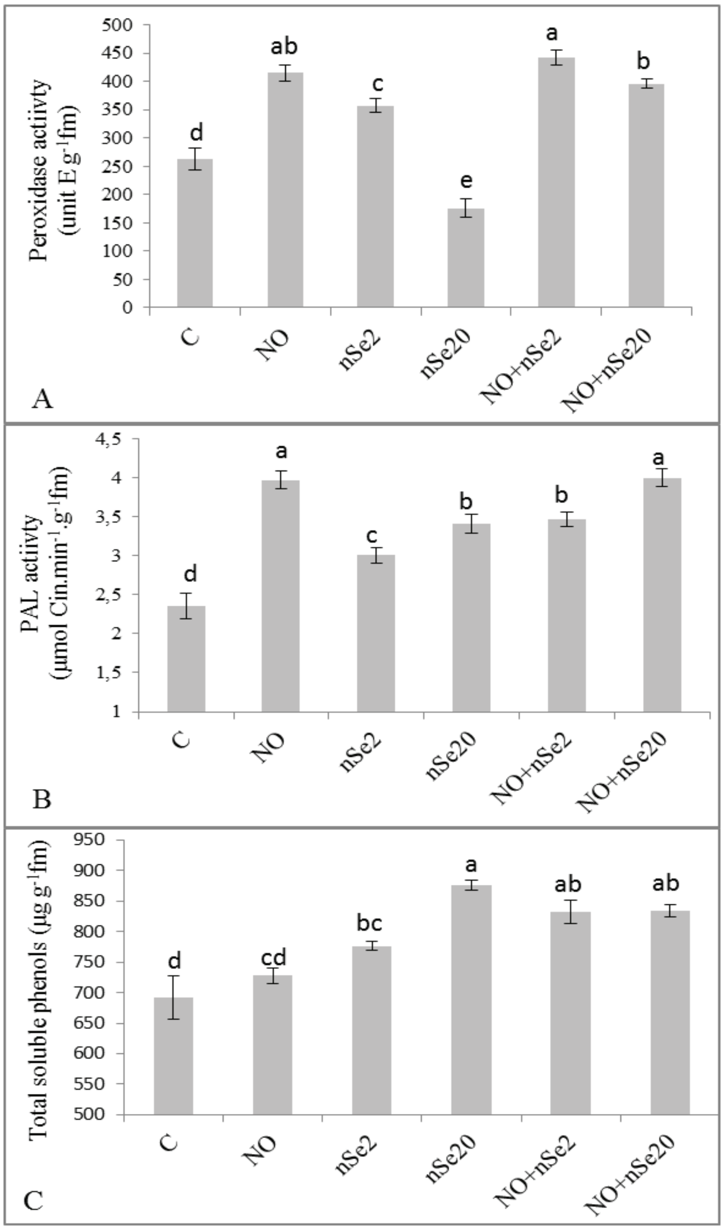

Figure 4: The recorded changes in the activities of peroxidase and PAL enzymes and total soluble phenols induced by the different concentrations of nSe and/or nitric oxide. C-control; NO- nitric oxide; nSe 2- nSe of 2 mg l^{-1}; nSe20- nSe of 20 mg l^{-1}; NO-nSe2- nitric oxide and nSe of 2 mg l^{-1}; NO-nSe20- nitric oxide and nSe of 20 mg l^{-1}.

3.4 Anatomical evaluation

In a complementary study, anatomical changes in the nSe and/or NO-treated plants were evaluated based on the cross-section of the basal stem (Figure 5). As it is presented in Figure 5, the toxicity of nSe (20 mg l^{-1}) treatment led to the severe cell destruction in the cortex layer of the basal stem (Figure 5E). However, the destructive impact of this treatment was partially alleviated by NO (Figure 5F). While, the application of nSe of 2 mg l^{-1} led to the only slightly destruction in collenchyma tissue (Figure 5C, D). Also, the NO strengthened the collenchyma tissue as it was depicted with the more colorfulness of this tissue in NO-treated plants. The present research provides the anatomical evidence on the toxicity of nSe for the first time on the basis of our knowledge. The found cellular destruction in the cortex layer may result from the ethylene accumulation as a cellular destruction agent, mainly via inducing the activity of hydrolytic enzymes (especially pectinase). Also, salicylic acid in combination with the ethylene may be considered as a triggering factor of the programmed cell death which is partially delayed by NO, the efficient signaling agent. The enhanced rate of ethylene synthesis results in the cell death and destruction (Gunawardena et al., 2001). Se provokes alterations in the phytohormones, including salicylic acid, jasmonic acid, and ethylene (Tamaoki et al., 2008), thereby affecting a cellular metabolism and patterns of gene expression. Also, NO may change an auxin metabolism (Sanz et al., 2014). Moreover, Se alters the endogenous NO status in plants (Lehotai et al., 2011). Plant defense system is modulated through the multiple networks of transduction pathways triggered by some critical signaling molecules, especially active oxygen species, NO, salicylic acid, jasmonic acid, and ethylene (Bouchez et al., 2007). Salicylic acid is involved in the induction process of the programmed cell death (Brodersen et al., 2005). Also, it is well known that ethylene may provoke the programmed cell death pathways and influences the activities of the hydrolytic enzymes, especially pectinase. There is evidence depicting NO acted as a protective antioxidant agent and delays the programmed cell death in barley aleurone cells (Beligni et al., 2002). Therefore, the recorded cellular destruction caused by nSe could be attributed to changes in the phytohormonal balances, especially ethylene, where this toxicity sign is partially alleviated by the applied exogenous NO, probably through influencing the hormonal balances and/or triggering the specific defense signaling.

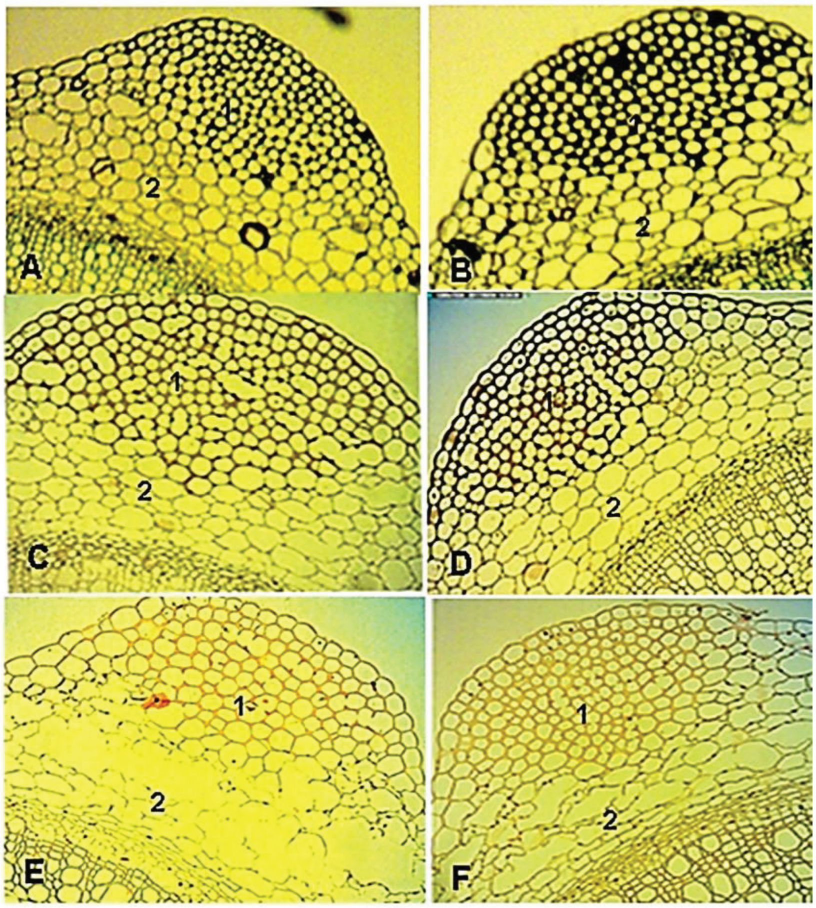

Figure 5: The recorded anatomical changes based on the cross-sections of the basal stem of peppermint plants supplemented by the different concentrations of nSe and/or NO. A-control; B- NO of 8 mg l^{-1}; C- nSe of 2 mg l^{-1}; D- nSe of 2 mg l^{-1} and NO of 8 mg l^{-1}; E- nSe of 20 mg l^{-1}; F- nSe of 20 mg l^{-1} and NO of 8 mg l^{-1}. 1 and 2 refer to the collenchyma tissue and cortex layer, respectively

4 CONCLUSION

The present study represents the valuable data on the potential benefits and phytotoxicity of nSe and the partially mitigating effects of NO. The obtained results clearly represented that nSe and/or NO in a dose-dependent manner has a considerable potency to affect the plant growth, anatomy, and metabolism. It seems that the simultaneous applications of the suitable concentrations of nSe and NO may be introduced as an alternative strategy to improve the plant growth, metabolism, and protection, taking account of the sustainable agriculture and environmental issues. Also, NO-mediated critical mechanisms, including a higher possible efficiency of photosynthesis, regulating the activities of key enzymes, modifying the nitrogen assimilation, enhancing in the proline content as a multifunctional protecting agent, and controlling the phytohormonal balances may responsible for mitigating the toxicity sign of the high dose of nSe. In addition, the anatomical evidence of the phytotoxicity of the high dose of nSe and the potential benefits of NO was provided for the first time. It is obvious that more concise studies are required to justify the exact involved mechanisms (especially at a molecular level).

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Divergence in hybrid rice parental lines detected by RAPD and ISSR markers

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ABSTRACT

Genetic distance between parental lines used in hybrid rice breeding program was estimated based on information from molecular markers data. Sixteen parents (5 CMS, 5 maintainers and 6 restorers) were analyzed with 15 random amplification polymorphic DNA (RAPD) and 20 inter simple sequence repeat (ISSR) marker. Out of 15 RAPD markers, 9 were polymorphic and 79 bands were generated, of which 28 bands were polymorphic (35 %). By using 10 out of 20 ISSR markers 86 bands were detected, of which 35 bands were polymorphic (41 %). Marker index (RAPD = 1.68; ISSR = 1.88) and percent of polymorphic bands indicated that ISSR markers were relatively more efficient in polymorphism detection. Cluster analysis of the parents based on Jaccard's genetic similarity and UPGMA method were revealed 3 groups. Restorer lines IR68061 and IR5931 with origin of the Philippines were found in distinct group. Results suggesting the cross between CMS lines Neda and Nemat with restorer lines IR68061 and IR5931, can be used as the best heterotic groups for exploration of heterosis.

Key words: rice; restorer; maintainer; cluster analysis; molecular marker

IZVLEČEK

LOČEVANJE HIBRIDNIH STARŠEVSKIH LINIJ RIŽA Z RAPD IN ISSR MARKERJI

Genetska oddaljenost med starševskimi linijami riža, ki so bile uporabljene v programu vzgoje hibridnega riža, je bila ocenjena na osnovi informacij iz podatkov, pridobljenih z molekularnimi markerji. Šestnajst staršev (5 CMS, 5 vzdrževalnih linij (vzdrževalci) in 6 linij, ki povrnejo fertilnost (obnovitelji) je bilo analiziranih s 15 RAPD in 20 ISSR markerji. Med 15 RAPD markerji jih je bilo 9 polimorfnih. Teh 9 markerjev je pomnožilo 79 fragmentov, med katerimi jih je bilo 28 polimorfnih (35 %). Z uporabo 10 od 20 ISSR markerjev so pomnožili 86 fragmentov, od katerih je bilo 35 polimorfnih (41 %). Indeks markerjev (RAPD = 1,68; ISSR = 1,88) in odstotek polimorfnih fragmentov sta pokazala, da so bili ISSR markerji relativno uspešnejši pri ugotavljanju polimorfnosti. Klasterska analiza staršev, ki je temeljila na Jaccardovi genetski podobnosti in na UPGMA metodi je pokazala tri skupine. Liniji obnoviteljev IR68061 in IR5931, z izvorom na Filipinih, sta tvorili jasno ločeno skupino. Rezulti nakazujejo, da bi križance med linijama CMS Neda in Nemat z linijama obnoviteljev IR68061 in IR5931 lahko uporabili kot najboljšo skupino križancev pri uporabi tega načina žlatnenja.

Ključne besede: riž; obnovitelej (restorer); vzdrževalec (maintainer); klasterska analiza; molekularni marker

1 INTRODUCTION

Evaluation of genetic diversity in the parental lines is associated with heterosis and performance in hybrid rice production (Wang, 2006). Cytoplasmic male sterility (CMS) systems have been greatly developed in production of hybrid seed and more than 90 % of the rice hybrids belong to wild abortive cytoplasmic source (Yao et al., 1997). Cytoplasmic male sterility is the most influential method for utilization of heterosis. Three different sources of CMS lines (Wild abortive, BaoTai and HongLian) have been characterized (Nematzadeh et al., 2010). Production and commercial exploitation of hybrid rice require wild abortive and nuclear restoration fertility (rf) system.

DNA markers have been utilized for the evaluation of genetic diversity, which could be used to predict heterosis prior to expensive field testing in breeding program (Zhao et al., 1999; Xiao et al., 1996). Among DNA markers, RAPD technique due to simple and low cost process and require no gene sequence information

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is considered to be a suitable approach for identification of cultivar, estimation of genetic diversity and genome mapping and tagging (Wang et al., 1995; Borokova et al., 1995; Raghunathachari et al., 2000). The technique has been used to study genetic diversity among cultivars of several crops like rice (Qian et al., 2001; Rabbani et al., 2008; Rahman et al., 2007; Raghunathachari et al., 2000), Barley (Hou et al., 2005) and tomato (Mansour et al., 2010).

In order to unravel some limitation of RAPD marker including dominant inheritance, low reproducibility and uncertainty in locus homology, Inter Simple Sequence Repeat (ISSR) marker can be used to determine genetic diversity. ISSR as a dominant marker involves PCR amplification of given microsatellite repeat which anchored at the 3′ or 5′ end by one to four arbitrary nucleotides using a single sequence repeat (SSR) motifs containing marker (Zietkiewicz et al., 1994). ISSR system has been successfully applied for evaluation of genetic diversity among rice cultivars and other crop plants (Godwin et al., 1997; Joshi et al., 2000; Qian et al., 2001; Wu et al., 2004).

The objective of the present study was to estimate the genetic diversity among 16 parental lines in hybrid rice breeding program using RAPD and ISSR markers.

2 MATERIALS AND METHODS

2.1 Plant materials and DNA extraction

Sixteen hybrid parental lines including 5 CMS lines, 5 maintainer lines (B-line) and 6 restorer lines (R-line) were used in this study (Table 1). Seeds were obtained from Genetics and Agricultural Biotechnology Institute of Tabarestan, Sari, Iran. Leaf tissues from parental lines were harvested from seedling grown in a greenhouse. Total genomic DNA was extracted following the method of Dellaporta et al. (1983), and its quality and quantity were estimated by spectrophotometry and gel electrophoresis, respectively.

Table1: List of parental lines (CMS, B and R lines) used in this study

Line name	Type	Origin
Nemat	CMS	Iran
Nemat	B-line	Iran
Neda	CMS	Iran
Neda	B-line	Iran
Dasht	CMS	Iran
Dasht	B-line	Iran
Champa	CMS	Iran
Champa	B-line	Iran
Amol-3	CMS	Iran
Amol-3	B-line	Iran
Sepidrod	R-line	Iran
Poya	R-line	Iran
Pajohesh	R-line	Iran
IR-50	R-line	The Philippines, IRRI
IR68061	R-line	The Philippines, IRRI
IR5931	R-line	The Philippines, IRRI

2.2 RAPD amplification and assay

The set of 15 random markers of Operon technologies Inc. were screened. From these, nine markers (Table 2) that produced sharp and scorable bands were selected and used for amplification. PCR aided amplification reaction were carried out in a 12.5 reaction volume containing 1x buffer PCR (containing 10 mM Tris-HCl (PH 8.8) and 50 mM KCl 0.8 %), 0.5 mM dNTPs, 5 pM RAPD marker, 2.5 mM $MgCl_2$, 30 ng genomic DNA, and 1 Unit of *Taq* DNA polymerase. The amplification was performed on a MJ Mini, BIO-RAD thermal cycler (USA) for initial step of 4 min at 94 °C, followed by 40 cycles of 60 s at 94 °C , 60 s at 35 °C , 60 s at 72 °C and a final 7 min extension step at 72 °C . The amplified products were separated by electrophoresis on 1.5 % agarose gels stained with ethidium bromide and visualized by illumination under UV light and photographed. Molecular weights were estimated using a DNA marker 100 bp plus (SM0321, Fermentas).

2.3 ISSR amplification and assay

A total of 20 ISSR markers were screened, in a preliminary experiment. From these, 10 markers were selected for their reproducibility and polymorphism (Table 2). The PCR reaction were performed in the same way as RAPD except for annealing temperature at 50-56 °C. Amplified product were loaded in 2 % agarose gels and were visualized under UV after staining with ethidium bromide. The size of amplified product was estimated using 100 bp plus (SM0321, Fermentas).

2.4 Data analysis

Polymorphic bands from dominant markers RAPD and ISSR were scored for the presence (1) or absence (0). Only clear PCR fragments were scored and entered into binary character matrix for further statistical analysis. The capacity of markers to detect polymorphic loci was measured using polymorphic information content (PIC) values and marker index (MI). PIC value was calculated as follows: PIC = Σ [2p_i (1- p_i)], where p_i is the frequency of *i*-th allele (Roldàn-Ruiz, 2000; Powell, 1996). The marker index of each marker was estimated using formula; MI = PIC × EMR. Effective multiplex ratio (EMR) is the number of polymorphic loci in the genotype, was calculated as; EMR = n × β, where n is the average number of amplified fragments, and β was estimated as; PB/(PB + MB), where PB and MB represent the polymorphic and monomorphic loci, respectively.

The binary data obtained by scoring the RAPD and ISSR were subjected to calculate Jaccard similarity coefficient. Coefficient estimated were used to construct SAHN (Sequential Agglomerative Hierarchical Non Overlapping) clustering using an Unweighted Pair Group Method with Arithmetic Average (UPGMA) in NTSYS software (Rohlf, 1994). In order to determine the best dendrogram analysis to the matrix, cophenetic correlation was performed based on Mantel test (Mantel, 1967).

3 RESULTS

3.1 RAPD analysis

Among 20 tested arbitrary markers for 16 CMS parental lines, 9 RAPD markers producing clear and polymorphic bands were selected. The number of bands produced by different markers varied from 4 to 12 with an average of 8.87. A total of 79 bands ranging from 200 to 2500 bp were scored, among which 28 bands were polymorphic (35 %). The number of polymorphic bands varied from 1 to 6 with an average of 3.11 (Table 2). Figure 1 shows an example of polymorphic bands of marker OPA11. PIC varied from 0.29 (OPA12) to 0.45 (OPA03) with average of 0.37. The marker index, a reflection of marker usefulness, was calculated for each RAPD marker. The average of MI value was 1.68 and ranged from a low of 0.88 (OPH06) to a high of 2.66 (OPH16).

Table 2: Polymorphism parameter detected with 10 ISSR and 9 RAPD markers in 16 parental lines of hybrid rice

Marker	marker sequence (5'-3')	TB	PB	MB	PPB (%)	PIC	EMR	MI
ISSR								
ISSR2	(GA)$_9$C	10	5	5	50	0.33	6.35	2.11
ISSR7	(GA)$_8$ C	15	7	8	47	0.37	6.80	2.54
ISSR8	(CT)$_8$ G	7	3	4	43	0.18	5.70	1.05
ISSR12	(GA)$_8$A	6	5	1	83	0.28	13.17	3.72
ISSR13	(TC)$_8$C	6	2	4	33	0.40	4.67	1.86
ISSR14	(TC)$_8$G	7	2	5	29	0.40	4.20	1.69
ISSR15	(AC)$_8$G	9	4	5	44	0.41	4.99	2.07
ISSR17	(AC)$_8$C	10	3	7	30	0.30	4.53	1.36
ISSR18	(ATC)$_6$T	8	2	6	25	0.25	3.84	0.95
ISSR19	(ATC)$_6$C	8	2	6	25	0.39	3.72	1.44
Total		86	35	51				
Avg./marker		8.6	3.5	5.1	40.9	0.33	5.80	1.88
RAPD								
OPA03	AGTCAGCCAC	12	3	9	25	0.45	3.44	1.53
OPA11	CAATCGCCGT	8	2	6	25	0.36	3.72	1.32
OPA12	TCGGCGATAG	9	5	4	56	0.29	6.05	1.76
OPA17	GACCGCTTGT	6	2	4	33	0.43	4.28	1.85
OPH03	AGACGTCCAC	10	4	6	40	0.31	5.40	1.66
OPH06	ACGCATCGCA	6	1	5	17	0.38	2.33	0.88
OPH16	TCTCAGCTGG	4	2	2	50	0.39	6.88	2.66
OPN01	CTCACGTTGG	14	6	9	43	0.37	5.95	2.18
OPN02	ACCAGGGGCA	10	3	7	30	0.35	3.75	1.30
Total		79	28	52				
Avg./marker		8.78	3.11	5.78	35.44	0.37	4.64	1.68

TB: total bands, PB: polymorphic bands, MB: monomorphic bands, PPB (%): percentage polymorphic bands, PIC: polymorphism information content, EMR: effective multiplex ratio, MI: marker index

3.2 ISSR analysis

Among 20 ISSR markers, 10 markers produced clear band profile. A total of 86 scorable bands with the average of 8.6 bands per marker were generated with polymorphism in 35 bands (41 %). The size of amplified products varied from 200 to 2000 bp. The number of polymorphic bands varied from 2 to 7 with the average of 3.5 polymorphic bands per marker (Table 2). ISSR pattern generated by marker ISSR15 in agarose gel is shown in Figure 1. The mean of PIC value for ISSR markers was 0.33 and varied from 0.18 (ISSR 8) to 0.41 (ISSR 15). The highest and the lowest MI value were 3.72 (ISSR 12) and 0.95 (ISSR 18), respectively with an average of 1.88.

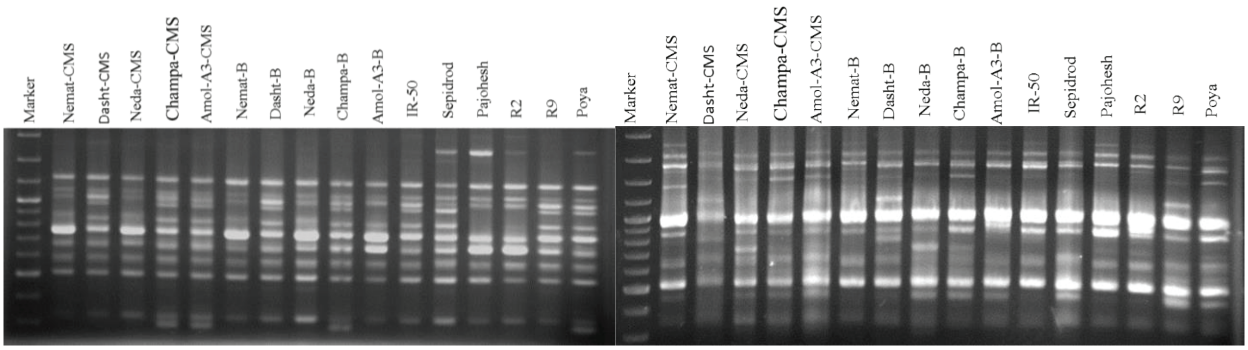

Figure 1: RAPD and ISSR amplification profiles of a 16 parental lines in rice, illustrated by marker OPA11 (left) and ISSR15 (right)

3.3 Genetic relationships

Similarity matrices obtained based on marker data using Jaccard´s coefficient pair-wise comparison between genotypes based on RAPD markers revealed that genetic similarity ranged from a maximum of 0.97 (between Neda-CMS and Neda-B) to minimum of 0.75 (between IR68061 and Dasht-CMS) and 0.77 (between Amol3CMS and IR5931), and based on ISSR markers from a maximum 0f 0.974 (between Champa-B and Amol3-B) to minimum of 0.76 (between IR5931 and Nemat-CMS) and 0.77 (between IR5931 and Neda-CMS).

Maximum genetic similarity of combination similarity coefficient was observed between Champa-B and AmolA3-B (0.965), and minimum was observed between Dasht-CMS and IR5931 (0.786). Average of similarity index in RAPD and ISSR markers were 0.866 and 0.869, respectively.

3.4 Cluster analysis

The cluster analysis based on Jaccard´s similarity coefficient through RAPD and ISSR data generated similar dendrogram topologies with some differences. The dendrogram based on RAPD analysis presented in Fig. 2. Cluster analysis based on RAPD data grouped 16 lines to 3 major groups. Group I consisted of four genotypes namely Nemat-CMS, Nemat-B, IR50 and Poya. The majority of the genotypes placed in group II, while two the Philippines' varieties formed a distinct cluster of III.

Cluster analysis based on ISSR data also formed three groups (Fig. 3). Nemat-B and Neda-B with their counterparts (Nemat-CMS and Neda-CMS) were put in cluster I. Cluster II consisted of Dasht-CMS, Dasht-B, Champa-CMS, Pajohesh, Amol3-B, Champa-B, IR50, Spidrod, Amol3-CMS and Poya. The Philippines' varieties IR68061 and IR5931 formed a distinct group (cluster III).

Matrices correlation coefficient were obtained using Mantel test, to compare the two Jaccard´s similarity matrices and the correlation value, was 0.61. The test indicated that cluster constructed based on RAPD and ISSR markers were approximately conserved (moderate degree of correlation).

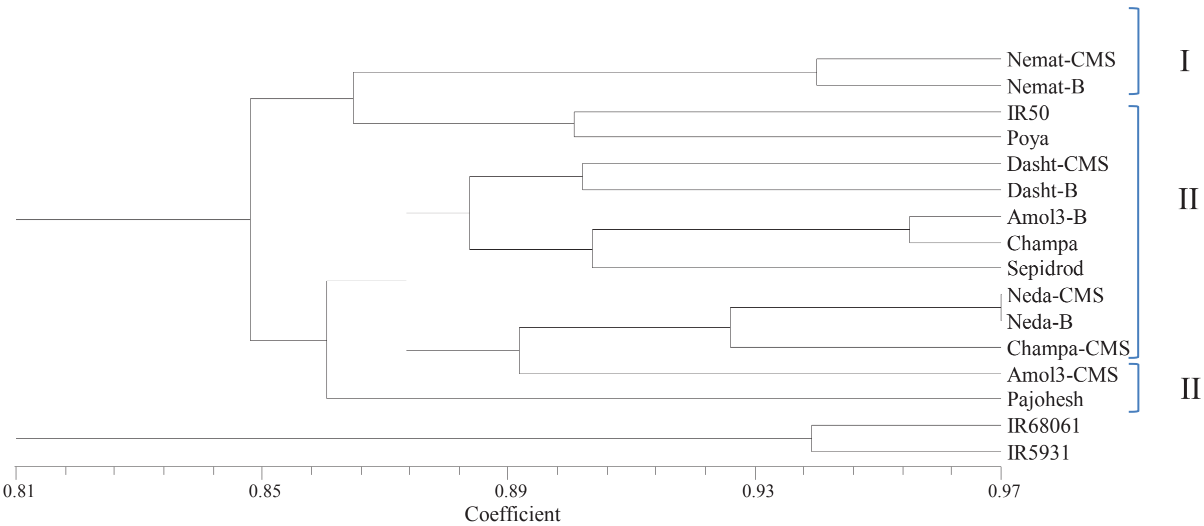

Figure 2: Cluster of 16 parental lines based on RAPD markers using the UPGMA methods

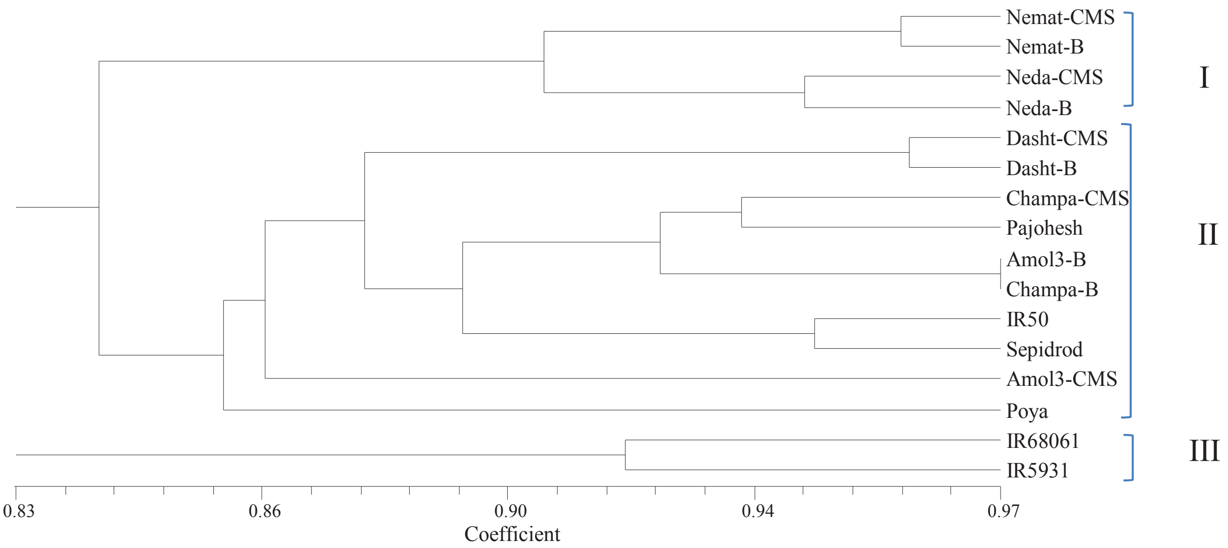

Figure 3: Cluster of 16 parental lines based on ISSR markers using the UPGMA methods

4 DISCUSSION

Prerequisite of performance and heterosis of hybrids is maximum diversity in parental lines, hence the grouping of parental lines based on genetic divergence is essential for breeders. In order to resolve the problems associated with morphological and biochemical markers, molecular markers considered as alternative tools for diversity investigations. Several markers such as SSR, AFLP, and RFLP have been used to determine genetic diversity between parental lines in rice (Subudhi et al., 1998; Zhang et al., 1997; Xu et al., 2002). In present investigation, RAPD and ISSR markers were employed to detect the genetic relationship between parental lines of hybrid rice (CMS, B and R lines).

The proportion of polymorphic bands revealed by ISSR (41 %) and RAPD (35 %), and mean MI for each of the markers (1.88 for ISSR and 1.68 for RAPD) suggested that ISSR marker systems have more discriminating power than RAPD markers in genetic diversity detection. Mean PIC for each of the markers was approximately similar (0.33 for ISSR and 0.37 for RAPD). Qian et al. (2001) also reported similar results in assess of genetic diversity in wild rice (O*ryza granulata* Nees & Arn. ex G.Watt.) population. Also, Ravi et al, (2005) reported SSR were more polymorphic than RAPD markers. A number of research groups reported that RAPD markers are more polymorphic than ISSR markers (Muthusamy et al., 2008; Kumar et al., 2014), which might be because of different frequency of microsatellite rich-region in various organisms and different amplification of genomic regions by each of marker system (Xu et al., 2002).

According to cluster topologies, the CMS lines were grouped in minimal distance from their maintainer lines such as Neda-CMS-Neda-B, Nemat-CMS-Nemat-B and Dasht-CMS-Dasht-B both in Fig 2 and Fig. 3, which suggested that nuclear genes of maintainer lines were similar with corresponding CMS lines. However, Champa-CMS and Amol3-CMS were not placed in minimal distances with their counterparts, Champa-B and Amol3-B. The reason behind this observation might be due to some degrees of contamination in multiplication process of these two CMS lines. This study involved thirteen hybrid parental lines from Iran along with three R lines from the Philippines (IR50, IR68061 and IR5931). IR50 had similarity with the genotypes of cluster I and cluster II using RAPD and ISSR markers, respectively. While the two Philippines lines, IR68061 and IR5931, formed a distinct group either in dendrograms using RAPD and ISSR data (Fig. 2 and Fig. 3).

The majority of lines were distributed into group I and II, however, R lines were grouped into distinct subgroup in all clusters. Restorer lines IR68061 and IR5931 formed distinct group III revealing highest genetic distance with other CMS and B lines. Genetic distance between restorer parents with IRRI origin and other Iranian lines suggested that these genotypes could be used to attain heterosis in further crosses. In group I, Neda and Nemat (CMS and maintainer lines) were tightly grouped together in molecular clustering studied with ISSR (Figure 3) which could be due to the existence of common pedigree in both cultivars

(Nematzadeh et al., 1997; 2001). Similar results were observed in assessing genetic variation using RAPD and SSR markers for predicting performance and heterosis of indica × japonica hybrids (Xiao et al., 1996).

Results obtained from this study suggested that, i: ISSR is superior to RAPD in assessing genetic distance and relationship between CMS and restorer lines. ii: based on genetic relationship, one can expect that the cross of CMS lines in group I (Neda and Nemat) with restorer lines in group III (IR68061 and IR5931), with the highest genetic distance, can develop the best heterotic groups for accessing strong heterosis. Therefore, hybrids of Neda-CMS × IR68061, Neda-CMS × IR5931, Nemat-CMS × IR68061 and Nemat-CMS × IR5931 can be suggested for experimental hybrid seed production. The heterotic group detection in parental lines with the aid of RAPD and ISSR markers could be effective tools to prevent the crosses between unrelated heterotic groups, thus reducing costs in hybrid seed production.

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Validation of the multiresidual GC-MS method for determining plant protection product residues in strawberries

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ABSTRACT

Gas chromatography coupled with mass spectrometry was used for the introduction and validation of the multiresidual method for determining of plant protection product residues in strawberries. During the validation procedure, limits of quantification were set and the method was checked for its recovery, linearity, repeatability, reproducibility and measurement uncertainty. An interlaboratory comparison was also performed to check the accuracy of the method. The method was proven to be fit for purpose. Afterwards 19 strawberry samples were analysed for the presence of plant protection product residues using the validated method. In the strawberries 5 active substances, all fungicides, were found: chlorothalonil, cyprodinil, fludioxonil, metalaxyl+metalaxyl-M and pyrimethanil. Residues of these active substances were in range 0.01 – 0.44 mg/kg. No cases exceeding the maximum residue levels were measured.

Key words: pesticide residues; GC-MS; strawberries; plant protection product residues; multiresidual method

IZVLEČEK

VALIDACIJA MULTIREZIDUALNE GC-MS METODE ZA DOLOČEVANJE OSTANKOV FITOFARMACEVTSKIH SREDSTEV V JAGODAH

Plinsko kromatografijo sklopljeno z masno spektrometrijo smo uporabili za vpeljavo in validacijo multirezidualne metode za določanje ostankov fitofarmacevtskih sredstev v jagodah. Med validacijo smo postavili meje kvantitativne določitve metode in preverili izkoristek, linearnost, ponovljivost, obnovljivost in merilno negotovost metode. Sodelovali smo tudi v medlaboratorijski primerjavi, da smo preverili točnost metode. Za metodo se je izkazalo, da ustreza namenu. Nato smo z validirano metodo ugotavljali prisotnost ostankov fitofarmacevtskih sredstev v 19 vzorcih jagod. V njih smo določili 5 aktivnih spojin: klorotalonil, ciprodinil, fludioksonil, meatalaksil + metalaksil-M in pirimetanil. Ostanki teh aktivnih snovi so se gibali v območju 0,01 – 0,44 mg/kg. Preseganja maksimalnih dovoljenih količin ostankov nismo izmerili.

Ključne besede: ostanki pesticidov; GC-MS; jagode; ostanki fitofarmacevtskih sredstev; multirezidualna metoda

1 INTRODUCTION

Fruit is an important part of our diet for its nutrition and health properties. To prevent the destruction of food crops by agricultural pests and to improve plant quality, plant protection products (PPPs) must be used in fruit production. While monitoring the PPP residues in fruit, vegetables and cereals, we noticed (Baša Česnik et al., 2009) that fruit contains the highest number of active compounds. Farmers need to protect fruit against rot, mould and insects, otherwise the fruit would not grow. Strawberries are mainly attacked by the diseases *Botrytis cinerea* (Persoon), *Colletotrichum acutatum* (J.H. Simmonds), *Oidium fragariae* (Harz) and *Mycospharella fragariae* ((Tul.) Lindau) and by the pests *Steneotarsonemus fragariae* (Banks, 1901), *Anthonomus rubi* (Herbst, 1795), and *Tetranychus urticae (*C. L. Koch, 1836*)* (Sójka et al., 2015). Therefore, the use of PPPs during strawberry growth is inevitable.

Unfortunately, PPP residues can have a negative impact on consumer health when they exceed the Maximum Residue Levels (MRLs). Therefore, the monitoring of PPP residues is necessary. For proper monitoring, efficient analytical methods are required, which enable analysis of large number of active substances and their residues at the same time.

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For determining the PPP residues in strawberries, a number of analytical methods were published. The first step in the methods is usually performed by liquid-liquid extraction, with three main solvents being used: ethylacetate (Berrada et al., 2006; Ferrer et al., 2005), acetonitrile –also known as the QuEChERS method (Bakirci et al., 2014; Lehotay et al., 2007) or acetone (Jardim et al., 2012; Stan, 2000). Our laboratory used acetone because of its high volatility and miscibility with the water present in strawberry matrices. For the better extraction of active substance residues, we added dichloromethane and petroleum ether to the acetone. In this way, a wide range of active substances from medium polar (e.g. diazinon and dimethoate) to non-polar (e.g. chlorpyrifos and cyhalothrin-lambda) were extracted. The extraction of PPP residues from the strawberry matrix is complicated because of its acidity. Therefore, in our laboratory, pH adjustment was used for better extraction efficiency, similar to the in QuEChERS method. CH_3COONa and acetic acid were added to the strawberry matrix, which enhanced the extraction efficiency of pH sensitive active compounds (e.g. pirimicarb and pyrimethanil).

For determining active substance residues, chromatography is usually used. Gas chromatographs (GC), used for non-polar to medium polar and volatile compounds, can be connected to a flame ionisation detector (FID), electron capture detector (ECD), nitrogen phosphor detector (NPD), flame photometric detector (FPD) or mass spectrometer (MS). In our laboratory, an MS was used as this is the only system that enables unequivocal qualitative and quantitative detection of active substance residues based on chromatographic retention time and mass spectra.

The purpose of this paper is to present the introduced, modified (pH adjustment) and then validated gas chromatography-mass spectrometry (GC-MS) method, which enables the qualitative and quantitative determination of a wide range of active compounds in strawberries and their residues in one chromatographic run. Statistical analyses for the obtained data were used: for linearity using the F test, for accuracy by checking recoveries and cooperation in interlaboratory comparisons, for precision according to ISO 5725 standard and for measurement uncertainty by multiplying the standard deviation by Student's t factor for 9 degrees of freedom and a 95% confidence level. Finally, method implementation in practice was performed.

2 MATERIALS AND METHODS

2.1 Materials

Chemicals:
Acetone (Merck), dichloromethane (Merck), ethyl acetate (Merck), cyclohexane (Merck) and petroleum ether (Merck) with p.a. grade and GC grade were used as solvents in our experiment. Similarly, only active substances (dr. Ehrenstorfer, Pestanal) with the highest available purity on the market (a minimum of 95 %) were used.

Preparation of the solutions:
Stock solutions in a mixture of ethyl acetate and cyclohexane in a ratio of 1 to 1 of the individual active substances were prepared in 25 ml volumetric flasks with concentrations of 625 μg pesticide ml^{-1}. From 53 stock solutions, two mixed solutions of all 53 active substances were prepared in 500 ml volumetric flasks: one at a concentration of 5 μg ml^{-1} and the other at the limit of quantification (LOQ) of the active substances. All the solutions used to determine the linearity and LOQs were prepared from the 5 μg ml^{-1} mixed solution with proper dilutions. For other validation parameters, both mixed solutions (5 μg ml^{-1} concentration and the concentration at LOQ) were used. For standard solutions, solvents of GC grade were used.

2.2 Procedure

To 20 g of homogenised blank matrix (milled strawberries, which contain no PPP residues) or homogenised sample, 2 g of anhydrous CH_3COONa was added. Afterwards 40 ml of acetone p.a. and 0.4 ml 100 % acetic acid were added. The mixture was homogenised for 2 minutes with mixer (Ultra-turrax T 25, Ika-Werke). Then 80 ml mixture of petroleum ether p.a. and dichloromethane p.a. at a ratio of 1:1 was added and mixed for another 2 minutes with a mixer. This mixture was filtered into the separatory funnel, containing 3 g of NaCl. The vessel was rinsed with 80 ml of a mixture of petroleum ether p.a. and dichloromethane p.a. at a ratio of 1:1 (v/v). The solvent was added to the separatory funnel, which was shaken for 1 minute. The upper organic phase was filtered through 15 g anhydrous Na_2SO4 in 500 ml Soxhlet flask. The lower water phase was re-extracted twice using the same procedure. Solvents were evaporated to approximately 2 ml on a rotavapor and dried with a nitrogen flow.

8 ml of a mixture of cyclohexane p.a. and ethyl acetate p.a. at a ratio 1:1 (v/v) were added to dry extract. After filtration through a 0.2 μm pore size filter, 5 ml of the extract was cleaned using a gel permeation

chromatograph, containing a column filled with bio-beds SX3. The flow of the mobile phase (ethyl acetate p.a. and cyclohexane p.a., v/v = 1:1) through the GPC column was 5 ml min^{-1}. The 90-200 ml of the eluate was collected into a Soxhlet flask, evaporated to approximately 2 ml on a rotavapor and dried with a nitrogen flow. To the dry eluate, 2 ml of the mixture of ethyl acetate p.a. and cyclohexane p.a. at a ratio of 1:1 (v/v) was added in case of sample preparation. In the case of the matrix match standards, 2 ml of the working solutions with proper concentrations were added.

2.3 Determination

Table 1: Chromatographic conditions of the GC (HP 6890)-MS (HP 5973) system:

Liner:	HP 5181-3316
Temperature of injector:	250 ºC
Injection type:	Pulsed Splitless
Precolumn:	2 m * 0,25 mm
Column:	HP 5 MS, 30 m * 0.25 mm, 0.25 µm film
Temperature gradient of column:	55 ºC 2 min 55 ºC – 130 ºC 25 ºC/min 130 ºC 1 min 130 ºC – 180 ºC 5 ºC/min 180 ºC 30 min 180 ºC – 230 ºC 20 ºC/min 230 ºC 16 min 230 ºC – 250 ºC 20 ºC/min 250 ºC 13 min 250 ºC – 280 ºC 20 ºC/min 280 ºC 20 min
Temperature of ion source:	230 ºC
Temperature of connector:	280 ºC
Temperature of detector:	150 ºC
Carrier gas:	Helium 6.0, 1.2 ml/min constant flow
Volume of injection:	1 µl

Table 2: Detection (selective ion monitoring):

active substance	**T**, Q_1, Q_2, Q3 **(m/z)**
aldrin	**263**, 265, 261
azinphos-methyl	**160**, 132, 105
azoxystrobin	**344,** 388, 345
bifenthrin	**181**, 165, 166
bromopropylate	**183,** 341, 185
bupirimate	**273**, 316, 208
captan	**79**, 107, 119, 149
chlorothalonil	**266**, 264, 268
chlorpropham	**213**, 127, 154
chlorpyriphos	**314**, 316, 197
chlorpyriphos-methyl	**286**, 288, 125
cyhalotrin-λ	**181**, 197, 208
cypermethrin (four isomers)	**181,** 163, 165
cyprodinil	**224,** 225, 210
DDT (5 isomers)	DDD-o,p: **235**, 237, 165
	DDD-p,p and DDT-o,p: **235**, 237, 165
	DDE-p,p: **318**, 246, 248
	DDT-p,p: **235**, 237, 165

active substance	**T**, Q_1, Q_2, Q3 **(m/z)**
deltamethrin	**181**, 251, 255
diazinon	**179**, 304, 199
dichlofluanid	**226,** 123, 167
dimethoate	**87**, 229, 143
diphenylamine	**169,** 167, 168
endrin	**263**, 261, 265
fenitrothion	**277**, 260, 109
fenthion	**278**, 279, 280
fludioxonil	**248**, 154, 127
folpet	**260**, 262, 130
HCH-alpha	**219**, 181, 183
heptachlor	**272**, 274, 270
heptenophos	**124**, 215, 250
iprodione	**314**, 316, 187
kresoxim-methyl	**116,** 206, 131
lindane	**183,** 219, 181
mecarbam	**131**, 159, 329
metalaxyl+metalaxyl-M	**249,** 206 , 234
methidathion	**145**, 85, 125
myclobutanil	**179,** 288, 150
parathion	**291**, 292, 235
penconazole	**248,** 159, 161
permethrin (2 isomers)	**183**, 163, 165
phosalone	**182**, 367, 121
pirimicarb	**166,** 238, 167
pirimiphos-methyl	**290**, 305, 276
propyzamide	**173,** 175, 145
pyridaphenthion	**199,** 340, 188
pyrimethanil	**198,** 199, 200
quinalphos	**146,** 298, 157
spiroxamine (2 isomers)	**100,** 126, 198
tolclofos-methyl	**265,** 267, 250
tolylfluanid	**238,** 137, 240
triadimefon	**208,** 210, 181
triadimenol (2 isomers)	**112,** 168, 128
triazophos	**161**, 162, 285
trifloxystrobin	**116,** 222, 186
vinclozolin	**285**, 124, 187

2.4 Sampling

Strawberry samples were randomly taken in May and June 2007 directly in the field after the expiration of pre-harvest interval. Samples originated from 6 production areas in Slovenia: Celje, Kranj, Ljubljana, Maribor, Murska Sobota and Novo mesto.

3 RESULTS AND DISCUSSION

The previous protocol for the determination of PPP residues in fruit and vegetables was published before (Baša Česnik et al., 2006). The disadvantage of this procedure was, that when it was used for strawberries, some active substances were not extracted at all. Recoveries of previous procedure were compared to recoveries of new procedure (the one that includes pH adjustment) for two parallel samples of blank strawberries (strawberries that contained no PPP residues) spiked at level 0.2 mg kg^{-1}. The new procedure differs from old procedure only in step where the anhydrous CH_3COONa and the 100 % acetic acid are added to the sample. pH adjustment enabled extraction of bupimirate, pirimicarb, pyrimethanil and spiroxamine, where recoveries were 0 without pH adjustment.

3.1 Linearity and limits of quantification

Linearity was verified using the matrix match standards (five repetitions for one concentration level, three to seven concentration levels for the calibration curve). The linearity and range were determined by linear regression using the F test. The linear model is fit and remains linear throughout the range presented in Table 1. The limits of quantification (LOQs) were estimated from chromatograms of the matrix match standards. LOQs were chosen at S/N = 10. The LOQ is the lowest value of the linearity range for particular active substance presented in Table 3.

Table 3: Linearity

active substance	linearity range (mg kg^{-1})	R^2	active substance	linearity range (mg kg^{-1})	R^2
aldrin	0.005-0.2	0.997	heptenophos	0.01-0.2	0.997
azinphos-methyl	0.01-0.2	0.989	iprodione	0.01-0.2	0.995
azoxystrobin	0.04-0.2	0.985	kresoxim-methyl	0.02-0.2	0.995
bifenthrin	0.01-0.2	0.997	lindane	0.01-0.2	0.997
bromopropylate	0.01-0.2	0.997	mecarbam	0.04-0.2	0.995
bupirimate	0.02-0.2	0.995	metalaxyl+metalaxyl-M	0.01-0.2	0.998
captan	0.1-0.2	0.994	methidathion	0.01-0.2	0.995
chlorothalonil	0.01-0.2	0.995	myclobutanil	0.05-0.2	0.996
chlorpropham	0.01-0.2	0.997	parathion	0.03-1.0	0.992
chlorpyriphos	0.01-0.2	0.997	penconazole	0.01-0.2	0.996
chlorpyriphos-methyl	0.02-0.2	0.997	permethrin	0.02-0.2	0.994
cyhalotrin-lambda	0.01-0.5	0.977	phosalone	0.01-0.2	0.993
cypermethrin	0.03-0.2	0.991	pirimicarb	0.01-0.2	0.997
cyprodinil	0.01-0.2	0.996	pirimiphos-methyl	0.01-0.2	0.998
DDT	0.05-1.0	0.997	propyzamide	0.01-0.2	0.997
deltamethrin	0.03-0.2	0.989	pyridaphenthion	0.01-1.0	0.991
diazinon	0.01-0.2	0.998	pyrimethanil	0.01-0.2	0.997
dichlofluanid	0.01-0.2	0.997	quinalphos	0.01-0.2	0.996

active substance	linearity range (mg kg^{-1})	R^2	active substance	linearity range (mg kg^{-1})	R^2
dimethoate	0.01-0.2	0.995	spiroxamine	0.02-1.0	0.993
diphenylamine	0.01-0.2	0.996	tolclofos-methyl	0.01-0.2	0.997
endrin	0.01-0.2	0.996	tolylfluanid	0.01-0.2	0.996
fenitrothion	0.01-1.0	0.991	triadimefon	0.02-0.2	0.997
fenthion	0.005-0.2	0.996	triadimenol	0.02-0.2	0.994
fludioxonil	0.01-0.2	0.992	triazophos	0.01-0.2	0.992
folpet	0.02-1.0	0.988	trifloxystrobin	0.03-0.2	0.996
HCH-alpha	0.005-0.2	0.997	vinclozolin	0.01-0.2	0.997
heptachlor	0.005-0.2	0.998			

3.2 Accuracy

Accuracy was verified by checking the recoveries. Ten extracts of spiked blank strawberry homogenate (milled strawberries that contained no PPP residues) were prepared for each spiking level in the shortest period possible. Each extract was injected twice. The average of the recoveries was calculated. According to the requirements for the method validation procedures (Document N° SANTE/11945/2015), acceptable mean recoveries are those within the range of 70-120 %, with an associated repeatability $RSD_r \leq 20$ %. Our recoveries of the spiking level at LOQ ranged from 96.6 % to 105.4 % with $RSD_r \leq 15$ %, except for HCH-alpha were the RSD_r was 23 %. At spiking level 0.2 mg kg^{-1}, recoveries ranged from 96.8 % to 99.9 % with $RSD_r \leq 13$ %.

According to the guidelines for single-laboratory validation (Alder et al., 2000), the acceptable mean recoveries:

- at level > 0.1 mg kg^{-1} ≤ 1 mg kg^{-1} are within the range 70-110 %, with an associated repeatability RSDr ≤ 15 %,
- at level > 0.01 mg kg^{-1} ≤ 0.1 mg kg^{-1} are within the range 70-120 %, with an associated repeatability RSDr ≤ 20 % and
- at level > 0.001 mg kg^{-1} ≤ 0.01 mg kg^{-1} are within the range 60-120 %, with an associated repeatability RSDr ≤ 30 %.

These requirements were achieved for all 53 active compounds. The results are given in Table 4.

Table 4: Recoveries for spiked strawberry blank matrix

active substance	spiking level (mg kg^{-1})	recovery (%)	RSD (%)	spiking level (mg kg^{-1})	recovery (%)	RSD (%)
aldrin	0.005	99.1	6.8	0.2	97.7	7.8
azinphos-methyl	0.01	98.9	11.4	0.2	99.9	12.2
azoxystrobin	0.04	98.8	14.5	0.2	99.8	12.2
bifenthrin	0.01	101.0	12.1	0.2	97.7	9.2
bromopropylate	0.01	101.3	13.8	0.2	97.6	9.3
bupirimate	0.02	103.2	13.6	0.2	97.5	9.6
captan	0.1	101.5	9.9	0.2	97.4	9.4
chlorothalonil	0.01	96.6	9.3	0.2	97.8	9.2
chlorpropham	0.01	100.6	8.6	0.2	97.6	8.2
chlorpyriphos	0.01	102.6	12.4	0.2	97.1	8.0
chlorpyriphos-methyl	0.02	102.1	9.6	0.2	97.5	7.8
cyhalotrin-lambda	0.01	99.8	8.4	0.2	97.3	10.6

active substance	spiking level (mg kg^{-1})	recovery (%)	RSD (%)	spiking level (mg kg^{-1})	recovery (%)	RSD (%)
cypermethrin	0.03	97.3	6.9	0.2	98.8	12.5
cyprodinil	0.01	103.1	12.1	0.2	97.5	9.3
DDT	0.05	101.6	9.8	1.0	97.4	9.1
deltamethrin	0.03	99.9	10.4	0.2	98.9	12.4
diazinon	0.01	104.2	12.0	0.2	97.8	7.4
dichlofluanid	0.01	100.1	8.8	0.2	97.4	8.1
dimethoate	0.01	102.7	10.9	0.2	97.9	8.9
diphenylamine	0.01	99.5	7.3	0.2	98.0	7.5
endrin	0.01	97.9	9.2	0.2	97.5	8.7
fenitrothion	0.01	100.1	8.3	0.2	97.0	10.2
fenthion	0.005	101.8	13.9	0.2	97.4	8.3
fludioxonil	0.01	99.3	11.8	0.2	99.3	11.3
folpet	0.02	101.7	11.2	0.2	97.6	10.7
HCH-alpha	0.005	100.9	23.0	0.2	97.8	7.5
heptachlor	0.005	99.8	7.0	0.2	97.9	7.5
heptenophos	0.01	101.2	8.4	0.2	97.9	7.9
iprodione	0.01	99.1	11.6	0.2	98.2	10.3
kresoxim-methyl	0.02	103.3	12.2	0.2	97.5	9.6
lindane	0.01	99.4	8.4	0.2	97.9	7.4
mecarbam	0.04	103.1	11.0	0.2	97.7	8.8
metalaxyl+metalaxyl-M	0.01	103.2	11.1	0.2	97.6	8.1
methidathion	0.01	103.5	12.0	0.2	98.0	9.8
myclobutanil	0.05	104.5	14.6	0.2	97.8	9.7
parathion	0.03	98.3	7.7	0.2	96.8	10.1
penconazole	0.01	104.9	10.2	0.2	97.7	9.1
permethrin	0.02	100.3	12.6	0.2	98.0	11.3
phosalone	0.01	101.3	11.5	0.2	98.1	10.9
pirimicarb	0.01	101.5	10.8	0.2	97.8	8.0
pirimiphos-methyl	0.01	103.6	12.1	0.2	97.9	8.2
propyzamide	0.01	102.1	9.0	0.2	97.4	8.4
pyridaphenthion	0.01	103.6	10.7	0.2	97.8	11.0
pyrimethanil	0.01	100.8	9.4	0.2	97.6	8.2
quinalphos	0.01	104.5	13.7	0.2	97.3	9.3
spiroxamine	0.03	102.2	10.9	0.2	97.4	8.1
tolclofos-methyl	0.01	101.6	8.3	0.2	97.8	7.8
tolylfluanid	0.01	100.1	9.7	0.2	97.2	8.7
triadimefon	0.02	101.5	10.6	0.2	97.3	8.8
triadimenol	0.02	105.4	10.6	0.2	97.6	9.9
triazophos	0.01	102.1	12.4	0.2	97.6	11.5
trifloxystrobin	0.03	102.9	13.4	0.2	97.8	9.9
vinclozolin	0.01	100.3	8.6	0.2	97.5	8.6

Table 5: Interlaboratory comparison results (in mg kg^{-1}) (BIPEA, 2015)

active substance	reference	tolerance	maximum	minimum	our result	z
azoxystrobin	0.053	0.027	0.080	0.026	0.056	0.22
bifenthrin	0.022	0.011	0.033	0.011	0.018	-0.73
cyhalotrin-lambda	0.064	0.032	0.096	0.032	0.062	-0.13
deltamethrin	0.166	0.076	0.242	0.090	0.164	-0.05
diphenylamine	0.129	0.062	0.191	0.067	0.112	-0.55
dimethoate	0.066	0.033	0.099	0.033	0.061	-0.3
fenitrothion	0.044	0.022	0.066	0.022	0.050	0.55
phosalone	0.163	0.075	0.238	0.088	0.158	-0.13
kresoxim-methyl	0.023	0.012	0.035	0.011	0.020	-0.5
lindane	0.146	0.068	0.214	0.078	0.140	-0.18
metalaxyl+metalaxyl-M	0.036	0.018	0.054	0.018	0.028	-0.89
myclobutanil	0.032	0.016	0.048	0.016	0.031	-0.13
pirimicarb	0.169	0.078	0.247	0.091	0.152	-0.44

Accuracy was also checked with participation in a proficiency testing scheme organised by BIPEA (Bureau interprofessionnel d´études analytiques). All the results were within the required range ($-2 \geq z \leq 2$). The results are presented in Table 3.

3.3 Precision

For the determination of precision (ISO 5725), i.e. repeatability and reproducibility, the extracts of spiked blank strawberry matrix were analysed at two concentration levels. Within the period of 10 days, two parallel extracts were prepared each day for each concentration level. Each one was injected once. Then the standard deviation of repeatability of the level and the standard deviation of reproducibility of the level were both calculated. The results are given in Table 6.

Table 6: Standard deviation of repeatability (s_r) and reproducibility (s_R) of the method

active substance	spiking level (mg kg^{-1})	means of the levels (mg kg^{-1})	s_r (mg kg^{-1})	s_R (mg kg^{-1})	spiking level (mg kg^{-1})	means of the levels (mg kg^{-1})	s_r (mg kg^{-1})	s_R (mg kg^{-1})
aldrin	0.005	0.0050	0.0002	0.0003	0.2	0.19	0.01	0.01
azinphos-methyl	0.01	0.010	0.001	0.001	0.2	0.19	0.02	0.02
azoxystrobin	0.04	0.038	0.005	0.006	0.2	0.19	0.02	0.02
bifenthrin	0.01	0.0098	0.0007	0.0008	0.2	0.19	0.01	0.01
bromopropylate	0.01	0.0097	0.0009	0.0009	0.2	0.19	0.01	0.01
bupirimate	0.02	0.020	0.001	0.001	0.2	0.19	0.01	0.01
captan	0.1	0.10	0.02	0.02	0.2	0.19	0.01	0.02
chlorothalonil	0.01	0.0099	0.0007	0.0007	0.2	0.19	0.01	0.01
chlorpropham	0.01	0.0098	0.0005	0.0006	0.2	0.19	0.01	0.01
chlorpyriphos	0.01	0.0098	0.0005	0.0007	0.2	0.19	0.01	0.01
chlorpyriphos-methyl	0.02	0.020	0.001	0.001	0.2	0.19	0.01	0.01
cyhalotrin-lambda	0.01	0.0095	0.0007	0.0009	0.2	0.19	0.01	0.01
cypermethrin	0.03	0.029	0.003	0.003	0.2	0.19	0.01	0.01
cyprodinil	0.01	0.0098	0.0006	0.0007	0.2	0.19	0.01	0.01
DDT	0.05	0.050	0.003	0.004	1.0	0.95	0.05	0.06
deltamethrin	0.03	0.029	0.003	0.003	0.2	0.19	0.02	0.02
diazinon	0.01	0.0098	0.0005	0.0006	0.2	0.19	0.01	0.01
dichlofluanid	0.01	0.0096	0.0006	0.0009	0.2	0.19	0.01	0.01
dimethoate	0.01	0.0097	0.0007	0.0008	0.2	0.19	0.01	0.01
diphenylamine	0.01	0.0099	0.0004	0.0005	0.2	0.19	0.01	0.01
endrin	0.01	0.0100	0.0006	0.0006	0.2	0.19	0.01	0.01
fenitrothion	0.01	0.0098	0.0007	0.0008	0.2	0.19	0.01	0.01
fenthion	0.005	0.0049	0.0003	0.0004	0.2	0.19	0.01	0.01
fludioxonil	0.01	0.010	0.001	0.001	0.2	0.19	0.01	0.02
folpet	0.02	0.020	0.004	0.004	0.2	0.19	0.01	0.02
HCH-alpha	0.005	0.0049	0.0002	0.0002	0.2	0.19	0.01	0.01
heptachlor	0.005	0.0050	0.0003	0.0003	0.2	0.19	0.01	0.01

active substance	spiking level (mg kg^{-1})	means of the levels (mg kg^{-1})	s_r (mg kg^{-1})	s_R (mg kg^{-1})	spiking level (mg kg^{-1})	means of the levels (mg kg^{-1})	s_r (mg kg^{-1})	s_R (mg kg^{-1})
heptenophos	0.01	0.0098	0.0004	0.0006	0.2	0.19	0.01	0.01
iprodione	0.01	0.0097	0.0009	0.0011	0.2	0.19	0.01	0.01
kresoxim-methyl	0.02	0.020	0.001	0.001	0.2	0.19	0.01	0.01
lindane	0.01	0.0100	0.0005	0.0005	0.2	0.19	0.01	0.01
mecarbam	0.04	0.039	0.003	0.003	0.2	0.19	0.01	0.01
metalaxyl+metalaxyl-M	0.01	0.0098	0.0004	0.0004	0.2	0.19	0.01	0.01
methidathion	0.01	0.0098	0.0009	0.0009	0.2	0.19	0.01	0.01
myclobutanil	0.05	0.049	0.004	0.004	0.2	0.19	0.01	0.01
parathion	0.03	0.029	0.002	0.002	0.2	0.19	0.01	0.01
penconazole	0.01	0.0097	0.0006	0.0007	0.2	0.19	0.01	0.01
permethrin	0.02	0.020	0.002	0.002	0.2	0.19	0.01	0.01
phosalone	0.01	0.0097	0.0009	0.0011	0.2	0.19	0.01	0.01
pirimicarb	0.01	0.0099	0.0006	0.0006	0.2	0.19	0.01	0.01
pirimiphos-methyl	0.01	0.0098	0.0005	0.0006	0.2	0.19	0.01	0.01
propyzamide	0.01	0.0098	0.0005	0.0005	0.2	0.19	0.01	0.01
pyridaphenthion	0.01	0.010	0.001	0.001	0.2	0.19	0.01	0.01
pyrimethanil	0.01	0.0098	0.0006	0.0006	0.2	0.19	0.01	0.01
quinalphos	0.01	0.0098	0.0007	0.0009	0.2	0.19	0.01	0.01
spiroxamine	0.03	0.0296	0.001	0.002	0.2	0.19	0.01	0.01
tolclofos-methyl	0.01	0.0099	0.0005	0.0005	0.2	0.19	0.01	0.01
tolylfluanid	0.01	0.010	0.001	0.001	0.2	0.19	0.01	0.01
triadimefon	0.02	0.020	0.001	0.001	0.2	0.19	0.01	0.01
triadimenol	0.02	0.0195	0.002	0.002	0.2	0.19	0.01	0.01
triazophos	0.01	0.0097	0.0008	0.0009	0.2	0.19	0.01	0.01
trifloxystrobin	0.03	0.029	0.002	0.003	0.2	0.19	0.01	0.01
vinclozolin	0.01	0.0099	0.0006	0.0006	0.2	0.19	0.01	0.01

3.4 Uncertainty of repeatability and uncertainty of reproducibility

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

$Ur = t_{95;\,9} \times s_r$; $UR = t_{95;\,9} \times s_R$

The results are presented in Table 7. The measurement uncertainty for PPP residues is set in the Official Gazette of the Republic of Slovenia (Republic of Slovenia, 2007). Its value is 50 %. With validation, analysts must prove that their measurement uncertainty is below or equal to the official measurement uncertainty.

3.5 Sample analysis

The method was checked in practice. 19 strawberry samples were analysed for the presence of all 53 validated active substances. 10 samples, which represent 52.6 % of all the analysed samples contained no residues. 5 active substances, all fungicides, were found: chlorothalonil, cyprodinil, fludioxonil, metalaxyl+metalaxyl-M and pyrimethanil. Other active substances were below the LOQ. The most frequently measured was cyprodinil, which was found in 8 samples, representing 42.1 % of all the analysed samples. The reason is probably that this substance is included in the PPP Switch 62.5 WG, which is the mixture of fungicides cyprodinil and fludioxonil used for strawberries and sold in Slovenia. 9 samples, which represent 47.4 % of all the analysed samples contained PPP residues in the range 0.01 – 0.44 mg/kg. Multiple residues (2 or more active substances) were found in 5 samples, representing 26.3 % of all the analysed samples. None of the substances exceeded the valid MRL. Therefore, the conclusion was drawn that farmers were using PPPs according to good agriculture practice described on the labels of the PPPs. Also, these strawberries presented no risk to consumers. The results are presented in Table 8.

Comparing our results with the literature we observed that PPP residues in strawberries in Slovenia are mainly comparable to observations of other authors. Jardim et al. (2012) found pesticide residues in Brazilia in 76.3 % of strawberry samples; 71.6 % of them had multiple residues and 13.5 % of them were exceeding the MRL. In Slovenia, the amount of positive samples was about 29 % lower, the amount of multiple residues was about 45 % lower and no MRL exceedances were observed. On the other hand Poulsen et al. (2017) reported that in Denmark, 37 % of the analysed samples contained multiple residues, which is approximately 11 % higher than in Slovenia.

In strawberry samples in Poland, Szpyrka et al. (2015) found cypermethrin, deltamethrin and trifloxystrobin among the active substances that we both analysed. On the other hand, again in strawberry samples in Poland, Sójka et al. (2015) found the fungicides cyprodinil (mean content 0.16 mg kg^{-1}), fludioxonil (mean content 0.115 mg kg^{-1}) and pyrimethanil (mean content 0.056 mg kg^{-1}), as well as the insecticide chlorpyrifos (mean content 0.012 mg kg^{-1}) among the active substances that we both analysed. The mean contents of cyprodinil and fludioxonil were comparable to ours, while the content of pyrimethanil was slightly lower. Chlorpyriphos was not found in our research. In protected strawberries Allen et al. (2015) found cyprodinil (mean content 0.062 mg kg^{-1}) and iprodione (mean content 0.055 mg kg^{-1}) among the active substances that we both analysed. The cyprodinil mean content was in the range of contents that we measured, while iprodione was not found in our research.

Table 7: Uncertainty of repeatability (U_r) and reproducibility (U_R) of the method

active substance	spiking level (mg kg^{-1})	U_r (mg kg^{-1})	U_r (%)	U_R (mg kg^{-1})	U_R (%)	spiking level (mg kg^{-1})	U_r (mg kg^{-1})	U_r (%)	U_R (mg kg^{-1})	U_R (%)
aldrin	0.005	0.0006	12.0	0.0006	12.0	0.2	0.02	10.0	0.02	10.0
azinphos-methyl	0.01	0.003	30.0	0.003	30.0	0.2	0.04	20.0	0.05	25.0
azoxystrobin	0.04	0.01	25.0	0.01	25.0	0.2	0.04	20.0	0.04	20.0
bifenthrin	0.01	0.002	20.0	0.002	20.0	0.2	0.02	10.0	0.03	15.0
bromopropylate	0.01	0.002	20.0	0.002	20.0	0.2	0.02	10.0	0.03	15.0
bupirimate	0.02	0.003	15.0	0.003	15.0	0.2	0.03	15.0	0.03	15.0
captan	0.1	0.04	40.0	0.04	40.0	0.2	0.04	20.0	0.1	50.0
chlorothalonil	0.01	0.002	20.0	0.002	20.0	0.2	0.03	15.0	0.03	15.0
chlorpropham	0.01	0.001	10.0	0.001	10.0	0.2	0.02	10.0	0.02	10.0
chlorpyriphos	0.01	0.001	10.0	0.002	20.0	0.2	0.02	10.0	0.02	10.0
chlorpyriphos-methyl	0.02	0.003	15.0	0.003	15.0	0.2	0.02	10.0	0.03	15.0
cyhalotrin-lambda	0.01	0.002	20.0	0.002	20.0	0.2	0.03	15.0	0.03	15.0
cypermethrin	0.03	0.007	23.3	0.007	23.3	0.2	0.03	15.0	0.03	15.0
cyprodinil	0.01	0.001	10.0	0.002	20.0	0.2	0.02	10.0	0.03	15.0
DDT	0.05	0.007	14.0	0.008	16.0	1.0	0.12	12.0	0.14	14.0
deltamethrin	0.03	0.006	20.0	0.006	20.0	0.2	0.04	20.0	0.04	20.0
diazinon	0.01	0.001	10.0	0.001	10.0	0.2	0.02	10.0	0.02	10.0
dichlofluanid	0.01	0.001	10.0	0.002	20.0	0.2	0.03	15.0	0.02	10.0
dimethoate	0.01	0.002	20.0	0.002	20.0	0.2	0.03	15.0	0.03	15.0
diphenylamine	0.01	0.0009	9.0	0.0011	11.0	0.2	0.02	10.0	0.02	10.0
endrin	0.01	0.001	10.0	0.001	10.0	0.2	0.02	10.0	0.03	15.0
fenitrothion	0.01	0.002	20.0	0.002	20.0	0.2	0.03	15.0	0.03	15.0
fenthion	0.005	0.0007	14.0	0.0008	16.0	0.2	0.02	10.0	0.02	10.0
fludioxonil	0.01	0.002	20.0	0.003	30.0	0.2	0.03	15.0	0.04	20.0
folpet	0.02	0.009	45.0	0.009	45.0	0.2	0.03	15.0	0.03	15.0
HCH-alpha	0.005	0.0005	10.0	0.0005	10.0	0.2	0.02	10.0	0.02	10.0
heptachlor	0.005	0.0006	12.0	0.0006	12.0	0.2	0.02	10.0	0.02	10.0

active substance	spiking level (mg kg^{-1})	U_r (mg kg^{-1})	U_r (%)	U_R (mg kg^{-1})	U_R (%)	spiking level (mg kg^{-1})	U_r (mg kg^{-1})	U_r (%)	U_R (mg kg^{-1})	U_R (%)
heptenophos	0.01	0.001	10.0	0.001	10.0	0.2	0.02	10.0	0.03	15.0
iprodione	0.01	0.002	20.0	0.002	20.0	0.2	0.03	15.0	0.03	15.0
kresoxim-methyl	0.02	0.003	15.0	0.003	15.0	0.2	0.03	15.0	0.03	15.0
lindane	0.01	0.001	10.0	0.001	10.0	0.2	0.02	10.0	0.02	10.0
mecarbam	0.04	0.007	17.5	0.007	17.5	0.2	0.02	10.0	0.03	15.0
metalaxyl+metalaxyl-M	0.01	0.0009	9.0	0.0010	10.0	0.2	0.02	10.0	0.03	15.0
methidathion	0.01	0.002	20.0	0.002	20.0	0.2	0.03	15.0	0.03	15.0
myclobutanil	0.05	0.009	18.0	0.009	18.0	0.2	0.02	10.0	0.03	15.0
parathion	0.03	0.005	16.7	0.005	16.7	0.2	0.03	15.0	0.03	15.0
penconazole	0.01	0.001	10.0	0.002	20.0	0.2	0.02	10.0	0.03	15.0
permethrin	0.02	0.004	20.0	0.005	25.0	0.2	0.03	15.0	0.03	15.0
phosalone	0.01	0.002	20.0	0.002	20.0	0.2	0.03	15.0	0.03	15.0
pirimicarb	0.01	0.001	10.0	0.001	10.0	0.2	0.03	15.0	0.03	15.0
pirimiphos-methyl	0.01	0.001	10.0	0.001	10.0	0.2	0.02	10.0	0.02	10.0
propyzamide	0.01	0.001	10.0	0.001	10.0	0.2	0.02	10.0	0.03	15.0
pyridaphenthion	0.01	0.002	20.0	0.003	30.0	0.2	0.03	15.0	0.03	15.0
pyrimethanil	0.01	0.001	10.0	0.001	10.0	0.2	0.02	10.0	0.03	15.0
quinalphos	0.01	0.002	20.0	0.002	20.0	0.2	0.02	10.0	0.02	10.0
spiroxamine	0.03	0.003	10.0	0.004	13.3	0.2	0.02	10.0	0.02	10.0
tolclofos-methyl	0.01	0.001	10.0	0.001	10.0	0.2	0.02	10.0	0.02	10.0
tolylfluanid	0.01	0.002	20.0	0.002	20.0	0.2	0.02	10.0	0.03	15.0
triadimefon	0.02	0.003	15.0	0.003	15.0	0.2	0.02	10.0	0.03	15.0
triadimenol	0.02	0.003	15.0	0.004	20.0	0.2	0.02	10.0	0.03	15.0
triazophos	0.01	0.002	20.0	0.002	20.0	0.2	0.03	15.0	0.03	15.0
trifloxystrobin	0.03	0.005	16.7	0.006	20.0	0.2	0.03	15.0	0.03	15.0
vinclozolin	0.01	0.001	10.0	0.001	10.0	0.2	0.02	10.0	0.02	10.0

Table 8: Contents of active substances found in 19 strawberry samples

	chlorothalonil (mg kg^{-1})	cyprodinil (mg kg^{-1})	fludioxonil (mg kg^{-1})	metalaxyl+metalaxyl-M (mg kg^{-1})	pyrimethanil (mg kg^{-1})
MRL (mg kg^{-1})	4.0	5.0	4.0	0.6	5.0
sample no.					
1	-	-	-	-	-
2	-	-	-	-	-
3	-	0.04	-	-	-
4	-	0.04	-	-	-
5	-	-	-	-	-
6	-	-	-	-	-
7	-	-	-	-	-
8	0.01	-	-	-	-
9	-	-	-	-	-
10	-	0.02	-	-	-
11	0.10	0.02	-	-	-
12	-	-	-	-	-
13	-	0.24	0.17	-	0.13
14	0.06	0.02	-	-	-
15	-	-	-	-	-
16	-	0.24	-	0.02	-
17	0.02	0.08	-	-	0.44
18	-	-	-	-	-
19	-	-	-	-	-

- means <LOQ
MRL is maximum residue level

4 CONCLUSIONS

According to the validation, the method is suitable for the determination of at least 53 active compounds and their residues in strawberries. The method could be expanded to more active substances. The system is linear with an R^2 higher or equal than 0.977. The LOQs range from 0.005 mg kg^{-1} for aldrin to 0.1 mg kg^{-1} for captan. Recoveries range from 96.6 % (chlorothalonil) to 105.4 % (triadimenol) at a spiking level equal to the LOQ. Uncertainty of reproducibility ranges from 10 % for vinclozolin to 50 % for captan. The method is fit for purpose and is accredited according to the SIST EN ISO/IEC 17025 standard by the Slovenian accreditation body SA.

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Vpliv namakanja v kombinaciji s tehnologijo nege ledine na razporeditev, migracijo ter vsebnost nitrata in bakra v tleh oljčnih nasadov

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IZVLEČEK

Vse pogostejši in intenzivnejši ekstremni vremenski dogodki (višje temperature – intenzivnost in pogostnost vročinskih valov, več daljših sušnih obdobij) ter vremensko pogojene bolezni in škodljivci, ki so v zadnjih letih povzročili največ škode v pridelavi oljk, opozarjajo na nujne spremembe v slovenskem oljkarstvu. Zaradi spoznanja, da imajo prilagoditve kmetijske pridelave na podnebne spremembe lahko negativne posledice na okolje (vodo, tla), smo z zasnovanim poskusom želeli ugotoviti dejanski učinek prilagoditev agrotehničnih ukrepov na dinamiko nitrata in bakra v tleh. Rezultati raziskave so pokazali, da namakanje v kombinaciji s tehnologijo nege ledine vpliva na razporeditev, migracijo in vsebnost nitrata in bakra v tleh oljčnih nasadov. Dodana voda lahko ob tem, da omogoča nemoten sprejem hranil v rastlino, izboljša tudi razmere za mineralizacijo in razgradnjo organske snovi, ki je močno odvisna od načina oskrbe in nege ledine.

Key words: tla; nitrat; baker; oljka; namakanje

ABSTRACT

THE EFFECT OF IRRIGATION IN COMBINATION WITH SOIL CULTIVATION ON DISTRIBUTION, MIGRATION AND CONTENT OF NITRATE AND COPPER IN THE SOILS OF OLIVE GROVES

The more frequent and intense extreme weather events (higher temperatures – the intensity and frequency of heat weaves, more and longer periods of drought) and weather-related diseases and pests, that have caused the greatest damage to olive production in the recent years, are a warning that urgent changes to Slovenian olive culture are needed. Due to the realisation that adaptations of agricultural production to climatic changes can have negative effects on the environment (water, soil), we conducted an experiment to determine the actual effect of adaptations of agro-technical management on the dynamics of nitrate and copper in the soil. The results of the study have shown that irrigation in combination with the technology of soil cultivation have effect on the allocation, migration and content of nitrate and copper in the soil of olive groves. Along with the fact that applied water allows the undisturbed absorption of nutrients into the plant, it can also improve the conditions for mineralisation and decomposition of organic matter, which is heavily dependent on the type of soil cultivation.

Ključne besede: **s**oil; nitrate; copper; olive; irrigation

1 UVOD

Slovenija predstavlja skrajno severno mejo, kjer se oljka lahko še goji na obsežnih zemljiščih (Sancin, 1990). Čeprav so za slovensko oljkarstvo značilna majhna kmetijska gospodarstva (85 % KMG kmetijsko gospodarstvo ima od 0,1 do 1 ha oljčnikov), posebno geografsko območje pridelave oljk omogoča pridelavo visoko kakovostnega oljčnega olja z zaščiteno označbo porekla »EDOOSI ZOP - Ekstra deviška oljčna olja Slovenske Istre« (Strategija za izvajanje resolucije…, 2014).

Vse pogostejši in intenzivnejši ekstremni vremenski dogodki (višje temperature – intenzivnost in pogostnost vročinskih valov, več daljših sušnih obdobij) ter vremensko pogojene bolezni in škodljivci, ki so v zadnjih letih povzročili največ škode v pridelavi oljk, opozarjajo na nujne spremembe v slovenskem oljkarstvu. Številni avtorji že poročajo o vplivih klimatskih sprememb na količino pridelka oljk in kakovost oljčnega olja (Dag in sod., 2014; Ozdemir, 2016). Ponti in sod. (2014) ter Kajfež-Bogataj (2005) opozarjajo, da se bodo stroški pridelave oljk, zaradi

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prilagajanja podnebnim spremembam in evropske kmetijske politike, ki vse bolj subvencionira intenzivno pridelavo kot ekološko pridelavo, povečali. To bo najbolj ogrozilo obstoj majhnih oljkarjev, katerih ohranjanje in razvoj imata pomembno vlogo pri poseljenosti podeželja, varovanju biotske pestrosti, kmetijskih tal in značilnosti kmetijske krajine.

Zaradi prilagoditev pridelave oljk na novo nastale razmere in vse večje potrebe po zagotavljanju zadostne količine kakovostnih in konstantnih pridelkov nekateri slovenski oljkarji že uporabljajo princip »kriznega namakanja«, kjer gre za časovno in količinsko nekontrolirano dodajanje vode (Pintar in sod., 2010; Podgornik & Bandelj, 2015). Nestrokovno namakanje v kombinaciji z neuravnoteženim gnojenjem (prekomernim odmerkom dušika), ki ne upošteva potreb rastline, fizikalnih lastnosti tal, evapotranspiracije ter časovne razporeditve in jakosti padavin, ima lahko negativni vpliv na okolje in vodne vire, saj obstaja nevarnost, da se dodana hranila izperejo v globlje plasti tal, podtalnico ter vodotoke in se tako vključijo v našo prehranjevalno verigo. Dušik se v obliki nitrata v slini ustne votline izredno hitro pretvori v nitrit (NO_2^-), ki pri motenem izločanju kisline v želodcu reagira z amini in tvori močne rakotvorne nitrozamine (Scharpf, 1991). Poleg tega lahko nitrit v krvi reagira s hemoglobinom in povzroči methemoglobinemijo dojenčkov (Consalter in sod., 1992). Zaradi negativnega učinka nitrata na zdravje ljudi je svetovna zdravstvena organizacija (WHO = World Health Organization) postavila mejno vrednost za količino nitrata v pitni vodi (50 mg NO_3^- kg^{-1}) (WHO, 1993), znanstveni odbor za živila (SCF = Scientific Committe for Food) pa mejo sprejemljivega dnevnega vnosa nitrata v človeško telo (do 3,65 mg NO_3^- kg^{-1} telesne teže) (Reports of the scientific committee for food, 1997).

Spremenjene podnebne razmere vse bolj vplivajo tudi na pojav, razvoj in širjenje rastlinskih bolezni (Sutherst in sod., 1995; Chakraborty in sod., 2000). Zaradi milih in vlažnih zim se v slovenskih oljčnikih v vse večjem obsegu pojavlja oljkova kozavost ali pavje oko (*Cycloconium oleaginum* Cast.), ki je najbolj znana in razširjena bolezen oljk na celotnem sredozemskem območju. Sorte oljk so na omenjeno bolezen različno občutljive. Med slovenskimi sortami je zelo občutljiva 'Istrska belica', ki je v slovenskih oljčnikih najbolj zastopana (70 % - oljčnih dreves). Poleg tega mora biti v sortni sestavi oljk, iz katerih je pridelano oljčno olje z oznako zaščiteno označbo porekla »EDOOSI ZOP - Ekstra deviška oljčna olja Slovenske Istre«, najmanj 30 % sorte 'Istrska belica'. Zaradi velikega pomena in zastopanosti sorte 'Istrska belica' v slovenskem prostoru, ugodnejših podnebnih razmer za razvoj rastlinskih bolezni in vse večjega pojava pavjega očesa na območju slovenskih oljčnikov obstaja bojazen prekomerne uporabe in kopičenja bakrovih pripravkov v tleh oljčnih nasadov. V Sloveniji je v skladu s Seznamom registriranih fitofarmacevtskih sredstev (2018) za zaščito oljk pred oljkovo kozavostjo ali pavjim očesom in oljkovo sivo pegavostjo (*Mycocentrospora cladosporioides* (Sacc.) P. Costa ex Deighton) edino registrirano in učinkovito sredstvo na osnovi bakrovega oksiklorida. Baker je pri majhnih koncentracijah esencialni element, ki je nujno potreben vsem živim organizmom (Gessa in Ciavatta, 2005). Velike koncentracije bakra, ki nastanejo v tleh zaradi uporabe in kopičenja bakrovih pripravkov za zaščito rastlin, pa toksično vplivajo na makro in mikro floro in favno ter tako na zmanjšano biološko aktivnost tal (Georgopoulos in sod., 2001; Besnard in sod., 2001).

Zaradi spoznanja, da imajo prilagoditve kmetijske pridelave na podnebne spremembe lahko negativne posledice na okolje (vodo, tla), smo z zasnovanim poskusom želeli ugotoviti dejanski učinek prilagoditev agrotehničnih ukrepov na dinamiko nitrata in bakra v tleh. Glede na to, da je obremenjenost okolja rezultat prepletanja različnih rab tal, smo želeli z raziskavo ovrednotiti vplive prilagoditve pridelave oljk na onesnaženost okolja in s tem pridobiti vpogled v problematiko prilagajanja kmetijske pridelave na podnebne spremembe. Nova spoznanja nam bodo omogočila okoljsko varno zamenjavo starih neučinkovitih agrotehničnih ukrepov in praks z novimi, ki omogočajo boljši neposreden odziv oljk na podnebne spremembe.

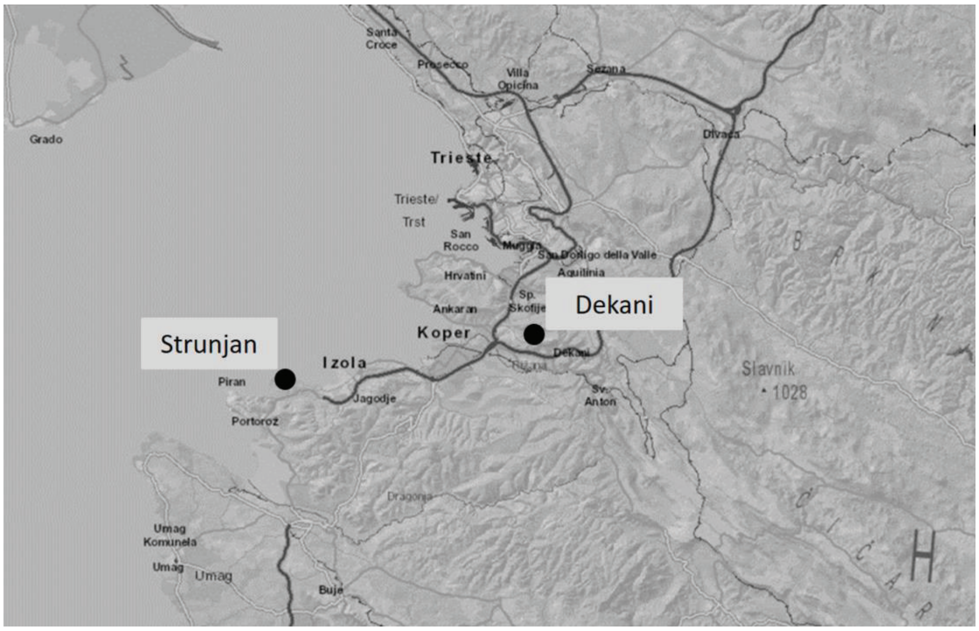
2 MATERIAL IN METODE

2.1 Opis poskusnih lokacij

V letu 2010 smo v oljčniku 'Istrska belica' na lokacijah Strunjan in Dekani zasnovali poskus spremljanja nitrata v odcedni vodi in tleh ter vsebnost bakra v tleh. Na vsaki izbrani lokaciji smo na namakanih in nenamakanih površinah od 1.8.2010 do 16.11.2011 spremljali vsebnost nitrata v odcedni vodi in tleh in od 19.8.2010 do 21.7.2011 vsebnost bakra v tleh.

V poskusnih oljčnikih smo 31.8.2010 izkopali pedološki profil do matične podlage, opisali morfološke lastnosti tal, opravili kemične in fizikalne analize ter tako določili lastnosti talnih horizontov posameznega pedološkega profila. S kvantitativnim in kvalitativnim ocenjevanjem ter opazovanjem talnih horizontov so bili določeni talni tipi opazovanih lokacij.

V času raziskave smo meteorološke podatke spremljali z avtomatsko meteorološko postajo SIAP+MICROS - Olimpo. V Dekanih (Slika 1) (nadmorska višina: 96 m, zemljepisna širina: 45°33,541', zemljepisna dolžina: 13°47,637') je meteorološka postaja nameščena neposredno v poskusnem oljčniku, medtem ko je v Strunjanu (Slika 1) (nadmorska višina: 175 m, zemljepisna širina: 45°30,732', zemljepisna dolžina: 13°37,896') od poskusne lokacije oddaljena 2 km zračne linije. V letu 2010 so bile količine padavin (Dekani: 1380 mm, Mala Seva: 1623 mm) nad dolgoletnim povprečjem (973 mm), medtem ko v letu 2011 niso presegle dolgoletnega povprečja, saj so dosegle le 57 % oz. 69 % (Dekani: 557 mm, Mala Seva: 676 mm) običajne količine padavin. Povprečne mesečne temperature zraka pa so tako v letu 2010 (Dekani: 14,0 °C, Mala Seva: 14,2 °C) kot v 2011 (Dekani: 14,7 °C, Mala Seva: 15,4 °C) presegle dolgoletno povprečje (13,7 °C).



Slika 1: Območje raziskave in lokaciji vzorčenja (Strunjan, Dekani) (Atlas okolja…2018)
Figure 1: Study area and location of sampling (Strunjan, Dekani) (Atlas okolja…2018)

Z namenom, da bi v poletnih mesecih nadomestili primanjkljaj vode v tleh, sta bila leta 2009 na del poskusnih površin nameščena kapljična namakalna sistema. Za zagotavljanje optimalnega namakalnega obroka, ki je bil enak 100 % evapotranspiraciji, sta bila na vsaki izbrani lokaciji vgrajena tenziometra – DL6 Tensimeter Data Logger, ki sta na podlagi zabeleženih polurnih podatkov avtomatsko prožila namakalni sistem. Količine dodane vode na poskusnih lokacijah so predstavljene v Preglednici 1.

Oljčnika sta zatravljena s krajevno prisotno travno rušo. V Dekanih je bila travna ruša košena glede na fenološki stadij rastline, medtem ko je bila travna ruša v Strunjanu mulčena (Preglednici 1).

V času naše raziskave so v Dekanih (13.2.2011) in Strunjanu (04.02.2011) v mesecu februarju dodali 37 kg N ha^{-1}. Tako v Dekanih kot v Strunjanu dodane količine dušika niso dosegle dovoljene mejne vrednosti letnega vnosa dušika (90 kg ha^{-1}), ki jo Tehnološka navodila za integrirano pridelavo (2011) predpisujejo za oljke.

Za zaščito oljk pred napadom glive *Spilocaea oleagina*, ki povzroča bolezen pavje oko, je bilo uporabljeno fitofarmacevtsko sredstvo Cuprablau-Z (aktivna snov – baker). Količine (kg ha^{-1}) dodane aktivne snovi so navedene v Preglednici 1.

Preglednica 1: Podatki o agrotehničnih ukrepih v poskusnih oljčnih nasadih
Table 1: Data of agrotechnical work in experimental olive groves

Lokacija	Čas aplikacije	Agrotehnični ukrep	Februar	Marec	April	Maj	Junij	Julij	Avgust	September	Oktober	November	Skupaj
Dekani	2010	baker (kg ha^{-1})										0,47	0,47
		datum košnje				15.5.				15.9.			/
		namakanje (mm)				11	91	84	33	16	8		243
	2011	baker (kg ha^{-1})			0,47							0,47	0,94
		datum košnje					1.6.			6.9.			/
		gnojenje (kg N ha^{-1})	37										37
		namakanje (mm)				50	57	85	164	70	150		576
Strunjan	2010	baker (kg ha^{-1})										1,4	1,4
		datum mulčenja						27.7.		09.9.			/
		namakanje (mm)	Dne 30.7.2010 je prišlo do okvare DL6 Tensimeter Data Logger-ja, zato podatki o porabi vode niso znani										
	2011	baker (kg ha^{-1})			1,40							1,40	2,8
		datum mulčenja				3.5.				30.9.			/
		gnojenje (kg N ha^{-1})	37										36
		namakanje (mm)	Dne 30. 7. 2010 je prišlo do okvare DL6 Tensimeter Data Logger-ja, zato podatki o porabi vode niso znani.										

2.2 Vzorčenje in analiza vsebnosti nitrata v talni vodi in tleh

Za spremljanje vsebnosti nitrata v talni vodi, ki je v tleh vezna s silo, manjšo od 0,33 bara, in s tem podvržena gravitacijskemu odtoku, smo izbrali keramične vakuumske svečke Pore water sampler – UMS GmbH, model SIC 20. Na vsakem izbranem vzorčnem mestu (1. vzorčno mesto – nenamakano Strunjan; 2. vzorčno mesto – namakano Strunjan; 3. vzorčno mesto – nenamakano Dekani; 4. vzorčno mesto – namakano Dekani) smo vkopali 6 keramičnih sveček, s pomočjo katerih smo 1-krat tedensko odvzeli vzorce perkolata oziroma odcedne vode na globini tal 40 cm. Vzorec perkolata je predstavljal povprečni vzorec 6 podvzorcev, pridobljenih iz 6 keramičnih sveček.

Ob vkopanih keramičnih svečkah smo 2-krat mesečno odvzeli tudi povprečne vzorce tal. Vzorčenje smo opravili v zgornjem talnem sloju do globine 50 cm. Vzorec tal (0,5 kg) je predstavljal povprečni vzorec, pridobljen iz 10 enakomerno razporejenih podvzorcev znotraj posameznega vzorčnega mesta.

Odvzete vzorce vode in tal smo takoj po vzorčenju shranili v zamrzovalno skrinjo (-16 °C). Na odvzetih vzorcih so bile opravljene analize za vsebnost nitratov. V vzorcih vode je bil nitrat določen direktno, medtem ko je bil nitrat v tleh določen v talnem ekstraktu. Za ekstrakcijo je bil uporabljen 0,01 mol l^{-1} $CaCl_2$. Vsebnosti nitrata v talni vodi in tleh so bile določene spektrofotometrično z aparatom Perkin Elmer UV/VIS Spectrometer Lambada 2, s FIAS sistemom vzorčenja. Analize nitrata v vodi so potekale po standardni metodi SIST ISO 14255 v laboratoriju Centra za pedologijo in varstvo okolja na Biotehniški fakulteti Univerze v Ljubljani.

2.3 Vzorčenje in analiza vsebnosti bakra v tleh

Ob vkopanih keramičnih svečkah smo poleg vzorcev za vsebnosti nitrata v tleh 1-krat mesečno odvzeli vzorce tal, v katerih smo ugotavljali vsebnost bakra. Vzorčenje smo opravili v zgornji talni plasti do globine 40 cm. Vzorec tal (3 kg) je predstavljal povprečni vzorec, pridobljen iz 25 enakomerno razporejenih podvzorcev znotraj posameznega vzorčnega mesta.

Na odvzetih vzorcih tal so bile takoj po vzorčenju izvedene analize bakra. Analize bakra so bile narejene po standardni metodi SIST ISO 17294, modif: 2003 v laboratoriju Inštituta za varstvo okolja Zavoda za zdravstveno varstvo Maribor.

3 REZULTATI IN DISKUSIJA

3.1 Lastnosti tal

Na podlagi rezultatov standardne pedološke analize smo tla v Dekanih uvrstili v globoka glineno-ilovnata tla, medtem ko se tla v Strunjanu uvrščajo v meljasto-glinena tla (Preglednica 2). Zgornji horizont poskusnih tal v Dekanih je zelo močno humozen (18,0 %), medtem ko so spodnji horizonti humozni do slabo humozni (3,1 % - 1,6 %). Humozna do slabo humozna tla so tudi tla v Strunjanu (3,3 % - 1,4 %). Reakcija tal se na obeh poskusnih lokacijah giblje v območju med pH 7,0 in 7,6, kar predstavlja optimalno okolje za mikrobiološke procese in ugodno vpliva na rast in razvoj oljk. Kationska izmenjalna kapaciteta se v poskusnih tleh giblje med (35 - 40 mmolc $100g^{-1}$), kar je značilno za mineralna tla (20 in 40 mmolc $100g^{-1}$) (Alloway, 1997). Po Kutileku in Nilsenu (1994) se tla v Strunjanu uvrščajo v tla z zelo slabo propustnostjo za vodo, saj se vrednosti koeficienta hidravlične prevodnosti gibljejo med 0,27 – 0,85 cm dan^{-1}, medtem ko se tla v Dekanih uvrščajo v slabo do srednje propustna tla za vodo (4,04 – 25,7 cm dan^{-1}).

Preglednica 2: Lastnosti tal poskusnih oljčnih nasadov
Table 2: Soil characteristics for the experimental olive groves

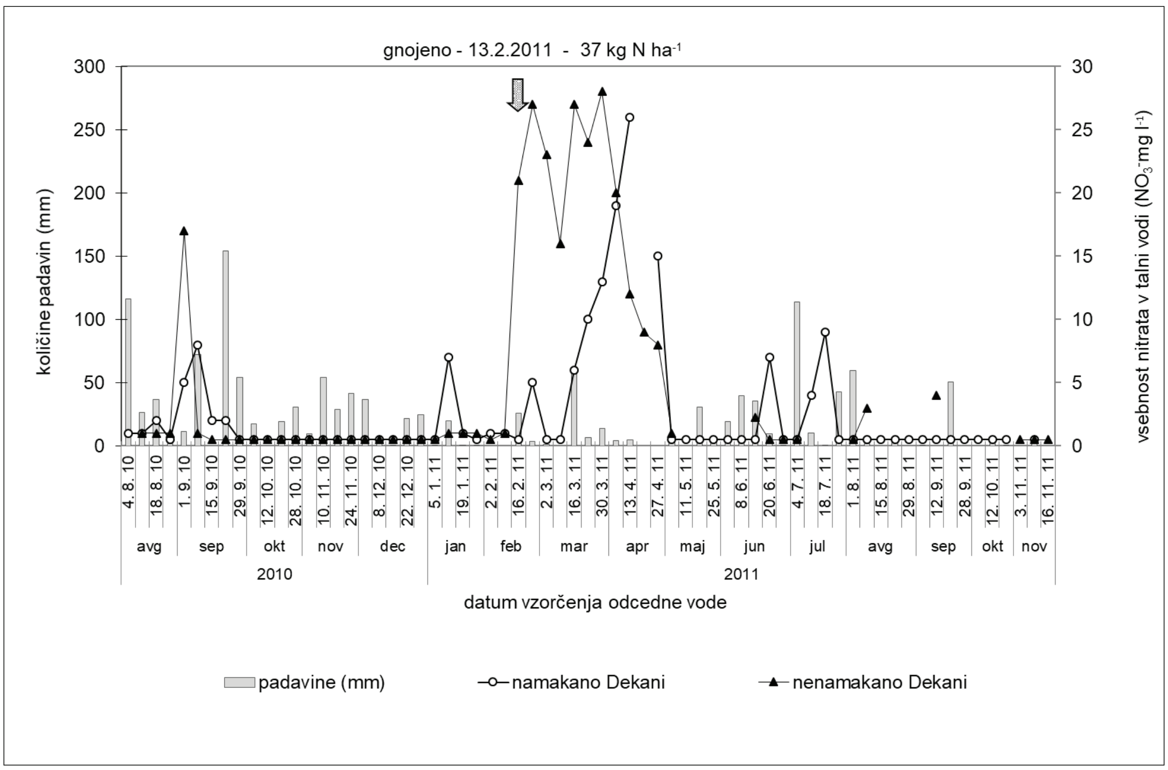
Vzorčno mesto	Horizont	Globina (cm)	Organska snov (%)	C/N- razmerje	Teksturni razred	Izmenljiva kapaciteta tal (mmolc+ kg^{-1})	Hidravlična prevodnost (cm dan^{-1})	Poljska kapaciteta tal (vol. %)	Točka venenja (vol. %)	pH
Dekani	Ah	0-2	18,0	14,4	I	40,8	/	32,33	18,56	7,0
	P1	2-24	3,1	15,0	GI	38,8	17,48			7,4
	P2	24-51	2,2	10,0	GI-I	39,2	25,07			7,4
	P3	51-74	1,6	11,3	GI	37,8	4,04			7,6
Strunjan	A1	0-8	3,3	10,0	MI-MGI	35,5	/	35,09	24,25	7,3
	P1	8-32	1,9	8,5	MI-MGI	36,9	0,27			7,4
	P2	32-73	1,4	7,3	MI-MGI	38,2	0,85			7,5

3.2 Dinamika nitrata v odcedni vodi

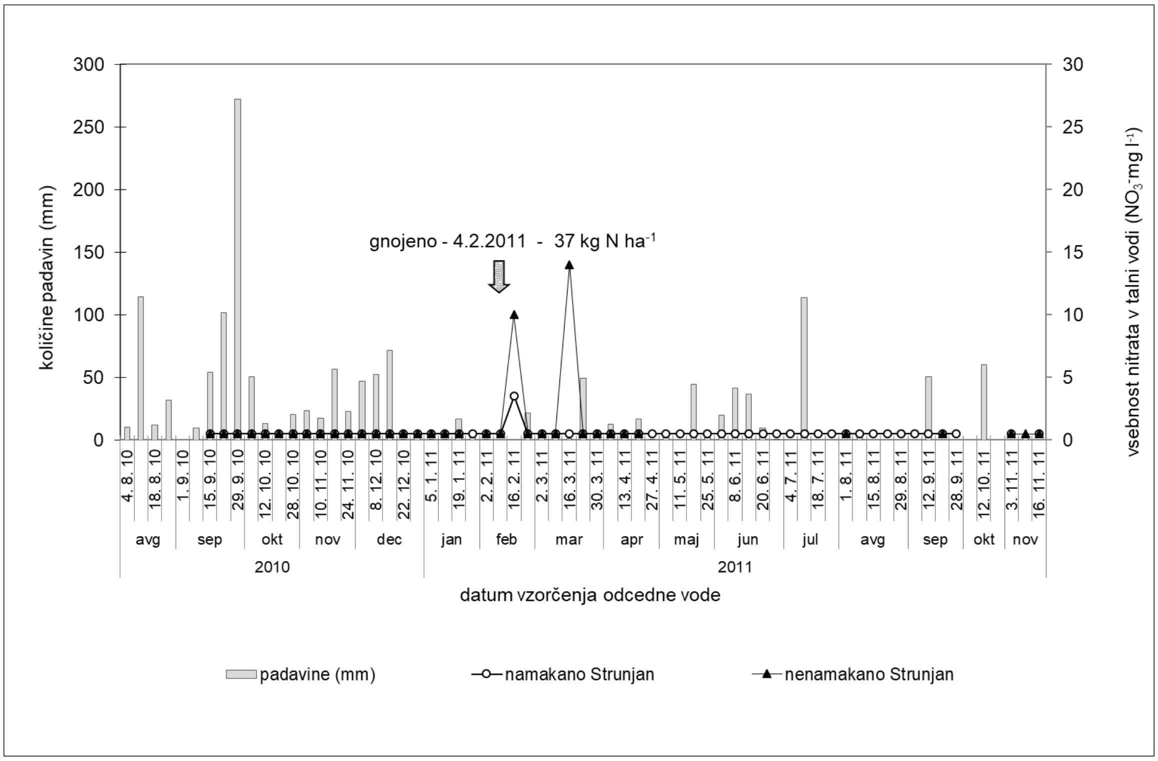
V večini odvzetih vzorcev tako v Dekanih kot v Strunjan so bile vsebnosti nitrata pod mejo detekcije. Največje izmerjene vsebnosti nitrata v vzorcih odcedne vode v Dekanih (nenamakano: 30. 3. 2011 – 28 mg NO_3^- l^{-1}; namakano: 13. 4. 2011 – 26 mg NO_3^- l^{-1}) in Strunjanu (nenamakano: 16. 3. 2011 – 14 mg NO_3^- l^{-1}; namakano:16. 3. 2011 – 3,5 mg NO_3^- l^{-1}) so bile izmerjene po gnojenju v času padavin, ki pa niso presegle mejne vrednosti (50 mg NO_3^- l^{-1}) za dobro kemijsko stanje podzemnih voda, ki ga predpisuje Uredba o standardnih kakovosti podzemne vode (Uradni list RS, št.100/2005).

Z analizo odcedne vode smo ugotovili, da so povprečne vsebnosti nitrata v vzorcih nenamakanih (5,36 mg NO_3^- l^{-1}) in namakanih površin (2,60 mg NO_3^- l^{-1}) v Dekanih, ki so bili odvzeti od 1. 7. 2010 do 16. 11. 2011, večje v primerjavi z vsebnostmi nitrata v perkolatu nenamakanih (1,14 mg NO_3^- l^{-1}) in namakanih (0,55 mg NO_3^- l^{-1}) površin v Strunjanu (Slika 2 in Slika 3). V Dekanih se zaradi pogoste košnje in neodstranjene travne ruše kopičijo velike količine odmrlih rastlinskih ostankov, ki predstavljajo velike zaloge organske snovi v tleh. V zgornjemu horizontu tal v Dekanih smo tako izmerili 18 % organske snovi, medtem ko so bile izmerjene vrednosti organske snovi v zgornjem sloju tal v Strunjanu znatno manjše (3,3 %). Čeprav imajo tla v Dekanih boljšo propustnost za vodo kot tla v Strunjanu in bi zato pričakovali večji »razredčitveni efekt«, se je v Dekanih v primerjavi z Strunjanom koncentracija nitrata v odcedni vodi večkrat povečala (september 2010, januar, junij, julij in september 2011) neodvisno od dodane količine gnojila. Glede na navedene rezultate in dejstva predvidevamo, da so bili v Dekanih izpolnjeni vsi pogoji (optimalna temperatura, pH, vlažnost in prezračenost tal) za proces mineralizacije organske snovi v tleh, ki se je razkrojila (mineralizirala) do osnovnih rastlinskih hranil – nitrata. Nastale količine nitrata so bile večje od potreb rastlin in so se tako izprale v globlje plasti tal.

Večja nihanja v vsebnosti nitrata smo zabeležili v perkolatu namakanih površin izven gnojilnega obdobja, iz česar lahko sklepamo, da je namakanje pomembno vplivalo na proces mineralizacije in razgradnjo organske snovi v tleh. Le to so potrdili tudi Valé in sod. (2007), ki poročajo o vplivu namakanja na mineralizacijo dušika v toplih klimatskih razmerah. Hkrati je potrebno poudariti, da dodana voda na namakanih površinah omogoča nemoten sprejem hranil v rastlino in lahko zaradi velikega pretoka vode redči nitrat v perkolirani vodi, medtem ko pa je voda v nenamakanih tleh v času suše vezana z večjo silo, kot jo zmorejo rastline, in se zato hranila ne porabijo, ampak se kopičijo v tleh in se ob povečanih padavinah izperejo v globlje plasti tal.



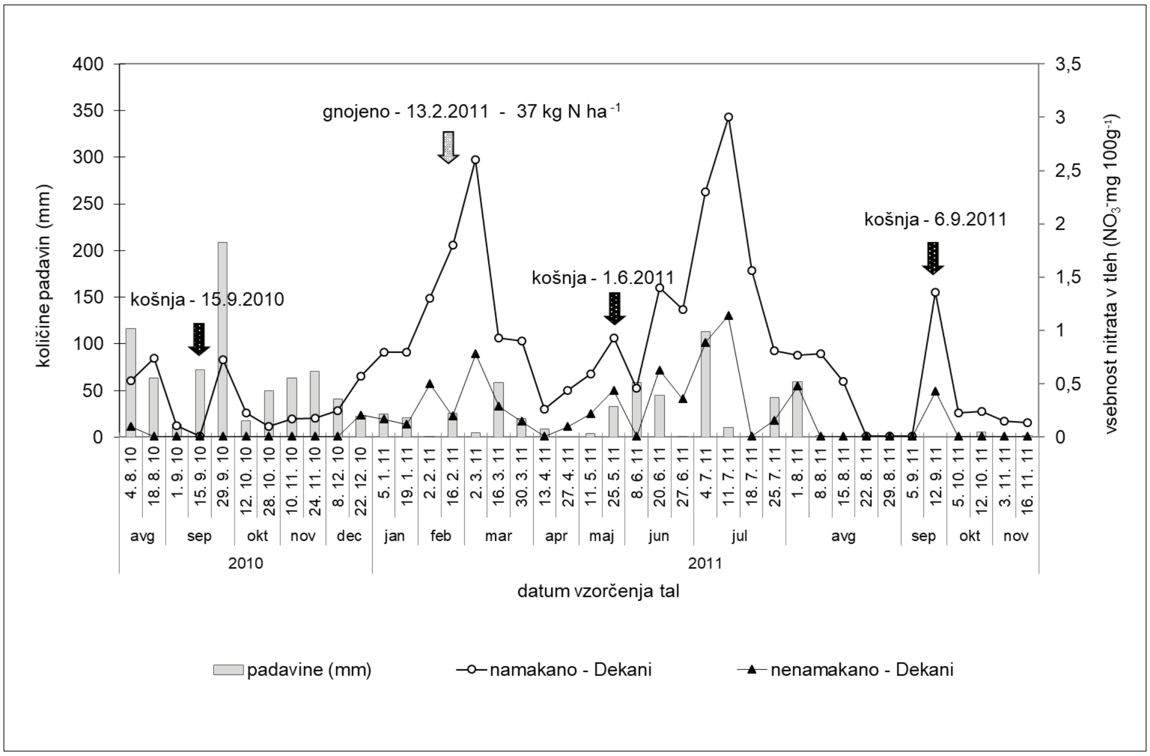
Slika 2: Padavine (mm) in povprečna koncentracija nitrata (NO_3^- mg l^{-1}) v odcedni vodi v Dekanih
Figure 2: Precipitation (mm) and average nitrate concentration (NO_3^- mg l^{-1}) in soil water samples for Dekani



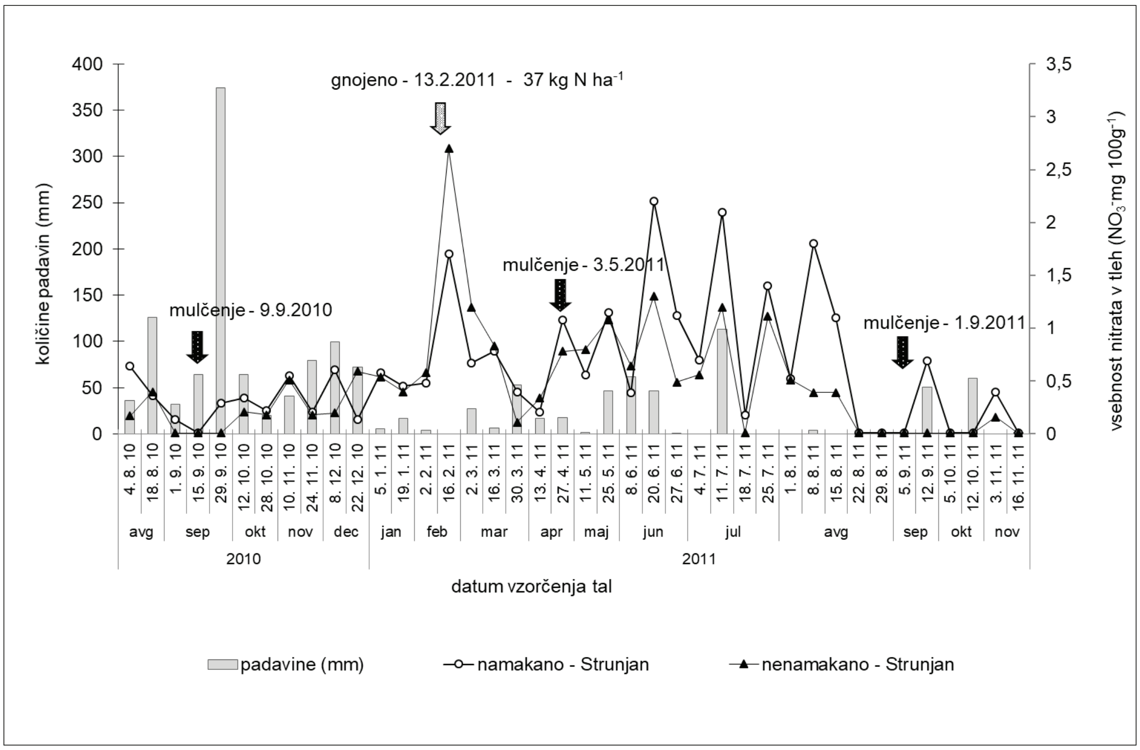
Slika 3: Padavine (mm) in povprečna koncentracija nitrata (NO_3^- mg l^{-1}) v odcedni vodi v Strunjanu
Figure 3: Precipitation (mm) and average nitrate concentration (NO_3^- mg l^{-1}) in soil water samples for Strunjan

3.3 Dinamika nitrata v tleh

Rezultati analize nitrata v tleh so pokazali, da so povprečne vsebnosti nitrata v času vegetacijske sezone v namakanih tleh višje (Dekani: 0,94 mg NO_3^- 100 g^{-1}; Strunjan: 0,51 mg NO_3^- 100 g^{-1}) kot v nenamakanih tleh (Dekani: 0,24 mg NO_3^- 100g^{-1}; Strunjan: 0,32 mg NO_3^- 100g^{-1}). Iz navedenih rezultatov in rezultatov analiz nitrata v odcedni vodi sklepamo, da namakanje pomembno vpliva na dostopnost nitrata v tleh. Pri tem je potrebno poudariti, da poleg namakanja, dejavnikov okolja in gnojenja na dostopnost dušika v tleh vpliva tudi način nege ledine oziroma obdelave tal. Iz slik 4 in 5 je razvidno, da se je tako v tleh, obdelanih z mulčenjem (Strunjan), kot v tleh s trajno ozelenitvijo (Dekani) vsebnost nitrata povečala po ukrepu nege ledine oz. obdelave tal. Gómez in sod. (2009) poročajo, da je trajna ozelenitev tal v oljčnih nasadih najprimernejši ukrep za varstvo tal in trajnostni način oskrbe tal. Izsledki španske raziskave so potrdili, da rastlinski pokrov zmanjša erozijo tal, zelena masa, ki jo po košnji travne ruše pustimo na tleh, pa povečuje vsebnost organske snovi in tako pozitivno vpliva na fizikalne, kemijske in biološke lastnosti tal (Moreno in sod., 2009). Hkrati pa Gucci in sod. (2012) poročajo, da je v intenzivnih oljčnih nasadih travna ruša konkurenca oljki za vodo in lahko v sušnih letih povzroči zmanjšanje pridelka. Vsekakor so za ovrednotenje vpliva načina nege ledine na rast in razvoj oljke ter rodovitnost tal potrebne nadaljnje raziskave, s katerimi bo določen optimalni način obdelave tal oljčnih nasadov v slovenskih klimatskih in talnih razmerah.



Slika 4: Padavine (mm) in povprečna koncentracija nitrata (NO_3^- mg 100 g^{-1}) v tleh v Dekanih
Figure 4: Precipitation (mm) and average nitrate concentration (NO_3^- mg 100 g^{-1}) in soil samples for Dekani

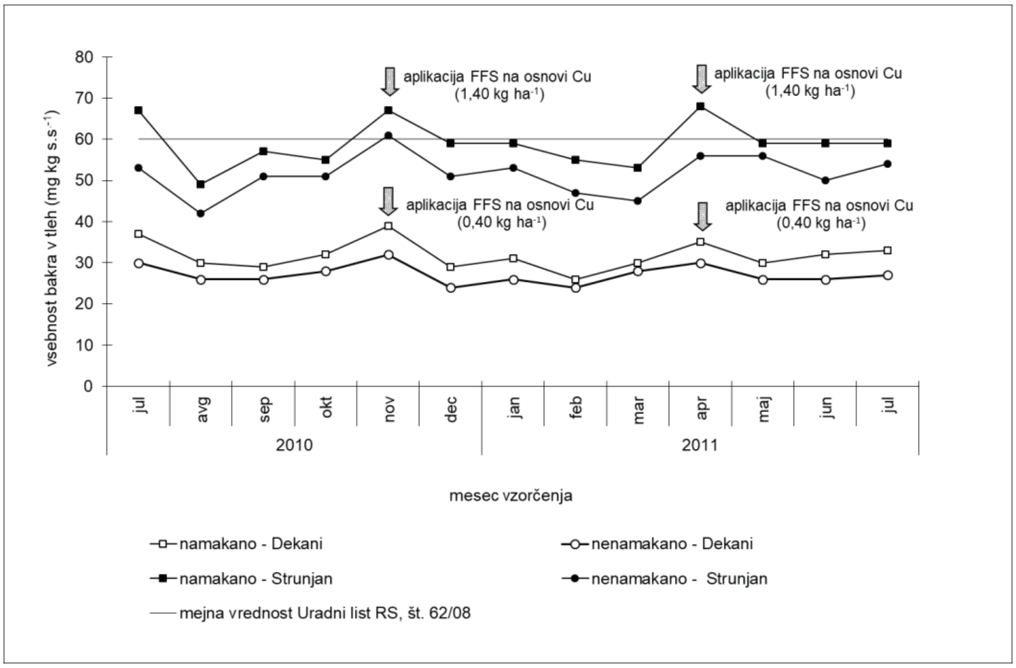


Slika 5: Padavine (mm) in povprečna koncentracija nitrata (NO_3^- mg 100 g^{-1}) v tleh v Strunjanu
Figure 5: Precipitation (mm) and average nitrate concentration (NO_3^- mg 100 g^{-1}) in soil samples for Strunjan

3.4 Dinamika bakra v tleh

V preučevanih tleh smo v Strunjanu tako v namakanih kot nenamakanih tleh zabeležili večje koncentracije bakra kot v namakanih in nenamakanih tleh na Dekanih (Slika 6). Koncentracije bakra v vzorcih, odvzetih v Dekanih, so bile od 24 do 39 mg kg^{-1} suhih tal. V vzorcih, odvzetih v Strunjanu, pa so bile vsebnosti bakra občutno večje (42 – 68 mg kg^{-1} suhih tal). Predpostavljamo, da so v Strunjanu večje vsebnosti bakra posledica dolgoletne kmetijske prakse varstva rastlin z bakrenimi pripravki in večjega deleža bakra, dodanega pri izvedbi fitosanitarnih ukrepov. Pri tem pa je potrebno poudariti, da količina dodanega čistega bakra v Strunjanu (1,40 kg ha^{-1}) ni presegla zgornje meje skupnega letnega vnosa čistega bakra (6 kg ha^{-1}), ki jo določa Uredba komisije ES 889/2008 v Prilogi II.

Največje vsebnosti bakra (21.7.2010 - 68 mg kg^{-1}; 27.10.2010 – 67 mg kg^{-1}; 21.4.2011 – 68 mg kg^{-1}) so bile izmerjene v namakanih tleh v Strunjanu in so presegle mejno vrednost za koncentracijo težkih kovin v tleh (60 mg kg^{-1}) (Uradni list RS. št. 62/08). Na območju vinogradov v submediteranskem delu Slovenije so že leta 2007 vsebnosti bakra v tleh (71 do 160 mg kg^{-1}) presegle opozorilne vrednosti (100 mg kg^{-1}) (Rusjan in sod., 2007), ki so bile določene z Uredbo o mejnih vrednostih vnosa nevarnih snovi in gnojil v tla (Uradni list RS, št. 19/17). Precej večje vrednosti so bile zabeležene na vinogradniških območjih Francije (Bordeaux 800 mg kg^{-1}) (Delas, 1963), kjer so več kot sto let za varstvo rastlin uporabljali bakrene pripravke. Res je, da je baker za rastline esencialni mikroelement in kot tak nujno potreben za rast in razvoj rastlin, vendar velike vsebnosti bakra povzročajo fitotoksičnost, kar se kaže v zmanjšani sposobnosti preživetja, zmanjšani rasti korenin, zaostanku v cvetenju, klorozi listov in zmanjšani tvorbi plodov ter manjši količini semen (Brun in sod., 2003; Alloway in Shorrocks, 1988).



Slika 6: Vsebnost bakra (mg kg^{-1}) v vzorcih tal v Strunjanu in Dekanih.
Figure 6: Content of copper (mg kg^{-1}) in soil samples for location Strunjan and Dekani.

Velik vpliv na vsebnost bakra v tleh ima tudi namakanje tal (Slika 6). Rezultati raziskave so pokazali, da je povprečna vsebnost bakra v namakanih tleh tako v Dekanih [1] (32 mg kg^{-1}) kot v Strunjanu (59 mg kg^{-1}) večja v primerjavi s povprečno vsebnostjo bakra v nenamakanih tleh (Dekani – 27 mg kg^{-1}; Strunjan – 51 mg kg^{-1}). Baker se v bazičnih karbonatnih tleh (tla nastala na flišu), kamor uvrščamo tudi poskusna tla v Dekanih in Strunjanu (slabo alkalna pH 7,0 - 7,6), zelo hitro in trdno veže na negativno površino talnih delcev – koloidov v obstojno obliko, kar močno vpliva na delež topnega in rastlinam dostopnega bakra v talni raztopini (Alloway in Shorrocks, 1988).

V vlažnih razmerah (padavine, namakanje) se ob nadaljnjem preperevanju bazični minerali izpirajo v nižje plasti tal. Posledica takih procesov je prehajanje vezanih bazičnih ionov v talno raztopino, njihova mesta pa zasedejo vodikovi ioni (H^+). Zaradi nadaljnjega izpiranja bazičnih ionov v nižje plasti tla niso več 100 %

zasedena z bazami in zato lahko absorbirani vodikovi ioni na površini talnih koloidov prehajajo v talno raztopino in znižujejo pH tal. Zaradi velike koncentracije vodikovih ionov H^+ v talni raztopini ti močno konkurirajo in izpodrivajo kovinske katione (bakra) iz vezanih mest na talnih koloidih. Posledično se z zniževanjem pH vrednosti talne raztopine veča delež bakra v talni raztopini in dostopnost bakra sami rastlini (Vitanović, 2012). Ti procesi so lahko vzrok, da je delež bakra v namakanih tleh nekoliko večji v primerjavi z vsebnostjo bakra v nenamakanih tleh. Iz zgoraj navedenega gre zaključiti, da so potrebne nadaljnje raziskave vsebnosti bakra v namakanih tleh, s katerimi bo natančneje ovrednoten vpliv dodane vode na kroženje bakra v tleh. Zaključimo lahko, da je poleg lastnosti tal (pH tal, kationske izmenjevalne kapacitete, železovih in manganovih kationov, vsebnosti organske snovi …) tudi vsebnost vode v tleh eden izmed pomembnejših dejavnikov, ki vpliva na vsebnost bakra v tleh.

4 SKLEPI

Rezultati raziskave so pokazali, da namakanje v kombinaciji s tehnologijo nege ledine vpliva na razporeditev, migracijo in vsebnost nitrata in bakra v tleh. Poleg tega, da dodana voda omogoča nemoten sprejem hranil v rastlino, lahko izboljša tudi razmere za mineralizacijo in razgradnjo organske snovi, ki je močno odvisna od načina oskrbe in nege ledine. S trajno ozelenitvijo in puščanjem rastlinskih ostankov na površini tal lahko povečamo zaloge organske snovi v tleh, kar v kombinaciji z optimalnimi okoljskimi razmerami vpliva na vsebnost nitrata. Za boljše razumevanje in ovrednotenje vpliva načina nege ledine na rast in razvoj oljke ter fizikalne, kemijske ter biološke lastnosti tal so potrebne nadaljnje raziskave, s katerimi bo določen optimalni način obdelave tal oljčnih nasadov v slovenskih klimatskih in talnih razmerah.

Ugotovljeno je bilo tudi, da je pri načrtovanju in uvajanju prilagoditvenih ukrepov na klimatske spremembe v kmetijski pridelavi potrebno upoštevati interakcijo med ukrepi ter vpliv le-teh na druge ranljive sisteme. Kljub temu, da je bila raziskava časovno in prostorsko omejena, nam bodo nova spoznanja omogočila okoljsko varno zamenjavo starih neučinkovitih agrotehničnih ukrepov in praks z novimi ter morda odkrila nove možnosti prilagoditev na podnebne spremembe. Za zagotavljanje varovanja okolja pri izvajanju prilagoditvenih ukrepov bi bilo potrebno preučiti tudi učinek prilagoditev agrotehničnih ukrepov na velikost in kakovost pridelka ter ekonomsko vzdržnost pridelave.

V slovenskih oljčnikih bi bilo potrebno, zaradi vse pogostejših in večjih napadov škodljivcev in bolezni, ki zahtevajo večkratno zaporedno tretiranje z bakrenimi pripravki ter blaženja posledic suše z namakanjem, ki močno vplivajo na dinamiko bakra v tleh, opraviti tudi inventarizacijo bakra v tleh. Le tako bi lahko preventivno preprečili degradacijske procese tal, ki so v svetu prepoznani kot ena največjih groženj okolju z direktnim vplivom na blagostanje prebivalstva.

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An assessment of population fluctuations of citrus pest woolly whitefly *Aleurothrixus floccosus* (Maskell, 1896) (Homoptera, Aleyrodidae) and its parasitoid *Cales noacki* Howard, 1907 (Hymenoptera, Aphelinidae): A case study from Northwestern Algeria

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ABSTRACT

The aim of this study was to assess the effectiveness of the parasitoid *Cales noacki* Howard, 1907 (Hymenoptera, Aphelinidae) in the biological control of the citrus pest woolly whitefly, *Aleurothrixus floccosus* (Maskell,1896) in Northwestern Algeria. In particular the pest and parasitoid population fluctuations under different environmental conditions were investigated. The study was conducted by examination and sampling of whitefly adult populations on young shoots and catches as well as its parasitoid during a 12-month period by counting the parasite and infested-live larvae. Results showed remarkable variations in abundance indices and infestation rates of larvae and adults that depended on the growth flush in foliage and meteorological conditions of the region. The woolly whitefly manifested three flight periods coinciding with three growth flushes of orange sap; in autumn towards the end of September followed by mid-November with the highest average abundance of 10 individuals per branch and a third flight period in mid-May. Phases of growth flush seemed to have an effect on the temporal distribution of adults over the sampling period from July to June. Pest emergence appeared favorable at 12-20 °C as the minimum interval temperature whereas the maximum varied between 25-33 °C with humidity levels of 50 % and 75 %. As for the incidence of natural enemies on whiteflies, despite their abundance, their impact was not optimum due to the large fluctuations in number of whiteflies. Although the pest-parasitoid complex appears to be important in the orchard, the biological regulation exerted by *C. noacki* while effective is insufficient for the complete neutralization of citrus woolly whitefly *A. floccosus*. An integrated approach is needed using additional natural enemies associated with the woolly whitefly.

Key words: citrus; biological control; population dynamics; *Aleurothrixus floccosus; Cales noacki;* phenology; Chlef Northwestern Algeria

IZVLEČEK

OVREDNOTENJE NIHANJA POPULACIJ ŠKODLJIVCA CITRUSOV, ŠČITKARJA *Aleurothrixus floccosus* (Maskell, 1896), IN NJEGOVEGA PARAZITOIDA *Cales noacki* Howard, 1907: VZORČNA ŠTUDIJA IZ SEVEROZAHODNE ALŽIRIJE

Namen raziskave je bil oceniti učinkovitost parazitoida *Cales noacki* Howard, 1907 (Hymenoptera, Aphelinidae) pri biotičnem zatiranju škodljivca citrusov, ščitkarja *Aleurothrixus floccosus* (Maskell, 1896), v severozohaodni Alžiriji. Še posebej je bilo preučevano nihanje populacij škodljivca in parazitoida v odvisnosti od različnih okoljskih razmer. V raziskavi so bile pregledane in vzorčene populacije odraslih osebkov ščitkarja na mladih poganjkih in v pasteh ter njihovi parazitoidi v obdobju 12-mesecev s štetjem parazitoida in napadenih, a živih ličink. Rezultati so pokazali znatne razlike v indeksih pogostosti in napadenosti ličink in odraslih, kar je bilo odvisno od dinamike rasti listov in meteoroloških razmer območja. Preučevani ščitkar je imel tri rodove, ki so sovpadali s tremi viški rasti in pretoka sokov pomarančevca in sicer konec septembra, v sredini novembra z največjim številom 10 osebkov na vejico, in tretji, v sredini maja. Obdobja rasti pomarančevca so vplivala na časovno pojavljanje odraslih osebkov v obdobju od julija do junija. Najnižji ustrezen temperaturni interval za pojav škodljivca je bil med 12 in 20 °C, najvišji pa med 25 in 33 °C pri relativni vlagi 50 in 75 %. Pojav naravnih sovražnikov ščitkarja kljub njegovi številčnosti ni bil optimalen zaradi velikega nihanja populacije škodljivca. Čeprav je kompleks škodljivca in parazitoida pomemben v sadovnjaku, je biotično uravnavanje s parazitoidom *C. noacki* nezadostno za celovito nevtralizacijo ščitkarja na citrusih. Zato je potreben integriran pristop z uporabo drugih naravnih sovražnikov ščitkarja *Aleurothrixus floccosus*.

Ključne besede: citrus; biotično varstvo; populacijska dinamika; *Aleurothrixus floccosus*; *Cales noacki*; fenologija; Chlef; severozahodna Alžirija

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1 INTRODUCTION

Citrus (*Citrus* spp.) constitutes the major group of fruits including oranges, grapefruits, trifoliate orange, mandarins, pummelo, citranges and lemon (Gebreslasie & Meresa, 2018). It serves as the main source of vitamins, mineral elements and sugar; hence, it controls the building process of the human body (Oviasogie et al., 2015). In Algeria, Chlef is the second most important citrus growing region with more than 5000 ha of which more than 90 % old plantations (Bellabas, 2011).

Gebreslasie and Meresa, (2018) reported that a decline in productivity of citrus can be attributed to several factors including fungal, bacterial, viral, nematode, and insect pests.

Citrus trees are attacked by several insect pests including the woolly whitefly, *Aleurothrixus floccosus* (Maskell, 1896) and the citrus whitefly, *Dialeurodes citri* (Ashmead, 1885) causing considerable economic damage (Yigit & Canhilal, 2005; Uygun & Satar, 2008; Giliomee & Millar, 2009; Tello Mercado et al., 2014; Abrol, 2015). The woolly whitefly was introduced accidentally in 1981 in western Algeria from Spain or Morocco (Doumandji & Doumandji-Mitiche, 1986). Citrus woolly whitefly's populations are pervasive and cause infestations with serious damages to citrus sector (Biche, 2012; Mahmoudi et al., 2017). *A. floccosus* Maskell is characterized with wings covered by white waxy substance. The adult male is slightly smaller than female attaining about 1.5 mm (Tello Mercado et al., 2014). The woolly whitefly produces copious amounts of sticky honeydew from the lower surface of leaves of lemon and other *Citrus* species (Grout et al., 2012).

Chemical control of crop pests is known for the negative effects on human health and environment as well as having low efficacy against *A. floccosus* (Katsoyannos et al., 1997; Nega, 2011). This resistance against insecticide is due to the wool-like wax filaments which cover 3rd and 4th instar nymphs and pupae of *A. floccosus* impeding penetration of insecticides (Katsoyannos et al., 1997). Integrated management of fruit pests including biological control is crucial to the economic success of many regions in the world (Kissayi et al., 2017).

The parasitoid *Cales noacki* Howard, 1907, (Hymenoptera, Aphelinidae) is an effective natural enemy and biological control agent of this pest (; Grout et al., 2012; Telli & Yiğit, 2012; Abrol, 2015).

There are few extensive reports which address the biological interaction of the woolly whitefly and its parasitoid *C. noacki* in the world (Ulusoy et al., 2003; ; Telli & Yiğit, 2012; Tello Mercado et al., 2014; Kissayi et al., 2017; Gebreslasie and Meresa, 2018).

The objective of this work was to assess the effectiveness of the parasitoid biological control agent *Cales noacki* Howard (Hymenoptera, Aphelinidae) against the citrus pest woolly whitefly, *Aleurothrixus floccosus* Maskell in Northwestern Algeria. The infestation rates of orange trees by *A. floccosus* and the parasitism rates by *C. noacki* were investigated in order to determine the favorable conditions for population growth and reproduction of *A. floccosus*.

The relation between adults' emergence and the appearance of the growth flush (i.e. young shoots) was followed to explain the close relationship between the number of generations per year and the growth flush. In addition, the parasitism activity by *C.noacki* was evaluated.

2 MATERIALS AND METHODS

2.1 Description of the case study area

This study was conducted in an orchard, occupying 4 ha, belonging to the citrus growing region of Chlef (36°05'53.3"N 1°13'02.4"E) situated in the northwest of Algeria (Figure 1). This area has a semi-arid bioclimatic stage with mild winters and dry periods extending from mid-April to October's end. The 12-year-old orchard was planted with orange trees *Citrus sinensis* L. of the Thomson Navel variety. Orchard maintenance (pruning, manual weeding and fertilisation) were done at the right time without phytosanitary treatments.

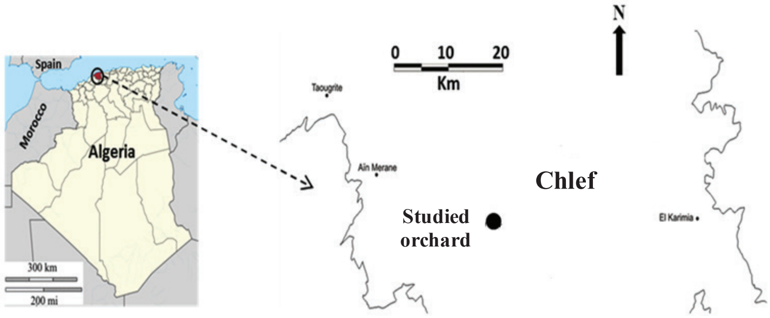

Figure 1: Geographical location of the case study orchard in the Chlef region of Northwestern Algeria

2.2 Population sampling of *A. floccosus*

The case study orchard was visited periodically each fortnight from July 2013 to June 2014. Ten whitefly-infested trees were randomly sampled at each fortnight. Four branches on different orientations carrying young shoots and elderly leaves per tree were observed for the sampling of adults and live larvae. In a related study Telli & Yiğit, (2012) considered the distribution of woolly whitefly on the four directions of citrus trees in Turkey.

Adult whiteflies as well as other circulating insects (i.e. entomofauna) within the orchard particularly the natural enemies of woolly whitefly, were caught through sticky traps. Three rectangular yellow plates Horiver-type (25 x 10 cm) were deposited within the canopy at a height of 1.5 m at a rate of one trap per tree at a distance of 50 m (Ekbom & Rumei, 1990). These traps were used to count the number of generations of several pests (Toorani & Abbasipour, 2017). In addition, a total of 50 leaves were randomly collected in the plot at each survey to investigate larvae, pupae and parasitized larvae per 1 cm^2.

2.3 Infestation rates by *A. floccosus*

The infestation rates of the global populations (T) were calculated according to the Townsend-Heuberger formula (Townsend & Heuberger, 1943) as follow:

T (%) = [(number of branches with presence of whiteflies × corresponding presence index / sum of branches with presence x 4) × 100]

The presence index was deduced from a scale of 0 to 4 and corresponds to intervals of abundance expressed as a percentage.
For the case of whiteflies:
(Index 1) < 25 % of presence
(Index 2) 25-50 % of presence
(Index 3) 50-75 % of presence
(Index 4) > 75 % of presence

2.4 Parasitism Rates by *C. noacki*

The parasitism rates were evaluated at each survey throughout the sampling period. Parasitized larvae were counted on a leaf area of 1 cm^2 under binocular magnifying glass at × 40 and the results were expressed in percent relatively to the total larvae on the observed surface.

The confirmation of the species of parasitoid emerged (*C. noacki*) was made from leaves carrying parasitized larvae collected and placed in rectangular boxes of dimensions (15 cm × 8 cm × 5 cm) containing cotton impregnated for emergence. Identification of the parasitoid was carried out using several identification keys (Doumandji & Doumandji-Mitiche, 1986; Goulet & Huber, 1993).

2.5 Phenology of orange tree in relation to climatic data

The periods of orange tree's growth flush during the study period were determined on the basis of the phenological growth stages described by Agusti et al. (1995). Meteorological data including rainfall amounts, average temperature values and relative humidity were obtained from the Boukadir meteorological station (5 km west of the orchard) belonging to the National Meteorological Office in Chlef.

2.6 Statistical analysis

The data were subjected to analysis of variance (ANOVA) for variable with normal distribution whereas the variables with abnormal distribution were subjected to the general linear model (G.L.M.). Differences at $P < 0.05$ were considered to be significant. The statistical analysis was accomplished using the computer software SPSS 16.0 for Windows (Chicago, IL, USA).

3 RESULTS

3.1 Fluctuations in abundance of *A. floccosus*

3.1.1 Temporal variations in abundance indices of larval & adult populations of woolly whitefly

The results of temporal variations in abundance indices of larval and adult populations of *A. floccosus* on the branches of *C. sinensis* of the studied orchard are given in the Figure 2.

The evolution of adult abundance indices of *A.floccosus* according to the three growth flush (1st gf, 2nd gf, 3rd gf), showed that the appearance of adults abundance peaks corresponding to the emergence of adults from pupae coincide with the appearance of young leaves of growth flush.

According to our survey, larvae index presents three significant abundance's peaks during the period of mid-November 2013, late February, and mid-June 2014 (Figure 2). In general, the analysis of fluctuations in adult abundance indices showed the existence of three peaks the most important among them was recorded in November 2013.

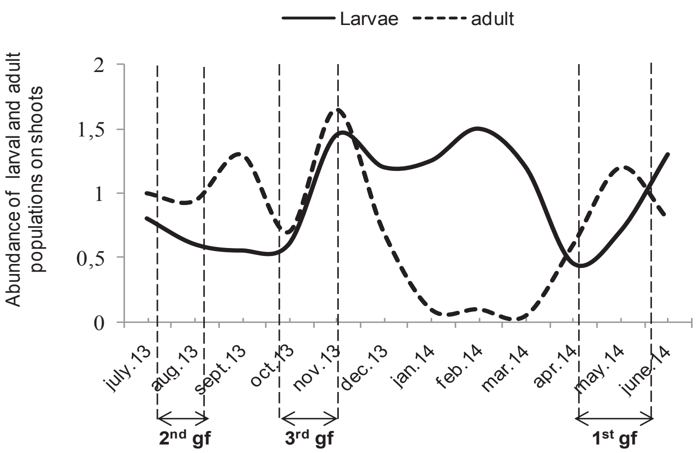

Figure 2: Temporal evolution of population abundance indices of *A. floccosus* on branches (gf: growth flush)

The evolution of adult *A. floccosus* caught through yellow traps revealed a stable distribution from July to late November, with a total ranging from 100 to 230 individuals per trap per month, except for mid-November attaining more than 275 individuals / trap / month.

In the last two months of the survey, a continuous evolution was observed in the adults recording about 600 adults / trap / month. The lowest enumeration of adults caught was recorded at end of March not exceeding 25 adults / traps / month (Figure 3). The first adults emerging from the pupae were noted in mid-June, end-October 2013 and early April 2014.

The analysis of the relationship between adult catches of woolly whitefly and orange's growth flush indicated that summer growth flush (1st gf) and autumn growth flush (3rd gf) coincided with the period of adult large trapping.

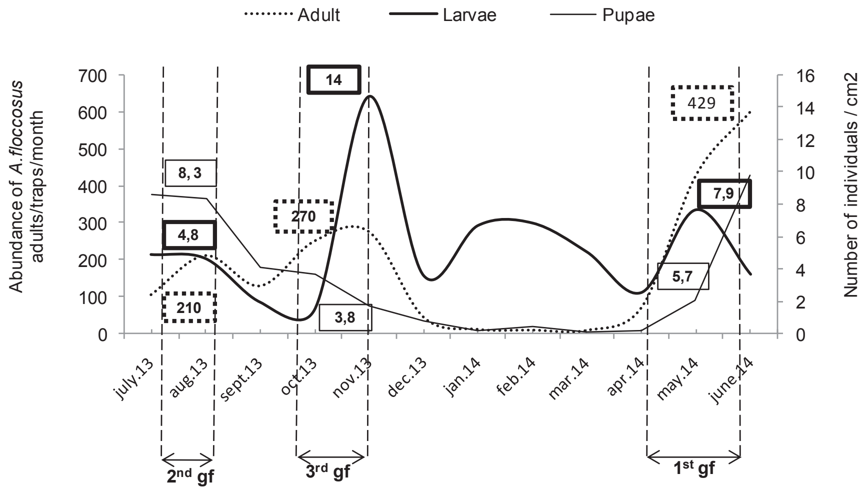

Figure 3: Evolution of the densities of the larval and pupal stages and adult catches by the sticky traps of the woolly whitefly. Absolute values shown in the graph represent abundance peaks of adults, larvae and pupae (gf: growth flush)

The evolution of *A. floccosus* biological stage was determined by counting the number of live larvae, pupariums on 1 cm^2 in 50 leaves. The results are shown in Figure 3. As can be seen from this figure, the live larvae are present throughout the year at all stages of foliage. In contrast, the pupae were almost absent during the period ranging from November 2013 to April 2014.

Furthermore, in 2013, from July to October, the density of the pupae of woolly whitefly was higher than those of live larvae. From November (maximum density) to the end of April, only live larvae were present. The density of larvae exhibited its second peak at the end of May followed by a significant change in the number of nymphs (Figure 3).

3.1.2 Variation in infestation rates of woolly whitefly

The larvae of the whitefly infested the leaves throughout the sampling period. A first phase of infestation by *A. floccosus* larvae was characterized by rate infestation ranging between 19 % to 39 % from early July 2013 to Mid-January 2014.

A second phase with more abundant infestation was observed in mid-February with an infestation rate of more than 80 %, then it reduced gradually to remain at a rate of 20 % during the month of April, a third stage of infestation was noted at the end of the sampling with a peak of more than 60 % (Figure 4).

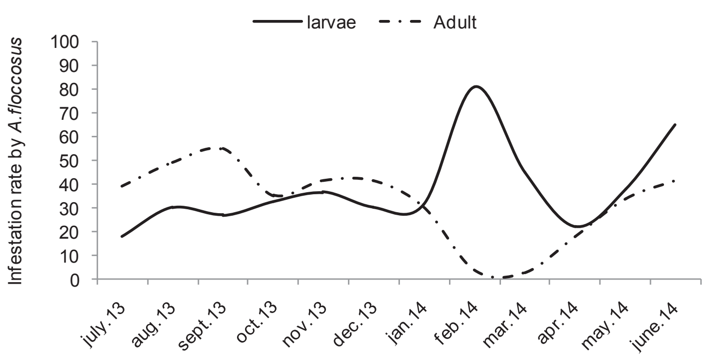

Figure 4: Temporal evolution of infestation rates of larvae and adults of *A. floccosus* on branches

Adults infesting the canopy, has shown three phases of infestation, the first phase displayed the highest infestation rate, exceeding 65 % in early September, coinciding with the 3rd growth flush (3rd gf) of 2013; the second period recorded low infestation rate from November to December and then decrease progressively to its lowest level from February to March; at the end of the sampling period, a rise in the infestation rate to a maximum of 40 % was observed (Figure 4).

3.1.3 Effect of exposure on spatiotemporal distribution of populations of *A. floccosus*

The evolution of abundance fluctuations of adult populations on branches with young leaves showed a preference for exposure to layed eggs on the underside of young leaves.

Indeed, adults emerging during the three flight periods recorded through the sampling period exhibited a remarkable preference for the Northern exposure for setting in the deposition of eggs, the southern exposure was sought by adults during the 2nd and 3rd peaks as well as the 1st peak with less importance (Figure 5).

On the other hand, it was noted that the larval distribution was not affected by either stage of leaves (old or young) not by their exposure during the sampling year (Figure 6).

The impact of different exposures/seasons on larval and adult distribution was analysed using ANOVA. The northern exposure of the canopy exhibited a significant effect on the distribution of adults on foliage comparatively to other exposures ($p < 0.05$). Similarly, the distribution of *A. floccosus* adults appeared to be influenced by the follow-up period ($p < 0.05$). However, the distributions of the larval populations seemed to be similar ($p > 0.05$) on different exposures.

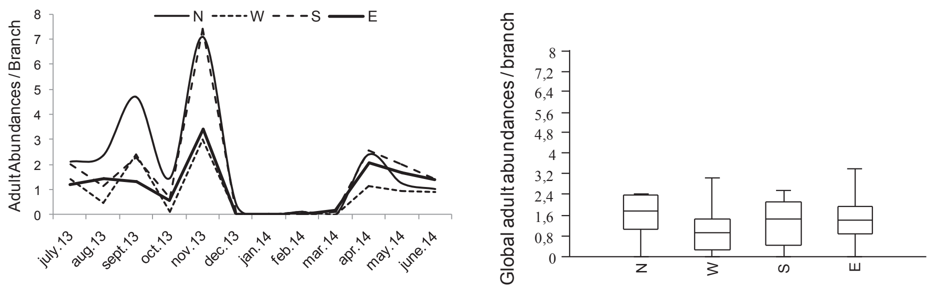

Figure 5: Effect of exposure on the distribution of adult populations (N: North, W: west, E: east, S: south)

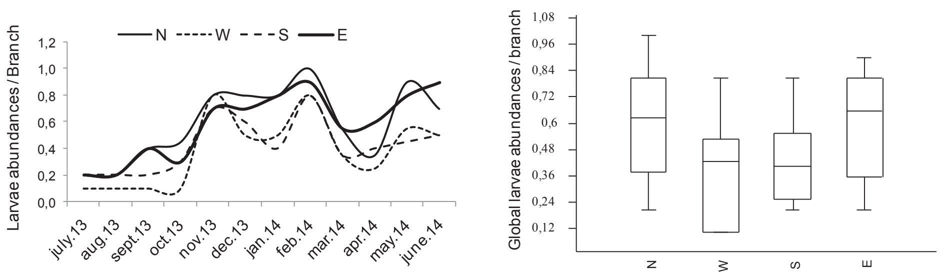

Figure 6: Effect of exposure on distribution of larval populations (N: North, W: west, E: east, S: south)

3.1.4 Evolution of parasitism by *C. noacki*

The temporal abundance and evolution of parasitism by *C. noacki* was followed during the sampling period. The results are presented in Figures 7.

As can be seen the parasitoid specific to *A. floccosus*, *Cales noacki*, showed three peaks of abundance, the most important was recorded at the end of sampling period during June 2014 with 16 individuals / trap / month (Figure 7). Nevertheless, for the rest of sampling period, few parasitoid adults were caught, not exceeding two individuals / trap / month.

Figure 7 shows *C. noacki*'s parasitism activity recorded throughout the sampling period, the highest activity was registered in July 2013 with more than 75 % of parasitism. This rate decreased gradually to a stable interval between 5 % and 30 % during the other months of the year. This rate of parasitism is calculated according to the number of parasitized larvae in relation to the total number of larvae per cm^2.

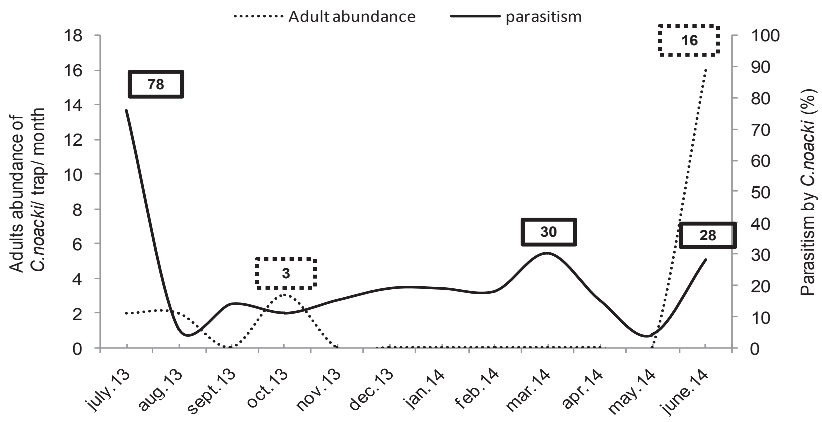

Figure 7 : Evolution of parasitism rate by *C. noacki* emerged from *A. floccosus* & adult catches in traps on orange trees

4 DISCUSSION

Ulusoy et al., (2003) reported that the woolly whitefly does not have a preference for specific citrus varieties. However, it has been observed mainly on orange trees in the Mediterranean region (Barbagallo et al*.,* 1986) where considerable damage for citriculture has been reported especially in Italy (Del-Bene & Gargani, 1991).

Furthermore, there are numerous studies on the population dynamics of *Aleurothrixus floccosus* (Onillon, 1973; Gebreslasie & Meresa, 2018).

In Algeria, few studies on this species have been carried out (Doumandji-Mitiche & Doumandji, 1988; Berkani, 1989). The current study has shown that there are three generations during a one year period. In contrast, Biche (2012) estimated that the woolly whitefly evolves in four to five generations per year, almost uninterrupted throughout the year with a simple slowdown noted during the colder months. The slowdown in infestation rates of larvae and adults of *A. floccosus* during the colder Algerian months of January to April was also noted in the present investigation (Figure 4).

Depending on the phonological characteristics of the *Citrus* (i.e. persistence of foliage, rhythmicity of appearance of growth flush), the dynamics of the pest populations are influenced by the host plant because of the qualitative and quantitative evolution of the resources offered (Bran & Onillon, 1978; Kissayi et al., 2017). This species spends the winter at the third or fourth instar larvae and sometimes at the egg stage, laid in the autumn by the last generation of adults.

Additionally, under the specific climatic conditions of the citrus region of Chlef, it has been noted that the first spring generation appears with the young shoots of the first growth flush of orange trees as a result of the first laying of the year. Ripa et al., (1999) and Giliomee & Millar, (2009) estimated that the underside of young leaves is preferred by the whitefly to feed and oviposit.

In another study, Onillon (1973) reported, that in early spring, the adults of the woolly whitefly prefer the young bitter-orange tree shoots resulting from a total transfer of the imaginal population on leaves of the first growth flush. These results are consistent with those cited by Abbassi (1980).

A second summer generation was recorded in the months of August coinciding with the 2nd summer growth flush. The latest generation and the largest in adults' number started with the new leaf outputs of the latest growth flush. However, Katsoyannos et al., (1997) have counted six overlapping generations in Athens. Grout et al., (2012), estimate that new infestations of *A. floccosus* are, habitually, associated with growth flushes.

The period from January to March corresponds to the hibernation period of the woolly whitefly in the current case study region where there was sometimes a total absence of adults; the lower faces of leaves were observed to be encrusted by larvae and exuviae, covered with sooty mould.

These findings were in accordance with those mentioned by Onillon (1973) and supported by the reports of Gebreslasie and Meresa, (2018).

The difference in the number of generations of *A.floccosus* between the region of Mitidja where it evolves in four to five generations (Doumandji-Mitiche & Doumandji, (1988); Biche, 2012) and the region of Chlef (three generations) can be explained by the specific climatic conditions of the Chlef province.

Indeed, the mean of minimum temperatures of the case study Chlef region varied between 12-20 °C and the maximum oscillating between 25-33 °C which was shown as favorable for the emergence of adults to trigger egg laying on the lower side of young leaves.

In support of this, France et al., (2011) estimated that the favorable average temperature for the development and reproduction of *A. floccosus* varies between 10.8 °C and 28.5 °C in northern Chile which is in the same range as observed in the current study.

The present results suggest that the rainfall pattern does not affect the whitefly population dynamics. The ideal moisture levels coinciding with the flight periods of adults varied between 50 % and 75 %.

Furthermore the yellow sticky traps can be considered as an effective tool for monitoring the population dynamics of crop pests, especially whiteflies (Prema et al., 2018). Adults caught by yellow traps accurately reflects the flight periods of woolly whitefly. The flight peaks effectively correspond to the generations of *A. floccosus*. In a related study, Prema et al., (2018) used different colored sticky traps including blue, white, yellow, red and green to monitor the movement of thrips (i.e. sucking pests similar to whiteflies) and the number of thrips catches at different growth stages of cotton growth. Results of their field trials confirmed that yellow sticky traps attracted more thrips compared to other colors.

In the existing study, in summer the number of adults caught reached more than 600 individuals per trap/month, in contrast to what was observed *in situ* where the autumn period was characterized by a large number of emerged adults.

This result could be due to the fact that the yellow sticky traps enable the capture of adults natives in the case study orchard as well as of the entomofauna circulating at the level of the orchards close to the study station.

The live larvae enumerated on 1 cm^2 of foliage showed that the latter occurred on the lower side of the leaves throughout the year, which explains the large number of adults caught by the traps or counted in the foliage.

The rate of parasitism by *C. noacki* was very high at 78 % during the month of July. In contrast this rate did not exceed 30 % during the other sampling periods. Katsoyannos et al., (1998), consider that in Greece, *C. noacki* is an effective natural enemy against woolly whitefly with parasitism rates exceeding 82 %.

In another report, Telli & Yiğit, (2012) estimated that rates of parasitism by *C. noacki* in Turkey ranged from 70 % to 88 %. Similarly, rates of parasitism reaching 90 % on clementine trees and 75 % on orange trees were reported by Doumandji and Doumandji-Mitiche (1986) for the Mitidja region in Algeria.

In a related study, a survey of *Aphelinidae* (Hymenoptera: Chalcidoidea) species associated with crop and forest pests in Morocco was conducted by Kissayi et al., (2017). The authors concluded that the knowledge of *Aphelinidae* especially in North Africa countries is still incomplete. They also argued that further study is required to implementing biological control of crop pests and taxonomic studies of parasitic wasps for sustainable agriculture in countries of the North Africa region.

The spatiotemporal distribution of the adults depends of climatic conditions, the phenology of the orange tree (growth flush) and exposure or orientation of the leaf. It has been demonstrated that the north and south exposure are the most populated by the adults of the woolly whitefly; this can be explained by the presence of a large number of young leaves favoring the laying and the installation of *A. floccosus* (Onillon, 1973; Meagher, 2008).

Additionally, the evolution of the curve of the larvae parasitized by *C. noacki* showed that the parasitism activity is very intense during the summer and spring period and is remarkably low during the winter period, which may be due to unfavorable climatic conditions. The decline in the rates of parasitism in the present work observed between November and the beginning of May can be explained by the influence of the wind shear at 3.9 - 4.4 m s^{-1} which was able to involuntary unhooking off the endoparasitic larvae, as they are fixed less firmly on the plant than those that are healthy.

Whereas, Ulusoy et al. (2003) found that the decline in parasitism may be due to the effect of frost, which destroyed parasitized larvae that lack the cottony mass that protects live healthy larvae.

In Algeria, several releases of the parasitoid *C. noacki* were carried out in 1985 and 1986, by Doumandji and Doumandji-Mitiche (1986). The parasitoid was able to settle in Mitidja and was able to limit the outbreak of *A. floccosus.* The presence of this parasitoid in the Chlef region can be explained by the adaptability of *C. noacki* which can makes it an effective tool for biological regulation against the woolly whitefly.

A similar case study of the insect pest complex of citrus, their natural enemies and their management at Keren in Eritrea has been reported by Husain et al*.,* (2017). Furthermore, Gebreslasie and Meresa, (2018) noted that many pests can attack citrus. This suggests that an integrated approach may be needed in the protection of citrus orchards in Algeria. While Husain et al., (2017) focused primarily on chemical control; this may not be the best way forward due to environmental concerns. Integrated biological control of citrus pests using various parasitoids is recommended.

5 CONCLUSIONS

The present study was the first detailed characterization of the relationship between woolly whitefly *A. floccosus,* the parasitoid *C. noacki* and the appearance of new citrus shoots in orange trees (‘Thomson Navel’) in the Chlef Northwestern Algerian region. The wooly whitefly pest prefers young shoots to settle and lay eggs.

Results showed remarkable variations in abundance indices and infestation rates of larvae and adults that depended on the growth flush in foliage and meteorological conditions of the region. The woolly whitefly manifested three flight periods coinciding with three growth flushes of orange sap; in autumn towards the end of September followed by mid-November with the highest average abundance of 10 individuals per branch and a third flight period in mid-May.

Phases of growth flush seemed to have an effect on the temporal distribution of adults over the sampling period from July to June. Pest emergence appeared favorable at 12-20 °C as the minimum interval temperature whereas the maximum varied between 25-33 °C with humidity levels of 50 % and 75 %. In the current case study region, as for the incidence of *C. noacki* on the wooly whitefly, their impact was not optimum due to the large fluctuations in number of whiteflies. Although the pest-parasitoid complex appears to be important in the orchard, the biological regulation exerted by *C. noacki* is insufficient for the complete neutralization of *A. floccosus*. An integrated approach is needed using additional natural enemies associated with the woolly whitefly.

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Combining ability and heterosis for some canola characteristics sown on recommended and late planting dates using biplot

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ABSTRACT

Canola (*Brassica napus* L.) is one of the most efficient oil-producing crops in arid and semi-arid regions of the world. In the current study, ten winter canola genotypes [seven genotypes as lines (Zarfam (L1), Talaye (L2), SLM046 (L3), Geronimo (L4), Modena (L5), Opera (L6) and Symbol (L7)] and three genotypes as testers [Okapi (T1), Licord (T2) and Orient (T3)] and their F1 hybrids (21 hybrids) were evaluated to determine the genetic parameters for grain yield, oil content, meal and seed glucosinolate contents under two different planting date [recommended (late September) and late planting (late October)]. According to combined analysis of variance there were significant differences among the genotypes for most studied traits. The genotype main effect and genotype × environment interaction (GGE) biplot method was used for analyzing line × tester design data. Among the lines, L5 showed high negative general combining ability (GCA) effect for meal glucosinolate content in both conditions whereas L1, L5 and L6 revealed high negative GCA effects for seed glucosinolate content in both planting date. From the results, it could be concluded that, hybridization between T1×L1, T1×L6 or T3×L5 is an efficient approach to release genotypes with low seed and meal glucosinolate content. Furthermore, to develop canola cultivars with higher seed and oil yield, hybridization between T1×L7 or T2×L7 is highly recommended. Improved oil content will be achieved if T1×L5, T2×L5 or T3×L6 hybrids are implemented into the breeding programs.

Key words: GGE biplot; heterosis; line × tester; polygon; grain yield; oil content

IZVLEČEK

SPOSOBNOST KOMBINIRANJA IN HETEROTIČNEGA UČINKA ZA NEKATERE LASTNOSTI OLJNE OGRŠČICE SEJANE OB PRIPOROČENEM IN POZNEM TERMINU Z UPORABO BIPLOTA

Oljna ogrščica (*Brassica napus* L.) je v sušnih in polsušnih območjih sveta ena izmed najbolj učinkovitih poljščin za proizvodnjo olja. V raziskavi je bilo ovrednoteno deset ozimnih genotipov oljne ogrščice [sedem genotipov kot linije (Zarfam (L1), Talaye (L2), SLM046 (L3), Geronimo (L4), Modena (L5), Opera (L6) in Symbol (L7)] in trije genotipi kot testerji [Okapi (T1), Licord (T2) and Orient (T3)] ter njihovi F1 križanci (21 križancev) za določitev genetskih parametrov za pridelek zrnja, vsebnost olja, vsebnost glukozinulatov v pogači in semenu v razmerah priporočene (konec septembra) in pozne setve (konec oktobra). Glede na kombinirano analizo variance so bile med genotipi značilne razlike za večino preučevanih znakov. Za analizo glavnega učinka genotipa in njegove interakcije z okoljem pri križancih linij in testerjev je bila uporablja metoda biplota. Med linijami je L5 pokazala zelo negativno splošno kombinacijsko sposobnost (GCA) za vsebnost glukozinulatov v pogači v obeh terminih setve, v istih razmerah so imele linije L1, L5 in L6 velik negativni GCA učinek na vsebnost glukozinulatov v semenu. Iz rezultatov bi lahko zaključili, da so križanja kot so T1×L1, T1×L6 ali T3×L5 učinkovit pristop za odbor genotipov z majhno vsebnostjo glukozinulatov v semenu in pogači. Še več, za vzgojo sort oljne ogrščice z večjim pridelkom semena in olja so križanja T1×L7 ali T2×L7 zelo priporočena. Izboljšano vsebnost olja bi lahko dosegli,če bi bila križanja T1×L5, T2×L5 ali T3×L6 vključena v žlahniteljske programe.

Ključne besede: GGE biplot; heterozis; linija × tester; polygon; pridelek zrnja; vsebnost olja

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1 INTRODUCTION

Canola (*Brassica napus* L.), a low-acid cultivar of rapeseed, is known as one of the most important edible oilseed crops across the world (Wang, 2005). Canola plays a starring role in the oilseed crops business. It has been reported that one percentage increase in canola seed oil is equivalent to 2.3~2.5 percentage increase in seed yield (Qian et al., 2009). Accordingly, a lot of effort has been put to breed high oil yield canola cultivars around the globe. It is well understood that the heterosis is an effective approach to increase crops' yield (Wang et al., 2009). A high-parent heterosis with 120 % for grain yield in rapeseed was reported by Brandle and McVetty (1989). In addition, Qi et al. (2003) reported the over parent percentage of grain yield per plant was 70.24 % (30.70-218.0 %). In most of the countries, hybridization plays a key role in the development of canola cultivars (Miller, 1999; Fu, 2000). However, little is known about the heterosis and its role in oil content in canola cultivars. For instance, Shen et al. (2005) pointed out that mid-parent heterosis for seed oil content differs from -1.55 % to 7.44 %. Therefore, further studies on heterosis in canola and its effects on oil content are helpful.

In order to improve canola seed yield, it is essential to have certain information on the nature of both general and specific combining ability (GCA, SCA) of candidate parents, used in hybridization. Moreover understanding the nature of genes action in controlling quantitative and qualitative traits is a critical aspect of breeding programs (Velasco & Becker, 1998). Numerous researches have been performed on combining ability and heterosis, most of these researches have shown significant effects of GCA and SCA on grain yield and oil content in canola suggesting that both additive and non-additive gene action play a crucial role in inheritance of these traits (Schierholt et al., 2001; Amiri-Oghan et al., 2009; Qian et al., 2009).

The line × tester approach is widely used in breeding both self and cross-pollinated crops to identify the most desirable parents. The crosses are chosen based on their GCA and SCA effects (Hinkelmann, 2012). On the other hand, combining ability analysis is a powerful tool to select the most desirable parental lines to release superior hybrids. The success in the hybridization programs strongly depends on the ability of the parents to perform desirable combinations (Hallauer & Miranda, 1981). Visual exploration of GCA and SCA effects through biplot approach could be beneficial for crop breeders in identifying the most suitable parents for hybridization. The biplot analysis method, which is used to graphically depict a two-dimensional dataset, was developed by Gabriel in 1971. However, some modifications have been made in the primary biplot analysis method over the time. For instance; Yan et al. (2000) proposed a special biplot as GGE (genotype main effect and genotype × environment interaction), developed from the first two principal components (PC1 and PC2) derived from principle component analysis of environment-centered yield data, for multi-environment trials.

In a study, Yan and Hunt (2002) applied the SREG2 model for graphic analysis of diallel data using biplot method. The SREG2 model consisted of two principal components namely PC1 and PC2, derived from tester-centered data, referred to as primary and secondary effects, respectively. In the line × tester data GGE biplot analysis, the "average yield" and "yield stability" are correspond to GCA and SCA of the parents, respectively (Yan et al., 2001). In other words, in the multi-environment trials data, genotypes are considered as lines and environment is considered as testers while it is reverse in the case of line × tester.

The objectives of this research were (i) to use the GGE biplot analysis for graphical interpretation of the line × tester data in canola and (ii) to determine the combining abilities of some canola parents for some important quantitative and quality traits.

2 MATERIALS AND METHODS

All the field experiments were carried out in research station of Seed and Plant Improvement Institute (SPII), Karaj, Iran in 2009 growing season. Seven winter canola lines including 'Zarfam' (L1), 'Talaye' (L2), 'SLM046' (L3), 'Geronimo' (L4), 'Modena' (L5), 'Opera' (L6) and 'Symbol' (L7), were crossed with three winter testers including 'Okapi' (T1), 'Licord' (T2), and 'Orient' (T3), based on the line × tester crossing scheme. The obtained seeds from 21 F1 hybrids and their parents were arranged and sown in a randomized complete block design with two replicates at two different planting dates: recommended planting date (29^{th} September, 2009), and late planting date (29^{th} October, 2009). Each plot consisted of four rows, 3 m long and 0.3 m apart. The distance between plants in the rows was 5 cm, providing a plant density of 240 plants per plot. The crop was grown according to local cultural practices and with uniform fertilization and plant protection treatments. At maturity stages, seed yield was determined from manually harvesting two middle rows of each plot and expressed as tons per hectare. The oil quantity (seed oil content) and quality (glucosinolate content in meal and seed) were measured using 3 g seed collected from each plot using nuclear magnetic resonance (NMR) machine (Bruker-Biospin, Karlsruhe, Germany) and gas chromatography (GC), respectively.

The normality of data were first confirmed by Anderson and Darling (1952) normality test using MINITAB version 14 (2005) statistical software. Pooled analysis of variance for combining ability was carried out according to the model presented by Elitriby et al. (1981). High-parent heterosis was estimated using mean values and t-test was applied for its significance test. The mean values for F1 hybrids and their parents were used to develop a line × tester dataset. The dataset was used to extract the first two principal components (PCs, see below). Data for the genotype's mean were subjected to line × tester analysis to estimate GCA and SCA effects using biplot analysis. And finally the following regression model (called SREG2, Yan and Kang, 2002) was developed:

$$\gamma_{ij} - \beta_j = \lambda_1 \xi_{i1} \eta_{j1} + \lambda_2 \xi_{i2} \eta_{j2} + \varepsilon_{ij}$$

where γ_{ij} is the genotypic value of the cross between line i and tester j; β_j is the mean of all crosses sharing tester j; λ_1 and λ_2 are the singular values for the first and second principal components (PCs); ξ_{i1} and ξ_{i2} are the PC1 and PC2 eigenvectors, respectively, for line i; η_{j1} and η_{j2} are the PC1 and PC2 eigenvectors, respectively, for tester j; and ε_{ij} is the model residual. All biplots were generated using the GGE biplot software package (Yan, 2001).

3 RESULTS AND DISCUSSION

According to the pooled analysis of variance, there were significant differences among environments in terms of oil yield and grain yield. However in case of the treatments, significant differences were found between parents and crosses in all traits (Table 1). The parents vs crosses, which represents average heterosis, was significant in all the traits except for oil content. Lines and line × tester were found to be significant in all the traits while testers were significant only in meal and seed glucosinolate contents. There were significant differences among treatments × environments in all the traits except for oil content. The significant interactions suggest that differences among lines, testers, and crosses are not constant from one condition to another. This type of interaction is more common and inevitable in agricultural and biological investigations (Yan & Kang, 2002).

The application of GGE biplot model demonstrated that the first two terms were relatively sufficient to explain the high proportion of variation in meal glucosinolate content in recommended and late planting dates by 80 % and 85 %, respectively. In the polygon view of the biplot, which is formed through connecting the markers of the vertex lines, the perpendicular lines were drawn from the biplot origin to each side of the polygon and divided the biplot into few sectors. In both planting dates, there were four vertex lines for meal glucosinolate content; L1, L3, L4 and L5 lines in the recommended planting and L3, L4, L5 and L6 lines in the late planting. These vertex lines identify the lines with the highest SCA for each tester. When recommended planting date was applied, the highest SCAs were related to L1 and T1, included in that sector, L4 and T2 and L5 T3 (Fig. 1A). In case of late planting date, the highest SCAs were related to L6 and T1, included in that sector, L3 and T2 and L5 and T3 (Fig. 1B). The significant interaction between genotype and environment is a common result in studies like this project, however, this result was not found between L5 and T3. Since SCA provides an estimate for non-additive gene action (Sprague & Tatum, 1942), these results were in agreement with those of Rameah et al. (2003), who stated that most of the total genetic variations in meal glucosinolate content in canola were due to non-additive gene effects.

Table 1: Combined analysis of variance for studied traits in rapeseed

Source of variations	df	Meal glucosinolate content (μmol gr^{-1} $meal^{-1}$)	Seed glucosinolate content (μmol $gr^{-1}seed^{-1}$)	Oil content (%)	Grain yield (t ha^{-1})
Environment (E)	1	13.86^{ns}	32.73^{ns}	0.15^{ns}	55.15^{**}
Replications/E	2	27.26	48.25	11.79	2.908
Treatments (G)	30	155.60^{**}	201.81^{**}	5.01^{**}	0.544^{**}
Parents (P)	9	23.40^{**}	36.40^{**}	2.81^{*}	0.554^{**}
P vs. Crosses (C)	1	169.65^{**}	169.21^{**}	1.25^{ns}	1.997^{**}
Crosses (C)	20	214.38^{**}	277.88^{**}	6.19^{**}	0.467^{**}
Lines (L)	6	156.34^{**}	145.49^{**}	5.48^{**}	0.707^{**}
Testers (T)	2	85.88^{**}	126.16^{**}	1.92^{ns}	0.081^{ns}
L× T	12	264.82^{**}	369.36^{**}	7.25^{**}	0.412^{**}
G× E	30	7.92^{**}	7.75^{*}	1.20^{ns}	0.436^{**}
P× E	9	2.30^{ns}	3.99^{ns}	0.53^{ns}	0.261^{*}
(P vs. C) × E	1	4.61^{ns}	7.26^{ns}	1.68^{ns}	0.195^{ns}
C × E	20	10.61^{**}	9.46^{**}	1.48^{ns}	0.527^{**}
L × E	6	9.86^{**}	7.07^{ns}	1.93^{ns}	0.704^{**}
T × E	2	13.99^{**}	15.29^{*}	3.27^{ns}	0.039^{ns}
L × T × E	12	10.42^{**}	9.69^{*}	0.96^{ns}	0.520^{**}
Error	60	2.49	4.26	1.31	0.113

**, * and ns, stands for significant at the 1 % and 5 % probability level and non-significant respectively

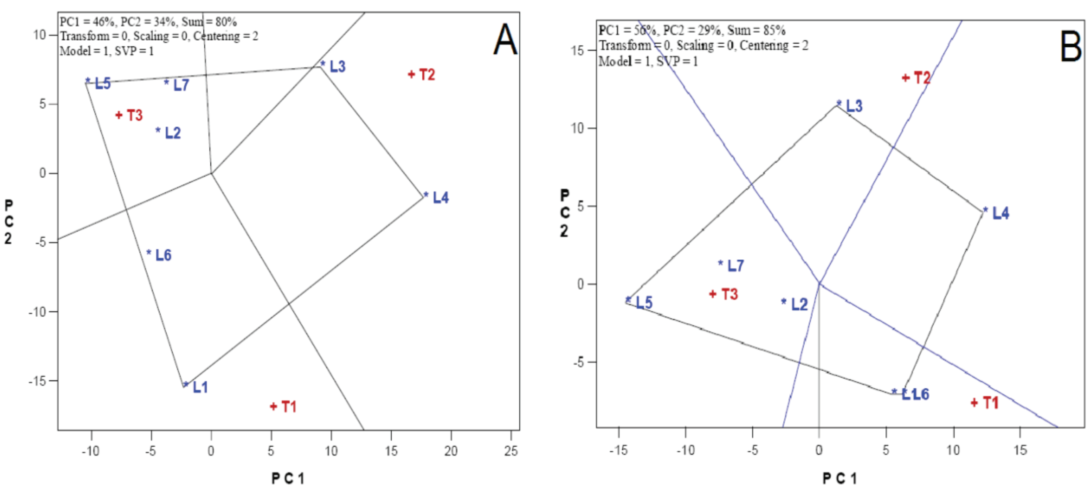

Figure 1: Polygon view of GGE biplot based on line×tester data of rapeseed for meal glucosinolate content in normal (A) and late planting (B) dates

As can be seen from Fig. 1A, there is high negative GCA effect for L5 line, whereas L3 and L4 showed positive GCA effects for meal glucosinolate content in recommended planting (RP) date. The L2, L6 and L7 lines appeared to have slightly negative GCA effects, and L1 line showed no GCA effect for meal glucosinolate content in recommended planting (RP) date (Fig. 1A). From Fig. 1B, L5 line showed high negative GCA effect, whereas L4 line showed positive GCA effects for meal glucosinolate content in late planting date. Moreover, L7 line indicated moderate, negative GCA effect. In addition, L1 and L6 lines showed moderate and positive GCA effects. The L2 and L3 lines had near zero GCA effect for meal glucosinolate content in late planting date (Fig. 1B). Thus, it appears that L5 line could be considered as superior combiner to develop genotypes with low meal glucosinolate content. Considering that GCA provides an estimate for additive gene actions (Griffing, 1956), the current results were in agreement with those of Marjanovic et al. (2007), who stated that most of the total genetic variability in meal glucosinolate content in canola were due to additive gene effects.

Biplot analysis was used to identify four distinct heterotic groups or sectors in both planting dates. Lines of each sector are suitable candidates for reaching high negative heterosis to produce genotypes with low glucosinolate content. This can be identified through original heterotic data (Table 2). The highest estimated negative heterosis for meal glucosinolate content was observed in combinations of T1 × L3, T1 × L7, T2 × L1, T2 × L6, T3 × L3 and T3 × L6, which were located in the distinct sectors in Fig. 1A and Fig. 1B. It is clear that polygon view of the biplot is a beneficial tool for detecting heterosis patterns in canola breeding materials.

The GGE biplot model described 83 and 88 % of total variation in seed glucosinolate content in both planting dates, respectively. In recommended planting date, there were four vertex lines including L1, L3, L4 and L5; and in the late planting date, there were five vertex lines including L1, L3, L4, L5 and L6. Therefore, the highest SCAs were related to L1 and T1, L3 and T2, and L5 and T3 in recommended planting date (Fig. 2A). Regarding late planting date, the highest SCAs were related to L3 and T2, included in that sector; L5 and T3 and L6 and T1 (Fig. 2B). Only T3 tester had different lines across two planting dates and the other testers did not indicate GE interaction.

In recommended planting date, the L1 and L6 lines showed high negative GCA effects, whereas L3 line showed positive GCA effect for seed glucosinolate content (Fig. 2A). In addition, in recommended planting date, moderate and positive GCA effects were found in L4 and L7 lines whereas, L2 and L5 lines showed no GCA effect for seed glucosinolate content (Fig. 2A). According to Fig. 2B, when it comes to late planting date, L1 and L6 lines showed high negative GCA effects, whereas L5 line showed positive GCA effects for seed glucosinolate content. Furthermore, L3 and L7 lines showed moderate and positive GCA effects, and L2 and L4 lines showed near zero GCA effect for seed glucosinolate content in late planting (Fig. 2B). Therefore, it appears that L1 and L6 lines could be considered as the good combiners to develop genotypes with low seed glucosinolate content. Similar to meal glucosinolate content, different heterotic groups can be identified from seed glucosinolate content biplots. There were four distinct groups in both planting dates. The highest negative heterosis for seed glucosinolate content was observed when T1×L3, T2×L1, T2×L6, T3×L3, T3×L6 and T3×L7 were combined (Table 2). The results are located in the distinct sectors in Fig. 2A and Fig. 2B.

Table 2: Estimates of high-parent heterosis in a line × tester experiments of rapeseed across two planting dates

	Meal glucosinolate content		Seed glucosinolate content		Oil content		Grain yield	
	NP	LP	NP	LP	NP	LP	NP	LP
T1× L1	195.1^{**}	74.0^{**}	150.3^{**}	102.4^{**}	-3.1^{**}	-4.6^{**}	21.4^{**}	-20.5^{**}
T1× L2	-31.0^{**}	18.5^{**}	-31.0^{**}	18.5^{**}	0.0^{ns}	-0.5^{ns}	13.6^{**}	12.3^{**}
T1× L3	-51.3^{**}	-55.6^{**}	-54.0^{**}	-55.6^{**}	-1.4^{ns}	-1.7^{ns}	-16.4^{**}	33.6^{**}
T1× L4	251.7^{**}	302.2^{**}	215.7^{**}	139.8^{**}	-4.4^{**}	-2.0^{ns}	-21.8^{**}	-5.9^{**}
T1× L5	31.1^{**}	-22.1^{**}	30.5^{**}	-22.0^{**}	1.1^{ns}	-2.2^{ns}	1.8^{**}	5.8^{**}
T1× L6	78.8^{**}	74.4^{**}	136.2^{**}	71.2^{**}	-1.8^{ns}	-2.5^{ns}	23.9^{**}	9.1^{**}
T1× L7	-64.1^{**}	-39.8^{**}	-64.1^{**}	-39.8^{**}	-0.9^{ns}	-0.4^{ns}	26.7^{**}	34.1^{**}
T2× L1	-55.9^{**}	-57.0^{**}	-55.9^{**}	-50.0^{**}	0.8^{ns}	1.4^{ns}	2.8^{**}	-13.1^{**}
T2× L2	-21.2^{**}	-33.4^{**}	-21.2^{**}	-40.3^{**}	-1.3^{ns}	1.7^{ns}	-23.0^{**}	-18.4^{**}
T2× L3	73.5^{**}	63.1^{**}	64.2^{**}	63.0^{**}	-0.8^{ns}	-2.3^{ns}	8.7^{**}	-0.2^{ns}
T2× L4	268.2^{**}	189.4^{**}	198.7^{**}	108.7^{**}	-4.7^{**}	-2.0^{ns}	-26.0^{**}	-3.6^{**}
T2× L5	14.6^{**}	-43.6^{**}	14.6^{**}	-13.4^{**}	1.9^{ns}	2.1^{ns}	-5.3^{**}	-23.6^{**}
T2× L6	-58.5^{**}	-56.6^{**}	-58.5^{**}	-56.6^{**}	5.2^{**}	4.7^{**}	-6.5^{**}	-13.3^{**}
T2× L7	-4.3^{ns}	-16.7^{**}	8.2^{**}	-1.4^{ns}	-0.4^{ns}	-4.2^{**}	42.4^{**}	-20.8^{**}
T3× L1	-26.3^{**}	-53.5^{**}	-26.2^{**}	-45.9^{**}	2.1^{*}	-0.7^{ns}	-11.5^{**}	-15.9^{**}
T3× L2	-46.0^{**}	-29.6^{**}	-43.9^{**}	-40.9^{**}	1.4^{ns}	-2.5^{ns}	28.8^{**}	28.9^{**}
T3× L3	-75.2^{**}	-64.8^{**}	-76.5^{**}	-57.8^{**}	3.3^{**}	2.3^{ns}	1.0^{*}	21.9^{**}
T3× L4	-22.3^{**}	5.0^{*}	-22.4^{**}	-11.9^{**}	0.4^{ns}	-2.9^{*}	-23.6^{**}	-2.6^{**}
T3× L5	338.4^{**}	248.1^{**}	270.4^{**}	192.3^{**}	-5.6^{**}	-9.7^{**}	15.8^{**}	1.4^{**}
T3× L6	-23.5^{**}	-63.3^{**}	-27.8^{**}	-63.3^{**}	4.1^{**}	6.8^{**}	38.9^{**}	5.8^{**}
T3× L7	-23.2^{**}	47.0^{**}	-23.2^{**}	7.2^{**}	4.8^{**}	0.1^{ns}	14.9^{**}	4.7^{**}
SE	1.67	1.48	2.05	2.08	1.01	1.27	0.40	0.26

NP: Normal planting; LP: Late planting
$^{**, *}$ and ^{ns}, stands for significant at the 1 % and 5 % probability level and non-significant respectively

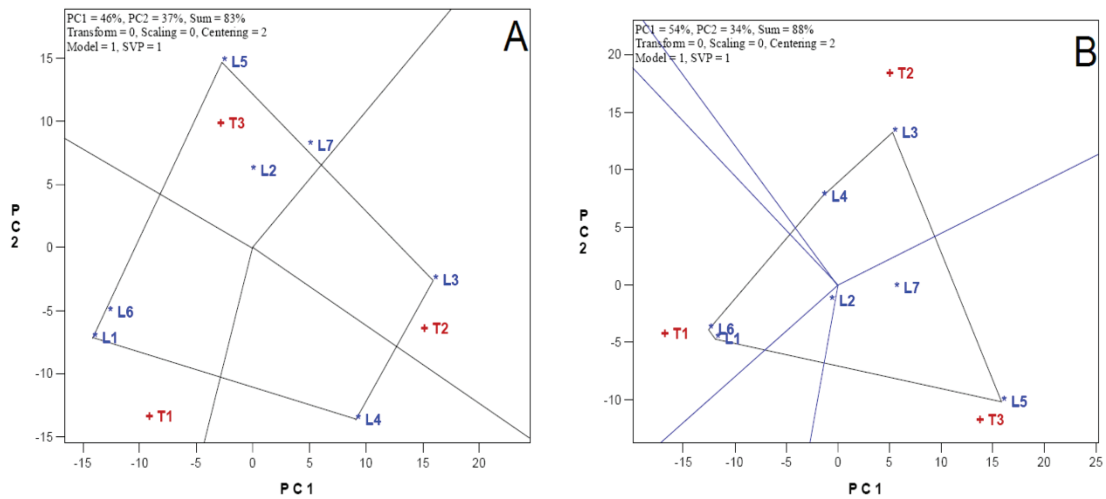

Figure 2: Polygon view of GGE biplot based on line×tester data of rapeseed for seed glucosinolates content in normal (A) and late planting (B) dates

According to the oil content GGE biplot model, the first two components explained 90 and 89 % of total variation, thus it seems to be sufficient as reducing the data dimensions. In the polygon view of the biplot there were four vertex lines in both planting dates, L4, L5, L6 and L7 lines in recommended planting date and L3, L5, L6 and L7 lines in late planting date. These vertex lines identify the lines with the highest SCA for each tester. In recommended planting date, the highest SCAs were related to L5 and T1, included in that sector, and T2 and L6 and T3 (Fig. 3A). In late planting date, the highest SCAs were related to L5 and T1, included in that sector; L6 and T2 and L6 and T3 (Fig. 3B). It is clear that there is G × E interaction in combination of each line with T2 tester for oil content (Fig. 3). Similar results emphasizing the importance of SCA effects in canola have already been reported by Marinković and Marjanović-Jeromela (2004) and Sabaghnia et al. (2010).

In recommended planting date, L5 line showed high positive GCA effect, whereas L4 line showed negative GCA effect for oil percentage. In addition, in the same planting date, L6 line indicated moderate and positive GCA effect, L7 line showed moderate and negative GCA effect, and L1, L2 and L3 lines revealed near zero GCA effect for oil percentage (Fig. 3A). Fig. 3B indicates that L6 line has high positive GCA effect, whereas L5 and L7 lines have negative GCA effect for oil percentage in late planting date. The results indicated that L1 and L3 lines have moderate and positive GCA effect, L2 line has moderate and negative GCA effect, and L4 line has no GCA effect for oil percentage in late planting date (Fig. 3B). Therefore, it seems that L6 line can be considered as an appropriate combiner for improving of oil percentage in canola seeds. A significant relationship between the GCA and hybrid performance in terms of oil content in canola has been reported by Qian et al. (2007), indicating possibility of hybrid performance prediction by GCA. Therefore, it appears that some parents, such as, L5 and L6 are able to produce hybrids with higher oil content. Different heterotic groups could be identified using biplot polygon. Lines located in each sector are known as appropriate candidates to be crossed with related testers to achieve high positive heterosis and high oil percentage in the seeds. According to table 2, the highest positive heterosis appeared in combinations of T2 × L6, T3 × L6 and T3 × L7 (Fig. 3A), and combinations of T2 × L6 and T3 × L6 (Fig. 3B), which were located in the same sectors in the biplots.

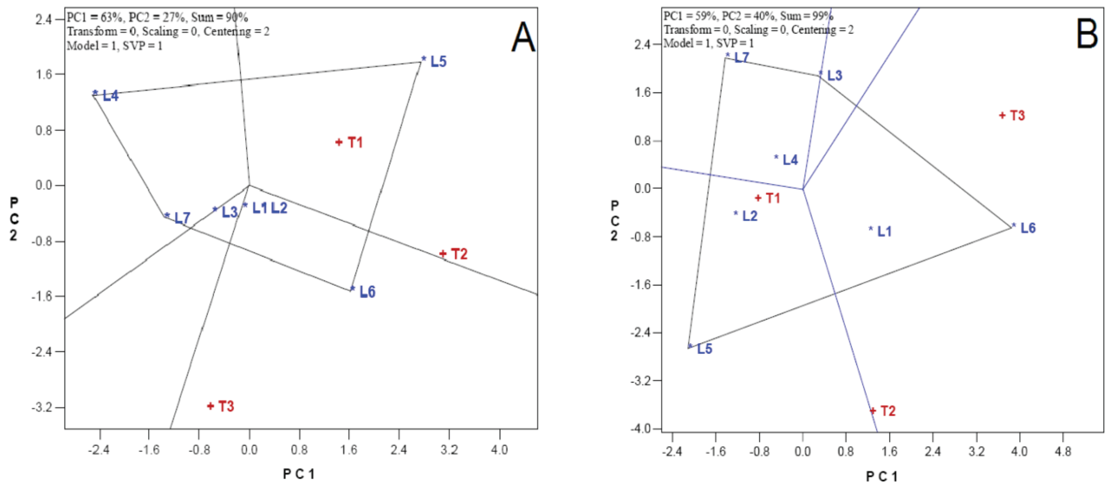

Figure 3: Polygon view of GGE biplot based on line×tester data of rapeseed for oil percent in normal (A) and late planting (B) dates

The application of GGE biplot model revealed that the first two components were relatively sufficient and explained 88 and 91 % of total variation in grain yield data for both planting dates. In the polygon view of the biplot, there were five vertex lines (L1, L2, L3, L4 and L7 lines) for grain yield in recommended planting date and six lines (L1, L2, L3, L4, L5 and L7) in late planting date. In late planting, L1 line showed the highest SCA with T1 tester, which were located in the same sector; L7 line showed the highest SCA with T2 tester and L3 line showed the highest SCA with T3 tester (Fig. 4A). In late planting date, L7 line indicated the highest SCA with T1 tester included in that sector; L2 line showed the highest SCA with T2 tester and L3 line showed the highest SCA with T3 tester (Fig. 4B). It is clear that there is G × E interaction in this dataset, nevertheless, this interaction was not observed in combination of L3 and T3 (Fig. 4).

From the results, the L3 and L7 lines showed high positive GCA effect, whereas L2 and L4 line indicated negative GCA effect in terms of grain yield in recommended planting date. In addition, L1 Line showed moderate and positive GCA effect, and L5 and L6 lines showed moderate and negative GCA effects in terms of grain yield in recommended planting date (Fig. 4A). Fig. 5B indicates that L7 line has high positive GCA effect; however L1 and L4 lines have negative GCA effect for grain yield in late planting date. Moreover, L3 and L5 lines indicated moderate and positive GCA effect, and L2 line showed moderate and negative GCA effect. Furthermore, L6 line showed near zero GCA effect for grain yield in late planting date (Fig. 4B). Therefore, it seems that L7 line can be used as the promising combiner for improving high grain yield genotypes. These findings for GCA effects for grain yield are in agreement with Thakur and Sagwal (1997) who reported similar GCA effects for grain yield.

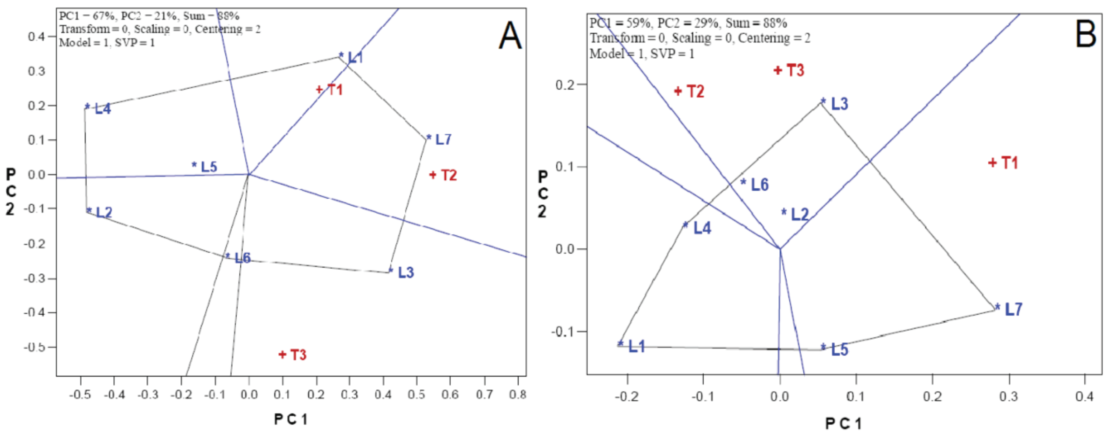

Figure 4: Polygon view of GGE biplot based on line×tester data of oilseed rape for grain yield in normal (A) and late planting (B) dates

The lines located in each sector are known as high potential candidates to be crossed with correspondent testers to gain high positive heterosis in breeding program to produce high oil yield genotypes. The highest positive heterosis values (Table 2) were observed in combinations of T1×L1, T1×L6, T1×L7, T2×L7, T3×L2 and T3×L6, as indicated in Fig. 4A, and in combinations of T1×L3, T1×L7, T3×L2 and T3×L3 in Fig. 3B, which were located in the same sectors in the biplots. Multivariate statistical methods such as GGE biplot have been introduced to explore the multi-directional aspects of data and to extract more information from interaction components. The GGE facilitates clustering of lines and testers into more cohesive groups based on the biological understanding of the L × T interaction. Identifying superior lines with favorable GCA and SCA effects is another importance of the method (Yan & Hunt, 2002). Sub-grouping of testers by the GGE method would be useful for selecting lines, because it helps researchers to exploit the interactions among lines and the subsets of testers (Yan & Kang, 2002). Application of GGE biplot is preferred for several reasons: (i), biplots are easy to use and to interpret, (ii), knowing GCA, SCA, and identifying heterotic groups may enhance our understanding to discover important patterns within the data and (iii), other information such as the best mating partner for each parent and groups of similar parents can be graphically visualized to rapid identification of suitable parents and crosses for further investigation.

4 CONCLUSIONS

Finally, it can be concluded that, to release low seed or meal glucosinolate genotypes, combinations of T1 × L1, T1 × L6 and T3 × L5 should be taken into account as preferential materials. Moreover, combinations of T1×L7 and T2×L7 to release high grain yield genotypes and combinations of T1 × L5, T2 × L5 and T3 × L6 to increase oil content in genotypes are suggested. With a few exceptions, all the studies showed presence of significant GCA and SCA effects for grain yield and oil content, indicating that both additive and non-additive gene action are involved in the inheritance of these characteristics. The current results also revealed that most of the characteristics were controlled by both additive and non-additive of gene action so that selection in promising hybrids could be used in hybrid rapeseed development with high oil yield. Moreover, simple phenotypic selection in high performance hybrids can also be effective in segregating generations. The presence of heterosis for the traits of interest indicates existence of the genetic potential for increasing grain yield by identifying heterotic groups and evaluating parents for their combining ability. Finally, the results indicated that both oil content and grain yield in canola, as the most important parameters for industrial use, can be improved.

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Salt overly sensitive 1 (*SOS1*) gene expression can be regulated via *Azospirillum brasilense* Sp7 in wheat seedlings under saline condition

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ABSTRACT

Salinity stress reduces plant growth via failure of physiological processes mainly due to the abundance of Na^+ ion. Salt overly sensitive (*SOS*) signaling pathway is considered as an important component of Na^+/K^+ homeostasis system in plants, especially under saline condition. Moreover, it is reported that wheat-*Azospirillum* associated has resulted in an enhanced salinity tolerance. To evaluate involvement of *Azospirillum* species in regulation of *SOS* signaling pathway, inoculated and none-inoculated wheat seedlings with *Azospirillum brasilense* Sp7 were grown for five days. Then uniform seedlings were transferred into saline hydroponic media with and without 200 mM NaCl. The relative expression of *TaSOS1* of root, sheath, and blade as well as Na^+/K^+ ratio was measured after 6, 24 and 48 hours since inoculated and non-inoculated seedling were transferred to NaCl media. Simultaneously Ca, Fe, proline content, root and shoot dry mass and soluble sugars were measured at 72 hour after application of NaCl. Result showed that salinity increased *TaSOS1* gene expression, Na^+, prolin and Na^+/K^+ ratio but Ca and Fe were decreased in root and shoot of wheat seedlings. Although *A. brasilense* Sp7 could improve salinity tolerance in wheat via reduction of Na uptake and upregulation of *TaSOS1* expression, but do not have any effect in sodium distribution within plant parts. Therefore, salinity could increase *TaSOS1* expression in the root, sheath and blade and *A. brasilense* Sp7 also could reduce the adverse effect of salinity via addition of over expression of *TaSOS1*.

Key words: *Azospirillum*; wheat; salinity; *TaSOS1*; Na^+/ K^+ ratio

IZVLEČEK

IZRAŽANJE NA SOL PREOBČUTLJIVEGA GENA (*SOS1*) BI PRI PŠENICI V RAZMERAH SLANOSTI LAHKO URAVNAVALI S SEVOM BAKTERIJE *Azospirillum brasilense* Sp7

Slanostni stres zmanjšuje rast rastlin preko odpovedi fizioloških procesov v glavnem zaradi Na^+ iona. Preobčutljiva (*SOS*) solna signalna pot predstavlja najvažnejši del Na^+/K^+ homeostaznega sistema v rastlinah v razmerah slanosti. Še več, poroča se, da je povezava pšenice z bakterijo iz rodu *Azospirillum* rezultirala v povečani odpornosti na slanost. Za ovrednotenje vloge bakterije iz rodu *Azospirillum* pri uravnavanju *SOS* signalne poti so bile gojene z bakterijo *Azospirillum brasilense* Sp7 inokulirane in neinokulirane sejanke pšenice za pet dni. Izenačene sejanke so bile prenešene v hidroponski medij z ali brez 200 mM NaCl. V presledkih 6, 24 in 48 ur po prenosu sejank v slan medij je bila merjena relativna ekspresija gena *TaSOS1* v koreninah, listni nožnici in listni ploskvi in Na^+/K^+ razmerje. Hkrati je bila 72 ur po prenosu v slan medij izmerjena vsebnost Ca, Fe in prolina, suha masa pogankov in vsebnost topnih sladkorjev v istih delih sejank. Rezultati so pokazali, da je slanost povečala ekspresijo gena *TaSOS1*, vsebnost Na^+, prolina in povečala količnik Na^+/K^+, a zmanjšala vsebnost Ca in Fe v koreninah in poganjkih sejank. Čeprav lahko bakterija *A. brasilense* Sp7 izboljša toleranco pšenice na slanost z zmanjšanjem privzema Na in povečano ekspresijo gena *TaSOS1* pa nima nobenega učinka na razporeditev natrija v rastlini. Slanost torej lahko poveča ekspresijo gena *TaSOS1* v koreninah in listih in inokulacija z bakterijo *A. brasilense* Sp7 lahko zmanjša škodljive učinke slanosti preko dodatnega vpliva na povečano ekspresijo *TaSOS1*.

Ključne besede: *Azospirillum*; pšenica; slanost; *TaSOS1*; Na^+/ K^+ razmerje

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1 INTRODUCTION

Wheat like some other crops cannot tolerate salinity (Tiwari et al., 2010). Salinity has inhibitory effects on wheat growth indexes such as root growth (Neumann, 1995), root/shoot ratio (El-Hendawy et al., 2005), and total dry matter (Pessarakli & Huber, 1991). The saline condition caused an increase in sodium in the root and shoot of plants (Xue et al., 2004), some osmoticum components such as proline, soluble sugars (Hamdia et al., 2004), as well as none-enzymatic (Norastehnia et al., 2014; Amini et. Al., 2017) and enzymatic antioxidant (Baniaghil et al., 2013). In contrast, seedling's dry mass and K^+ content in the roots and shoots of wheat cultivars (Akbarimoghaddam et al., 2011; Lekshmy et al., 2013) and maize (Turan, 2008) decreased by increasing salinity.

It has been reported that plant growth promotion rhizobacteria (PGPRs) could induce more mineral uptake (Askary et al., 2009), phytohormones production (Kang et al. 2014) and consequently better tolerance to abiotic stresses (Vargas et al., 2012). El-Dengawy et al. (2011) reported that inoculation of Carob seedlings (*Ceratonia siliqua* L.) with *Azospirillium lipoferum* (Beijerinck) Tarrand et al. (ATCC® 29707™) under saline condition could improve seedling growth rate, K^+/Na^+ ratio and root characters. In addition, Upadhyay et al. (2012) reported that when wheat co-inoculated with two PGPR strains (*Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 SU47 and *Arthrobacter* sp. SU18) were grown under different salinity conditions (2–6 dS m^{-1}), an increase in dry biomass, total soluble sugars and proline were observed, meanwhile sodium of wheat leaves was reduced in co-inoculated plants under saline conditions.

Creus et al. (1997) also confirmed the reduction of adverse effects of salinity and osmotic stresses on height and dry mass of wheat plants when inoculation with *Azospirillium brasilense* Tarrand, Krieg & Döbereiner, 1978. Vargas et al. (2012) also showed the upregulation of ethylene receptors genes expression in rice plants upregulated due to the inoculation with *A. brasilense* Sp245 as well as an increase in transcripts of some genes involved in nutrition uptake in response to *A. brasilense*. *A. brasilense* could change the pH of soil via proton efflux and increase availably of plant nutrients in wheat cultivars (Amooaghaie et al., 2002). Meanwhile, change in character of rhizosphere due to PGPRs activities and vis versa change in qurom sensing of bacteria due to the change of rhizosphere is a coincide fenomena which affect plant growth and development in stress condition (Pakdaman et al., 2014).

Among the salt overly sensitive (*SOS*) genes, SOS1 is a Na^+/H^+ antiporter located in the cell membrane. Its role has already been demonstrated in cytosolic Na^+ homeostasis by different researchers (Ramezani et al., 2013; Sathee et al., 2015; Shi et al., 2002; Shi & Zhu, 2002; Yadav et al., 2012). It has been reported that *TaSOS1* experssion was up-regulated in the root and the shoot of wheat seedlings under saline condition. For example, Yadav et al. (2012) showed that over expression of *SOS1* gene in transgenic tobacco caused a higher salt tolerance, elevated seed germination, addition of root and shoot length, less dry mass reduction, higher K^+/Na^+ ratio and more sugars relative to wild-type plants. It has been suggested that the SOS signaling pathway has an important role in ion (Na^+ and K^+) homeostasis and salt tolerance under saline condition.

For instance, *TaSOS1* expression in transgenic *Saccharomyces cerevisiae* Meyen *ex* E.C. Hansen (which was already salt sensitive and had high cellular Na^+ content) caused reduction of Na^+ and addition of K^+ of cells (Xu et al., 2008). In addition, Feki et al. (2014) indicated that the *Arabidopsis sos1-1* mutant is hypersensitive to both Na^+ and Li^+ ions, but its hypersensitivity would have disappeared using *TaSOS1* gene. At this condition, better germination and more robust seedling growth, greater water retention capacity, retained low Na^+ and high K^+ in their shoots and roots were observed in nutrient solution containing Na^+ and Li^+ salts. Their work and complementary studies revealed that *TaSOS1* upregulated the ion homeostasis and helped in salinity tolerance (Ramezani et al., 2013).

It has been reported that PGPRs such as *Azospirillum* species can improve wheat growth and productivity under salinity condition (Upadhyay et al., 2011) and co-inoculation of *A. brasilense* and *Rhizobium meliloti* (Dangeard 1926) De Lajudie *et al*. 1994, comb. nov. in different wheat cultivar also showed an enhancement of root colonization and nitrogenize activity (Askary et al., 2008). Help of PGPRs in saline condition in one hand, and increasing 10 % annually saline areas due to the low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor cultivation in the other hand, create needs for more research in dual effects of salinity and PGPRs.

It is known that *TaSOS1* expression is up-regulated in salt tolerant wheat cultivars (Ramezani et al. 2013) and *A. brasilense* also increased tolerance to salinity. However, it is not known that *Azospirillum* species increase salinity tolerance via up or down regulation of *TaSOS1* gene in wheat seedlings under salinity condition or this phenomena has been done by different process. Therefore, the aim of this study was to evaluate *SOS1* gene expression, Na, K, Ca and Fe uptake, sugar and proline content and biomass production of root, sheath and blade of wheat cultivars inoculated with *A. brasilense* Sp7 under salinity condition to know more about the effect of *A. brasilense* Sp7 and *TaSOS1* involvment.

2 MATERIALS AND METHODS

2.1 Preparation of inoculant and seeding

Standard strain of *A. brasilense* Sp7 was obtained from NCIMB Company, in Germany and then cultured in an NFb liquid medium supplemented with NH_4Cl (0.25 g l^{-1}) at 30 °C (Baldani & Döbereiner, 1980) in Erlenmeyer flasks for 48 h using a rotary shaker at 200 rpm (logarithmic phase). A high density bacterial culture was obtained by centrifuging at 1000 g for 10 min and was then washed with sterile saline phosphate buffer. Finally, desired concentration (10^7 CFU ml^{-1} of *A. brasilense* Sp7) was prepared from the media.

Seeds of a winter semitolerant wheat cultivar named Sardary (*Triticum aestivum*'Sardari') were obtained from Institute of Agricultural and Research of Isfahan in Iran. Seeds were surface sterilized by dipping in 95 % ethanol for 2 min and then in 1 % sodium hypochlorite (NaOCl) for 1 min followed by six washes in sterile distilled water (Öğüt et al., 2005). Then, sterilized seeds were transferred to autoclaved water agar medium and were kept at 25 ℃ for germination. After 24 hour, uniform germinated seeds were divided into two groups. The first group was inoculated by submerging the germinated seeds in the solution containing 10^7 CFU ml^{-1} of *A. brasilense* Sp7 and the second group treated without bacteria as control. To verify the inoculation success, the root segments stained with tetrazolium chloride dye and also cross section of inoculated root were prepared. After 3 hour, all seedlings (inoculated and none inoculated) were transferred into pots containing sterile perlite and then irrigated with 1/4 strength of Hoagland's nutrient solution (Hoagland & Arnon, 1950). The pots were kept for 5 days in a glasshouse under photoperiod 16/8 h (light/dark) at 25±2 °C. Then, 200 mM of NaCl (as one dose) was added as salinity treatment into only half of the plants in each group via irrigation water to have inoculated and non-inoculated plants under salinity and optimum saline condition. This experiment was conducted in randomized block design with three replicates.

Samples of roots, sheaths and leaves were collected at 6, 24 and 48 h after salinity applied. Some of the collected plant samples were used for Na and K analysis and some other immediately frozen in liquid nitrogen for Real-time quantitative PCR. Simultaneously, some of the plants was allowed to growth up to 72 hours and then their plant parts were collected for Ca, Fe, dry mass, soluble sugar, proline and root and shoot length analysis.

2.2 Real-time quantitative PCR

The total RNA was isolated from frozen roots, sheaths and leaves using Iraizol reagent (RNA biotech, Iran). After RNA extraction, samples were treated with DNase. Then, the first stranded cDNA was synthesized using the M-MLV reverse transcriptase (Fermatas). Real-time PCR was performed in triplicate using SYBER Green Master Mix (RNA Biotech, Iran). Gene-specific primers were designed for an 110 bp fragment of *TaSOS1* (Gen Bank Accession No. AY326952). The primer pair was 5'-GGGATGATGAGGAACTTGGG-3' in sense direction and 5'-CTTGTCAGGAACATCGTGGG-3' in anti-sense direction. The primer pair for the housekeeping gene, actin, (Gen Bank Accession No. GI:48927617) was 5'-GTTCCAATCTATGAGGGATACACGC-3' in sense direction and 5'-GAACCTCCACTGAGAACAACATTACC-3' in anti-sense direction with an amplification length of 422 bp (Xu et al., 2008). The PCR conditions were 94 °C for 4 min followed by 40 cycles of 94 °C for 10 s, 62 °C for 40 s, 72 °C for 60 s, followed by 7 min at 72 °C. Serial dilutions of cDNA were used to obtain optimized standard curve amplification efficiency and the best cDNA concentration for real-time PCR was obtained. The relative expression ratio of target and reference genes were calculated based on its real time efficiencies (E) and crossing point difference (ΔCp) of sample versus control as well as reference versus control, respectively (Pfaffl, 2001).

2.3 Dry mass, Na^+ and K^+ determination

Each plant part (root, sheath and blade) was weighted separately and then 100 mg of dried mass of each sample was digested with 10 ml 3 % (w/v) aqueous sulfosalicylic acid for 24 hours. Extracted samples were filtered with Whatman No. 1 filter paper. Then, Na^+ and K^+ concentrations were measured using flame photometric (Perkin-Elmer Coleman 51-ca), using related standard curves for sodium and potassium.

2.4 Determination of soluble sugar content

Soluble sugar content was determined using phenol-sulphuric method (Dubois et al., 1956). To do so, 0.01 g of dried plant sample was extracted in distilled water and centrifuged at 3000 rpm for 10 min. The extract (0.5 ml) was treated with 0.5 ml phenol (5 %) and 2.5 ml pure sulphuric acid and then after a mild vortex, their absorbance was measured at 490 nm using Shimadzu double beam UV-visible Spectrophotometer.

2.5 Determination of Ca and iron content

Dry mass (100 mg) of each plant sample (roots and shoots separately) was digested in 3 ml of a 1- 4 (v/v) mixture of 37 % (v/v) HCl and 65 % (v/v) HNO3 in Teflon cylinders for 7 h at 140 °C. After adjustment of volume to 10 ml with deionized water, Ca and Fe was determined using an atomic absorption spectrophotometer (AAS, Shimadzu model 6200).

2.6 Determination of proline content

Roots and shoots proline were determined using Bates et al. (1973) method. 100 mg of fresh plant samples was homogenized with 4 ml sulfosalicylic acid (3.0 %) in a mortar. The suspension was centrifuged at room temperature at 3000 rpm for 5 min. The supernatant was mixed well with 4 ml acidic ninhydrin reagent and the reaction mixture was vortexed and the content was placed in a boiling water bath for 60 min. Then, the content was cooled in the ice bath and the mixture was extracted with 4 ml of toluene. The light absorbance of toluene layer was recorded at 520 nm using Shimadzu spectrophotometer (Shimadzu UV-160, Japan) and the concentration of unknown samples was calculated using respected standard curve.

2.7 Statistical analysis

The experimental design was completely randomized design with 3 replicates and MSTAT-C software was used for ANOVA. Duncan multiple range test was used (at 5 % level of significance) to compare the mean values of measured indexes. Excel was used to draw the necessary graphs.

3 RESULTS AND DISCUSSION

3.1 Growth, Ca, Fe, and soluble sugars

The results of length, dry mass and soluble sugars of roots and shoots of inoculated and non-inoculated wheat plants under saline and non-saline conditions are presented in Fig. 1. Root length of 8 days old plants was increased significantly in response to inoculation under saline and non-saline conditions. Simultaneously, shoot length was increased significantly under non-saline condition, while no significant difference was seen between inoculated and non-inoculated plant's shoot length under saline condition.

Root dry mass of inoculated plants was increased significantly under saline (25.8 %) and non-saline conditions (30.1 %) in comparison to control plants (non-inoculated and non-saline condition). However, shoot dry mass of inoculated plants did not improve either under saline or non-saline conditions in a short period of time. In contrast, root and shoot soluble sugars were affected by saline condition and shoot soluble sugars in inoculated (16.7 %) and non-inoculated (13.2 %) plants were increased significantly under saline condition.

Calcium concentration in the roots and shoots of inoculated plants not exposed to salinity was increased by 14.1 and 10.63 %, respectively, when compared to control plants (Fig 2). Salinity caused a significant reduction in Ca content of both roots (38.7 %) and shoots (31.26 %) of seedlings. However, inoculation improved Ca concentration in the root and shoot but its amount was still less than the control plants.

In inoculated seedlings, the amount of Fe in the roots and shoots (Fig 2) was the highest (0.51 and 0.21 mg g^{-1} DM, respectively) meanwhile, a significant reduction was observed in Fe content of the roots (23.68 %) and the shoots (23.68 %) of plants treated NaCl. Inoculation couldn't help the roots to uptake more Fe under salinity condition.

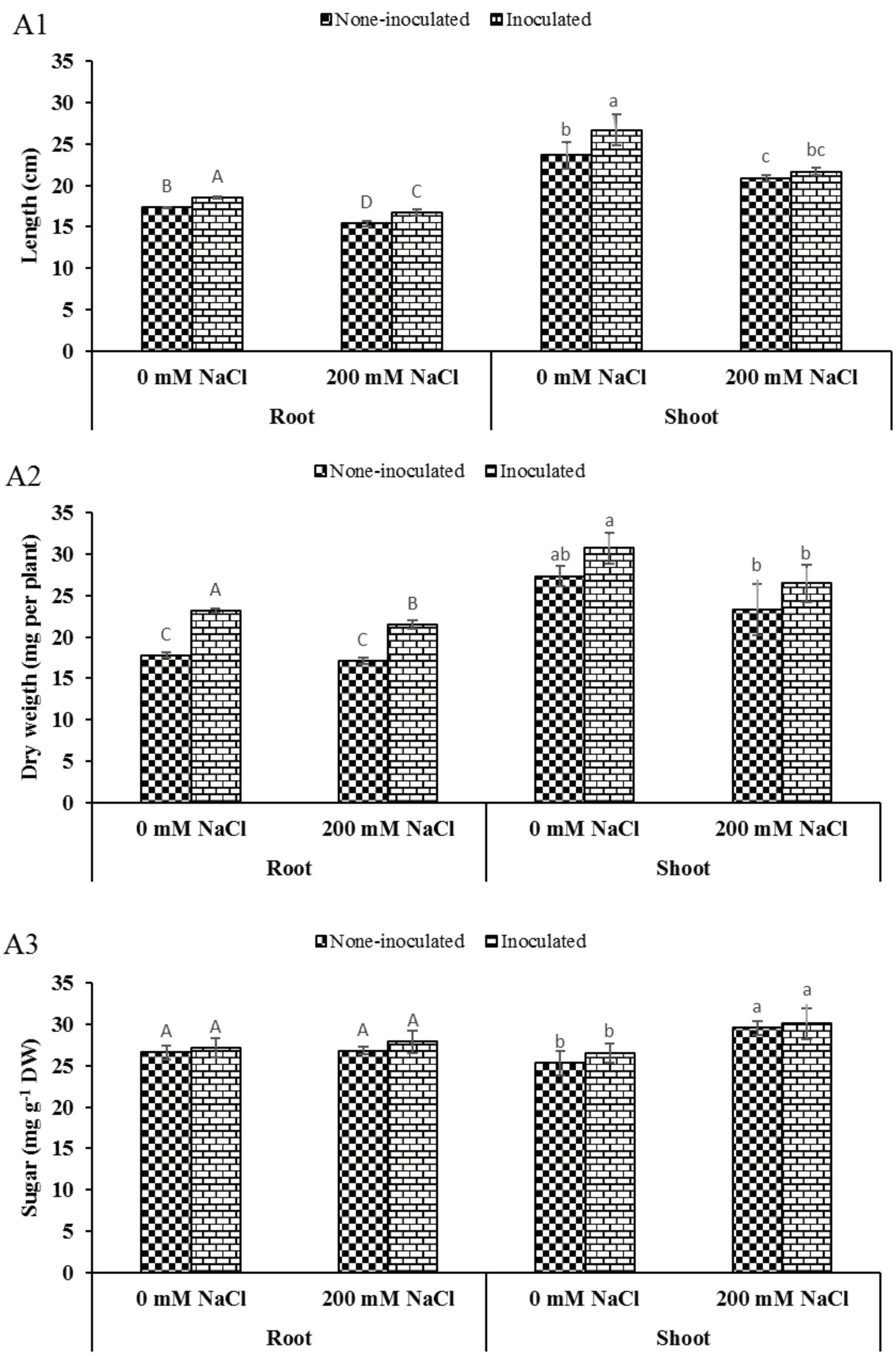

Figure 1: Length (A1); dry mass, DM (A2) and soluble sugars (A3) of root and shoot of wheat plants ('Sardari') in non-inoculated and inoculated with *A. brasilense* Sp7 (10^7 CFU ml^{-1}) grown in saline (200 mmol NaCl) and non-saline conditions. Wheat was grown in 16 h light 8 h dark, photon density 650 µmol m^{-2} s^{-1} and temperature of 25 ºC. Plant samples were collected 72 hours after salt application. Each value represents the mean of three measurements ±SE. Different letters represent significant differences at 5 % level of significance

Excess of sodium chloride can damage plant growth and development through reducing water and nutrients uptake as well as negative effects on biochemical processes (Akbarimoghaddam et al., 2011). Numerous researchers have reported that growth and morphological indexes such as root length (El-Hendawy et al., 2005), leaf area (Hamdia et al., 2004) as well as soluble sugars, proline (Tavakoli et al., 2016), Ca and Fe content (Askary et al., 2017) are affected significantly by saline condition (Turan, 2008). The result of this study showed that in non-inoculated plant, salinity causes a reduction in root and shoot length. One of the mechanisms that is chosen by plants to cope with environmental stress such as salinity is the accumulation of some organic molecular (such as soluble carbohydrates and proline). It appears that increase in soluble carbohydrates and proline content in the root of wheat seedlings probably cause the better osmotic adjustment and maintained cell turgor for better growth under salinity. However, the soluble sugar of the root and shoot of inoculated and non-inoculated wheat seedlings were increased due to salinity. Meanwhile, proline was increased just in the root of non-inoculated seedlings. This result is similar to the results obtained by Maghsoudi and Arvin (2010). They reported a significant reduction in dry matter of susceptible wheat varieties under saline condition, when compared to the salt tolerate wheat cultivars. Moreover, the results of this study showed that inoculation of wheat plants with *A. brasilense* Sp7 under saline and non-saline conditions had no significant effect on the amount of root and shoot soluble sugars in short period of time. But, inoculation increased significantly dry mass and length of the roots. Similar results were reported by different researchers. Zarea et al. (2012) showed that *Azospirillum* had no effect on soluble sugars content of wheat plants. In similar work Hamdia et al. (2004) reported that root dry mass and root length of maize were increased significantly in response to inoculation with *A. brasilense*. However, growth reduction under saline condition may be either due to lowering the external water potential or ion toxicity on metabolic processes. Other reports indicated that *Azospirillum* Spp. may produce various plant growth regulators that increase plant growth indexes (dry mass, root and shoot length), nitrogen fixation, and absorption of water and minerals (El-Dengawy et al., 2011, Askary et al., 2017). In this study, Ca and Fe content of root and shoot seedlings significantly reduced under salt stress while inoculation with *A. brasilense* shown some improvement in Ca uptake. Upadhyay et al. (2011) reported that some of the native strains of bacteria which separated from the wheat rhizosphere of the soils were able to establish salt tolerance by bacterial secretion such as exopolysaccharides that affect the mineral availability.

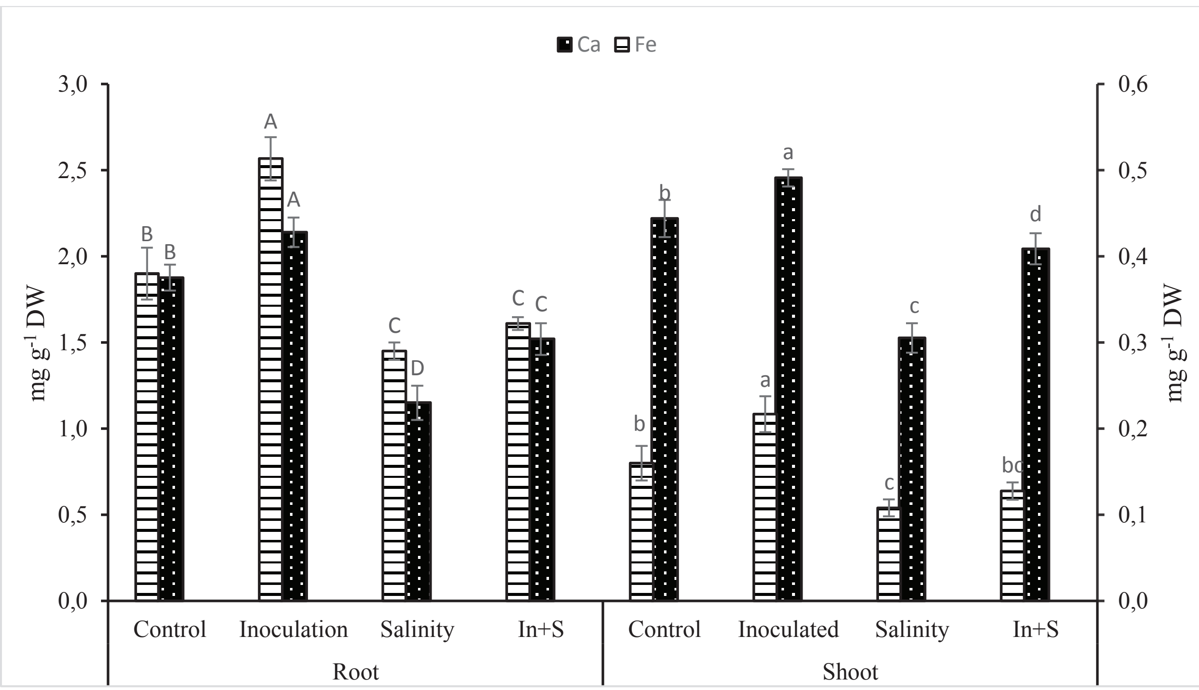

Figure 2: Effect of inoculation and salinity on average Ca and Fe content of roots and shoots of wheat seedlings. Differences in small (shoot) and cap (root) letters on the bar graph indicated difference in their mean (n = 3) values based on Duncan's multiple range tests

3.2 Sodium, potassium and Na^+/K^+ ratio

In control condition, sodium content of roots, sheaths and leaf blades showed an increasing trend over time, and so, the sodium content was the highest at 48 hour (at the end of experiment) after salinity applied (Table 1), it means sodium was accumulated during the experiment in all plant parts. The sodium content of roots, sheaths and leaf blades were also increased in same pattern in non-inoculated plants under saline condition as compared to control plants. Under inoculation and non-saline condition, the sodium content of root, sheath and leaf blade were significantly less than control plants. Although salinity raised sodium accumulation in root, sheath and leaf blade of inoculated plants over time, their sodium content were still significantly less than non-inoculated plants that were grown under saline condition. Moreover, the maximum sodium content in root, sheath and leaf blade of non-inoculated plants which exposed to 200 mmol NaCl was 7.94, 4.44 and 2.70 mg g^{-1} DM, respectively at 48 hour after salinity applied. According to the result (Table 1), we observed that wheat inoculation by *A. brasilense* could help to prevent the sodium entrance to the roots of plants (17.22 % at non-saline and 9.5 % at saline conditions) meanwhile didn't have considerable effect on sodium allocation within plant parts.

Although sodium content was increased in inoculated plants under saline condition, it was significantly less than sodium content of non-inoculated plants which grown under saline condition. In addition, we observed that *A. brasilense* could help to prevent sodium uptake by root of inoculated wheat plant. This might be due to producing and secretion of bacterial exopolysaccharides to the root environment and reducing the availability of Na^+ for plant uptake. This result is consistent with the results presented by Upadhyay et al. (2011). They indicated that some of the native strains of bacterial which separated from the wheat rhizosphere in the soils of Varanasi and India were able to establish salt tolerance by bacterial secretion such as exopolysaccharides.

Previous studies revealed that sodium accumulation in germinated seeds was increased gradually under saline condition (Akbarimoghaddam et al., 2011; Hamdia et al., 2004). In addition, El-Dengawy et al. (2011) showed that Carob seedlings (*Ceratonia siliqua* L.) inoculated with *A. lipoferum* under saline condition improved the addition of sodium-to-potassium ratio and gave better root characteristics. Al these results show that establishing a cooperation system between wheat and *Azospirillum* help to facilitate better growth through direct or indirect mechanisms (Hamdia et al., 2004; Nadeem et al., 2006).

Table 1: Mean values of Na^+ content (mg g^{-1} D M) in inoculated and non-inoculated wheat plants with *Azospirillum brasilense* SP7 (10^7 CFU ml^{-1}) grown under saline (200 mmol NaCl) and non-saline conditions for 6, 24 and 48 h. The growth condition was light with photon density 650 µmol m^{-2} s^{-1} and temperature of 25 ºC. Different letters in each plant parts separately represent a significant difference at 5 % level of significance.

	6 h			24 h			48 h		
	Root	Sheath	Blade	Root	Sheath	Blade	Root	Sheath	Blade
None inoculated									
Control	3.94^{E}	1.38^{H}	1.11^{G}	4.22^{DE}	1.89^{E}	1.24^{F}	4.37^{D}	1.81^{EF}	1.38^{E}
Salinity	4.56^{D}	2.28^{D}	1.28^{EF}	6.94^{B}	3.94^{B}	1.91^{C}	7.94^{A}	4.44^{A}	2.70^{A}
Inoculated									
Control	3.45^{G}	1.09^{I}	0.86^{H}	3.69^{FG}	1.50^{GH}	1.03^{G}	3.54^{G}	1.63^{FG}	1.29^{EF}
Salinity	4.47^{D}	2.25^{D}	1.06^{G}	5.99^{C}	3.33^{C}	1.60^{D}	7.12^{B}	3.36^{C}	2.55^{B}

Table 2: Mean (n = 3) values of K^+ content (mg g^{-1} D.M) in inoculated and non-inoculated wheat plants with *Azospirillum brasilense* (10^7 CFU ml^{-1}) grown under saline (200 mmol NaCl) and non-saline conditions for 6, 24 and 48 h. The growth conditions were light with photon density 650 µmol m^{-2} s^{-1} and temperature of 25 ºC. Different letters in each plant parts represent significant differences at 5 % level of significance.

	6 h			24 h			48 h		
	Root	Sheath	Blade	Root	Sheath	Blade	Root	Sheath	Blade
None inoculated									
Control	1.20^{DE}	2.47^{G}	2.06^{H}	1.23^{D}	3.53^{BC}	2.98^{E}	1.28^{DE}	3.69^{B}	3.75^{C}
Salinity	0.999^{G}	2.02^{H}	1.97^{H}	1.05^{FG}	3.04^{DF}	2.71^{FG}	1.03^{FG}	2.94^{EF}	3.85^{C}
Inoculated									
Control	1.51^{C}	2.79^{F}	2.57^{G}	1.52^{C}	3.74^{B}	3.46^{D}	2.16^{A}	4.15^{A}	5.03^{A}
Salinity	1.12^{EF}	2.16^{H}	2.08^{H}	1.29^{D}	3.22^{D}	2.76^{F}	1.24^{D}	3.44^{C}	4.11^{B}

Table 3: Mean values of Na^+/K^+ ratio in inoculated and non-inoculated wheat plants with *Azospirillum brasilense* (10^7 CFU ml^{-1}) grown under saline (200 mmol NaCl) and non-saline conditions for 6, 24 and 48 h. The growth conditions were light with photon density 650 µmol m^{-2} s^{-1} and temperature of 25 ºC. Different letters in each plant parts represent significant differences at 5 % level of significance.

	6 h			24 h			48 h		
	Root	Sheath	Blade	Root	Sheath	Blade	Root	Sheath	Blade
None inoculated									
Control	3.28^{FG}	0.56^{E}	0.54^{DE}	3.51^{F}	0.54^{E}	0.42^{F}	3.41^{F}	0.49^{E}	0.37^{G}
Salinity	4.56^{D}	1.13^{C}	0.65^{B}	6.61^{B}	1.30^{B}	0.71^{A}	7.70^{A}	1.51^{A}	0.70^{A}
Inoculated									
Control	2.30^{GH}	0.39^{F}	0.34^{GH}	2.44^{GH}	0.40^{F}	0.30^{HI}	1.64^{I}	0.39^{F}	0.26^{I}
Salinity	3.98^{E}	1.04^{D}	0.51^{E}	4.67^{D}	1.03^{D}	0.58^{CD}	5.74^{C}	0.98^{D}	0.62^{BC}

Potassium content of plant's root in treated and non-treated wheat seedlings with *A. brasilense Sp7* and *Azospirillum* plus salinity was higher compared to salt stressed plants alone (Table 2). Also, potassium content of plant's sheath and leaf blade in all treated and non-treated wheat seedlings showed an increasing trend over time (Table 2). Under saline condition, potassium accumulation decreased in root and sheath as compared to control plants while potassium content of leaf blade was preserved. The reduction of potassium content was more severe in root at 48 hour after salinity applied (46.6 %). Potassium accumulation of root, sheath and leaf blade in control plants were 1.94, 3.69 and 3.75 mg g^{-1} DM, respectively. While potassium content decreased to 1.03, 3.04 and 3.85 mg g^{-1} DM when salinity applied. However, with inoculation, potassium content increased to 2.16, 4.15 and 5.03 mg g^{-1} DM in root, sheath and leaf blade, respectively. In dual effect (salinity and inoculation) the potassium content was higher than control condition but less than in inoculated plants. The average potassium content of whole plant was 3.13 (control), 2.64 (salinity), 3.78 (inoculation) and 3.47 mg g^{-1} DM for dual effects of inoculation and salinity.

In root and sheath of non-treated plants, Na^+/K^+ ratio didn’t show variation over time while this ratio in leaf blade was significantly decreased over time (Table 3). Under saline condition, the Na^+/K^+ ratio of non-inoculated plant's root, sheath and leaf blade was higher than control plants and also showed an increasing trend over time. Moreover, the maximum Na^+/K^+ ratio in non-inoculated plants was seen in root at 48 hour after salinity applied (278 %). Under non-saline condition, Na^+/K^+ ratio of root, sheath and leaf blade in inoculated plants were less than non-inoculated plants. Although the Na^+/K^+ ratio of inoculated plants under saline condition was more than control plants, but it was less than non-inoculated plants treated with salinity. Moreover, the maximum reduction (29.73 %) of Na^+/K^+ ratio was observed in leaf blade of inoculated plants not exposed to NaCl as compared to control plants.

Under saline and non-inoculated condition, potassium content of root and sheath showed a significant reduction compare to control plants. Meanwhile, potassium content of inoculated plants was more than that of non-inoculated plants under saline condition. In addition, under non-saline condition, potassium content of all plant parts was increased due to inoculation. The results of this study show that the wheat plant inoculation with *A. brasilense* Sp7 has a significant effect on K^+ accumulation under saline and non-saline conditions. Under saline condition, the competition between uptake of sodium and potassium by non-inoculated plants favored sodium ions (Wakeel, 2013), but the wheat seedlings inoculated with *Azospirillum* increases potassium uptake. Therefore, sodium entry into the cell and potassium leakage out of the cell is decreased; this leads to a reduction of Na^+/K^+ ratio (Fraile-Escanciano et al., 2010; Hamdia et al., 2004; Upadhyay et al., 2011). Ardakani et al. (2011), showed that the potassium content of inoculated wheat plants by *A. brasilense* was increased and causes better mineral nutrient uptake. The results of this study are also similar to Askary et al. (2009), who showed that *A. brasilense* Sp7 improves potassium, phosphorus and nitrogen uptake by different wheat cultivars. In addition, the results are the same as Omar et al. (2009), who showed that Na^+/K^+ ratio of wheat plant after inoculation with *A. brasilense* was decreased due to increasing K^+ and limiting Na^+ uptake. In inoculated and non-inoculated plants, Na^+/K^+ ratio of leaf blade significantly was lower than the root. This might be due to less sodium accumulation in leaf blade that have already confirmed by Davenport et al. (2005).

3.3 Proline

Inoculated seedlings had lower proline concentration in the root and the shoot when compared to seedlings which were exposed to saline conditions. The lowest amount of proline (0.4 µmol g^{-1} FM) was measured at the root of inoculated plants not exposed to salinity ($P < 0.05$, Fig. 2). Salinity didn't have a significant effect on shoot proline, meanwhile, the maximum content of proline was observed in the root of salt-affected that non-inoculated with *Azospirillum* ($P < 0.05$, Fig. 3). Proline production is one of the mechanisms that enable the plant to tolerate adverse effect of environmental stresses. Proline is thought to contribute to osmotic adjustment, detoxification of ROS, and protection of membrane integrity (Heuer, 2010).

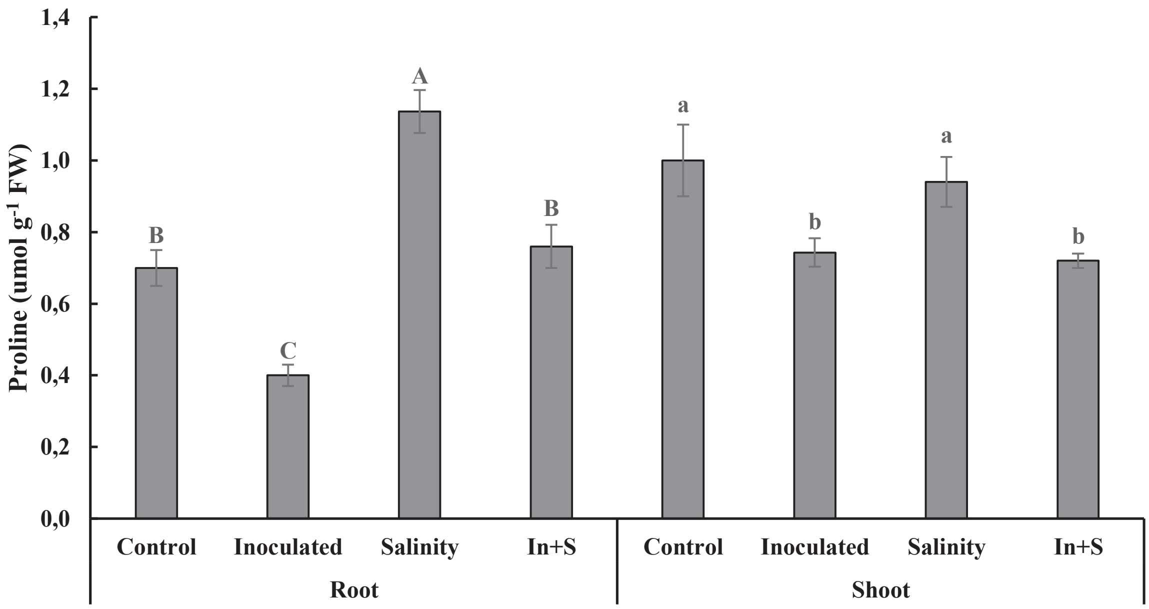

Figure 3: Effect of inoculation and salinity on average (n = 3) proline content of roots and shoots of wheat seedlings. Differences in small and cap letters on the bar graph indicated difference in their mean values based on Duncan's multiple range tests.

3.4 Relative *TaSOS1* (*Triticum aestivum* salt overly sensitive 1) gene expression

In control plants, the relative expression ratio of root *TaSOS1* didn't show any variation in its transcript level over time (Table 4) while a considerable reduction in *TaSOS1* expression was observed in sheath and leaf blade. When wheat seedlings exposed to 200 mmol NaCl and inoculated with *A. brasilense* Sp7 (10^7 CFU ml^{-1}), *TaSOS1* gene expression at mRNA level varied differently in different plant parts.

Root: Due to salt stress, *TaSOS1* over-expression in non-inoculated plant's root as compared to the control plants (non-inoculated and non-saline condition) was observed. Although the maximum (224 %) over-expression happened at 24 h after salinity applied, its value declined almost by 50 % afterward (at 48 h). In non-saline condition, the root *TaSOS1* relative expression of inoculated plants showed an up-regulation trend during the experiment. While, inoculated roots under salinity condition showed a considerable up-regulation (260 %) at 24 h and then down-regulated at 48 h after salinity applied but still the *TaSOS1* expression was higher than its control plant. In addition, *TaSOS1* over expression was higher in inoculated plants, than non-inoculated under non-saline condition.

Sheath: In non-inoculated plants, the relative expression ratio of *TaSOS1* was increased immediately when NaCl was added, then its value was reduced at 24 h, and finally reached to its maximum (120 %) at 48 hour. In non-saline condition, inoculation caused a significant increase in sos*1* gene expression as compared to control plants and reached to its maximum at 48 h (188.4 %). In saline and inoculated condition, the relative expression of *TaSOS1* was increased in sheath by 119 % when compared to its corresponded control plants. However, *TaSOS1* up-regulation due to dual treatments (salinity and inoculation) was significantly higher than inoculation or saline condition.

Leaf blade: There was a significant increase (almost 14 %) in *TaSOS1* expression after salinity applied as compared to non-treated plants. In inoculated and non-saline condition, the relative expression of *TaSOS1* showed an increasing trend and reached to 1.66 at 48 h. However, in saline condition, the expression of *TaSOS1* in inoculated plants was higher than control plants at 24 (70 %) and 48 h (27 %) after salinity applied. The *TaSOS1* mRNA level was higher in inoculated plants treated with NaCl as compared to control plants and also seedlings exposed to salinity.

Table 4: *TaSOS1* gene relative expression inoculated and non-inoculated wheat plants with *Azospirillum brasilense* (10^7 CFU ml^{-1}) grown under saline (200 mmol NaCl) and non-saline conditions for 6, 24 and 48 h. The plants were grown under the light density of 650 µmol m^{-2} s^{-1} and temperature of 25 ºC. Each value represents the mean of three individual measurements ±SE. Different letters in each plant parts represent significant differences at 5 % level of significance.

	6 h			24 h			48 h		
	Root	Sheath	Leaf	Root	Sheath	Leaf	Root	Sheath	Leaf
None inoculated									
Control	0.660^{G}	0.696^{E}_{F}	1.010^{D}	0.557_{G}	0.737^{E}	0.818^{F}	0.594^{G}	0.576^{F}	0.908^{E}_{F}
Salinity	1.163^{D}_{E}	0.911^{D}	1.011^{D}	1.805_{B}	0.741^{E}	0.936^{D}_{E}	1.262^{CD}_{E}	1.262^{B}	1.026^{D}
Inoculated									
Control	0.885^{F}	0.931^{D}	0.884^{E}_{F}	1.135_{E}	1.015^{C}_{D}	1.181^{C}	1.190^{DE}	1.660^{A}	1.661^{A}
Salinity	1.365^{C}	0.893^{D}	0.869^{E}_{F}	2.01^{A}	0.923^{D}	1.390^{B}	1.282^{CD}	1.153^{B}_{C}	1.155^{C}

The relative expression ratio of *TaSOS1* in different plant parts shown that the highest up-regulation of *TaSOS1* was observed in root at 24 h whereas the highest in sheath and leaf blade was happend at 48 h after salinity applied (with a delay) as compared to the root.

The result of this study showed that addition of sodium in the root rhizosphere and inoculation of wheat seedlings with *A. brasilense* Sp7 cause an increase in *TaSOS1* expression. This result is similar to that obtained by Ramezani et al. (2013), who showed that under saline condition *TaSOS1* and *TaSOS4* gene expression would increase in such cases. Moreover, Xu et al. (2008) showed that after 3 h of salt stress implementation, *TaSOS1* expression of root was increased immediately and then decreased. But, these changes occurred in the leaf with lower intensity and with a delay (after 9 h of salt stress).

Wheat seedling treated with *A. brasilense* and salinity showed that *TaSOS1* expression was increased in compared to non-inoculated and saline condition. Therefore, it can be concluded that *A. brasilense* can increase *TaSOS1* expression under saline and non-saline condition and can help sodium and potassium ions to be adjusted in plant. Numerous studies have already showed that gene expression changes in host plant after inoculation with plant growth-promoting rhizobacteria (PGPR). For instance, Vargas et al. (2012), showed that rice ethylene receptor gene expression was increased after inoculation with *A. brasilense* Sp245. In addition, it was reported that some genes involved in nutrient uptake were increased when wheat plant inoculation with *A. brasilense* (Camilios-Neto et al., 2014).

It seems that the tolerance of wheat plant to high concentration of salt is related to their ability to prevent Na uptake, avoid accumulation of toxic levels of sodium, regulation of osmotic pressure and maintaining adequate amount of potassium especially in the leaf blade. To achieve these goals, some genes related to such indexes should be express differently, e.g. *TaSOS1* which is a regulator for Na^+ uptake and upregulated at salinity condition. Establishment of an associated relationship between wheat plants and PGPR such as *A. brasilense* may help to regulate expression of such genes (e.g. *SOS1*) and consequently regulate the balance of cytosolic sodium and potassium. This is coincidence with, reduction of Na^+ availability for plant uptake via secretion of bacterial exopolysaccharides to the root environment.

4 CONCLUSION

This study showed that *TaSOS1* gene expression, growth, and biochemical indexes increased due to inoculation and saline conditions. Furthermore, the highest up-regulation of *TaSOS1* was observed in sheath and leaf blade with a delay as compared to the root. Also, *A. brasilense* had an important role in preventing the sodium entry into the plant. Further research might explore plasma membrane *TaSOS1* antiporter proteins by proteomic techniques in inoculated wheat seedlings under saline condition.

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An overview of applications in pineapple agroindustrial residues

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ABSTRACT

Industrial food production causes a high amount of waste. This waste must be taken to a suitable location where it can be further processing. During industrial processing of the pineapple, about 50 % of the mass of the fruit ends up being discarded becoming a residue. Researchers have studied these residues in order to add value to these by-products, to reduce disposal costs and guarantee environmental sustainability. This work investigates the development characteristics of research on agroindustrial residues of pineapple based on bibliometric methods to explore the structure of knowledge in this field over the years, according to the year of publication, periodicals, country, authors, area of knowledge, institutions, keywords, subject type, and citation analysis. In total 927 articles were found and after a careful analysis and selection of papers, 364 articles remained of which 82 % were published only in the last decade. Most studies focused on agricultural and biological sciences. About 1183 authors from 50 different countries contributed to this subject, in which India has the largest number of publications. The results obtained with this study, highlighting the different uses for pineapple residues, can provide valuable information for researchers interested in the field of agroindustrial wastes.

Key words: bibliometric analysis; residues; agroindustrial wastes; pineapple; waste management

IZVLEČEK

PREGLED UPORABE AGROINDUSTRIJSKIH OSTANKOV ANANASA

Industrijska proizvodnja hrane povzroča velike količine odpadkov. Odpadki morajo biti spravljeni na primernem mestu, kjer so lahko nadalje obdelani. Med industrijsko predelavo ananasa postane okrog 50 % mase odpadek. Ti ostanki so bili preučevani z vidika, da bi tem stranskim produktom dodali vrednost, zmanjšali stroške odvoza in zagotovili okoljsko vzdržnost. Prispevek preučuje razvojne značilnosti raziskav, ki se ukvarjajo s preučevanjem agroindustrijskih ostankov ananasa na osnovi bibliometričnih metod z namenom, da se prikaže znanje na tem področju v zadnjih letih glede na leto publikacije, vrsto revij, države, avtorjev, področij znanja, institucijo, ključne besede, tem raziskav in analizo citiranja. Celokupno je bilo najdenih 927 člankov. Po skrbni analizi in izboru objav je ostalo 364 člankov, od katerih je bilo 82 % objavljenih v zadnjem desetletju. Večina raziskav je bila usmerjena v agronomske in biološke vede. Okrog 1183 avtorjev iz 50 držav je prispevalo svoj delež, med njimi ima Indija največje število publikacij. Izsledki, dobljeni v terj raziskavi, osvetljujejo različne rabe ostankov ananasa, kar lahko prispeva pomembne informacije raziskovalcem, ki ji zanima področje agroindustrijskih odpadkov.

Ključne besede: bibliometrična anliza; ostanki; agroindustrijski odpadki; ananas; upravljanje z odpadki

1 INTRODUCTION

Brazil is one of the largest fruit producers in the world. In terms of total volume, pineapple is one of the most important fruit crops (FAO, 2015). The crop represents, according to data from the Brazilian Institute of Geography and Statistics (IBGE, 2017), the third largest fruit product, being surpassed only by oranges and bananas. The total planted area of pineapple in 2016 was 100,238 hectares, of which 67,254 hectares were

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harvested. Production in this same year reached 1,734,627 tons, obtaining an average yield of 25,792 kg·ha^{-1} (IBGE, 2017). Advantages such as geographic location, wide availability of arable land and climatic conditions favor the leading role of pineapple cultivation, ensuring jobs and contributing significantly to the national economy (Brito Neto et al., 2008; Morgado et al., 2004; Silva, 2016).

Industrial food production causes a high amount of waste, such as bagasse, husks and pulp residues of the fruits. This waste must be taken to a suitable location, which in general adds a costly procedure to the industry. In addition to the cost of treating this material, many of which are of low efficiency, there are still risks for the continuity of environmental pollution (Timofiecsyk & Pawlowsky, 2000). Since the residues produced have great potential for reuse, together with the concern for the environment, numerous studies have been carried out with the intention of taking advantage of them (Borges et al., 2004; Ferrari et al., 2004; Zhang et al., 2007). Thus, it is possible to reduce environmental pollution, and increase sustainability, along with obtaining new products with higher added value (Pelizer et al., 2007).

During industrial processing of the pineapple, the crown and stem are cut prior to desquamation. Subsequently, the shell and core are removed, leaving only the pulp. This residue usually corresponds to about 50 % of the fruit mass, representing a growing environmental problem due to microbial deterioration (Ketnawa et al., 2012). The other parts of the plant, such as the stem, roots, and leaves, are generally discarded in the field as agricultural residues, representing high waste since these components also make up the fruit (Fagundes & Fagundes, 2010).

Numerous academic studies are being carried out with the aim of making some use of the agroindustrial residues of pineapple: protein enrichment (Alexandre et al., 2013; Navid et al., 2010; Díaz-Vela et al., 2017), vinegar manufacturing (Madurai et al., 2010; Isitua & Ibeh, 2010), extracting bromelain (Ketnawa et al., 2012; Manosroi et al., 2014) enzyme production (Selvakumar & Sivashanmugam, 2017), preparation of activated carbon nanosheets (Sodtipinta et al., 2017), production of cellulose nanocrystals (CN) (Santos et al., 2013), represent some types of use. These documents summarize ways of taking advantage of agroindustrial residues of pineapples plants from different perspectives, but a bibliometric approach still was not applied. Thus, no study presented a comprehensive picture of this type of exploitation due to its research restrictions.

The search for indicators to quantify scientific activity and technological knowledge has increased over the years. In Brazilian literature, for example, it was mediated by the need for the government and scientific community to have instruments to promote guidelines, incentive programs and evaluation of science and technology development in Brazil (Mugnaini et al., 2004; Allen, 1969). According to Wang et al. (2014), bibliometric is a very useful tool for mapping the literature around a research, using statistical and quantitative analyzes to demonstrate patterns of productivity of articles in a given field of research, institution or country throughout the time. The bibliometric approach has been widely used in several studies, such as the use of nanotechnology in agriculture (Stopar, 2016), the analysis of scientific production related to organic agriculture (Aleixandre et al., 2015), use of energy from biomass and its interaction with the environment (Mao et al., 2018), evaluation of the evolution of the topic of food waste (Chen et al., 2016), verification of publication trends concerning natural fibers (fiber crops or fiber plants) (Bartol & Mackiewicz-Talarczyk, 2015), evaluation of the distribution of research related to rural tourism (Hočevar & Bartol, 2016).

Thus, the purpose of this work was to quantitatively and qualitatively evaluate the scientific literature on the use of agroindustrial residues of pineapples using bibliometric analysis which results provide valuable information to assist researchers in selecting potential research fields, identifying suitable institutions to evolve their studies, and contacting researchers for collaboration filling existing gaps in this field of research.

2 MATERIALS AND METHODS

2.1 Methods

Initially a literature review was carried out on the Scopus database, with the international scientific production on the use of agroindustrial residues of pineapples as its guideline, opting for the search of articles as the only type of document.

In this citation database, the search was used in the section of title, abstract and keywords. The following keywords together with Boolean operators were used as search strategies, only on scientific articles: TITLE-ABS-KEY ((pineapple*) AND (waste* OR residue* OR bagasse* OR skin* OR crown* OR peel* OR core*)) AND (LIMIT-TO (DOCTYPE, "ar")).

After the bibliographic review, the material was analyzed. Once all publications were identified, a pre-selection was made, according to the theme regarding the use of pineapple agroindustrial residue guiding the study, and previously defined inclusion and exclusion criteria. All languages of publication were considered.

The collected data were organized, coded, tabulated and submitted to statistical analysis with the Scopus base itself, and statistics/network using VOSviewer software (Van Eck & Waltman, 2018; Van Eck & Waltman, 2014). For the purpose of analysis, the following variables were chosen: year of publication, periodicals, country, authors, area of knowledge, institutions, keywords, type of subject and citation analysis.

2.2 Review of documents

After searching the Scopus database, 927 articles were found in total. Articles generally provide more original search results and more information about the authors and their affiliations, and only those documents were used in the analysis.

All articles have been evaluated by the titles, abstracts and information contained in the publication. In this phase, the documents that did not correspond specifically to the object of study were identified. This stems from the fact that the use of the keywords results in a general and broad search, covering any kind of subject that contains these questions at random. Thus, scientific documents dealing with pineapple in a general manner were excluded, such as those related to the use of parts of pineapple plant (leaf and stem), pineapple fruit quality, physico-chemical properties, pesticide residues in pineapple and peeling of the fruit.

This filtering process eliminated 563 publications, resulting in 364 articles, which were exported in CSV format for bibliometric analysis.

2.3 Analysis tools

In this study, the Scopus database was used. VOSviewer (Van Eck & Waltman, 2018; Van Eck & Waltman, 2014) was used as tool for analysis and visualization of the network.

Scopus is a global citation database covering science, technology, medicine, social sciences, arts and humanities (Elsevier, 2018). This database has a vast collection of more than 71 million records and 23,700 titles from more than 5,000 international publishers. It also has intelligent tools that allow the tracing, analysis and visualization of searches, which can be done by title of the article, abstract, keywords, authors, affiliation, language, ISSN, DOI, among others (Elsevier, 2018).

VOSviewer is a software used for the creation, visualization and investigation of bibliometric maps from network data using the VOS (Visualization of Similarities) mapping technique. It is possible to use file data from databases such as Web of Science, Scopus, RIS, PubMed, Crossref JSON to build networks. It is worth noting that VOSviewer is not only restricted to the analysis of bibliometric networks, it is possible to expand its use for the elaboration, visualization and analysis of maps made up of any type of network data.

Different publications report applications of VOSviewer, such as in the importance of climate change for the production of tea (verification of the *Camellia sinensis* (L.) Kuntze) (Marx et al., 2017), in the analysis of the research landscape of precision agriculture in Italy (Costa et al., 2017), in the review of publications dealing with climate change and viticulture (Marx et al., 2017), in the verification of publications related to nanocellulose (Milanez et al., 2016).

3 RESULTS AND DISCUSSION

3.1 Publication trend

The trend of publication related to the use of agroindustrial residues of pineapples is shown in Figure 1.

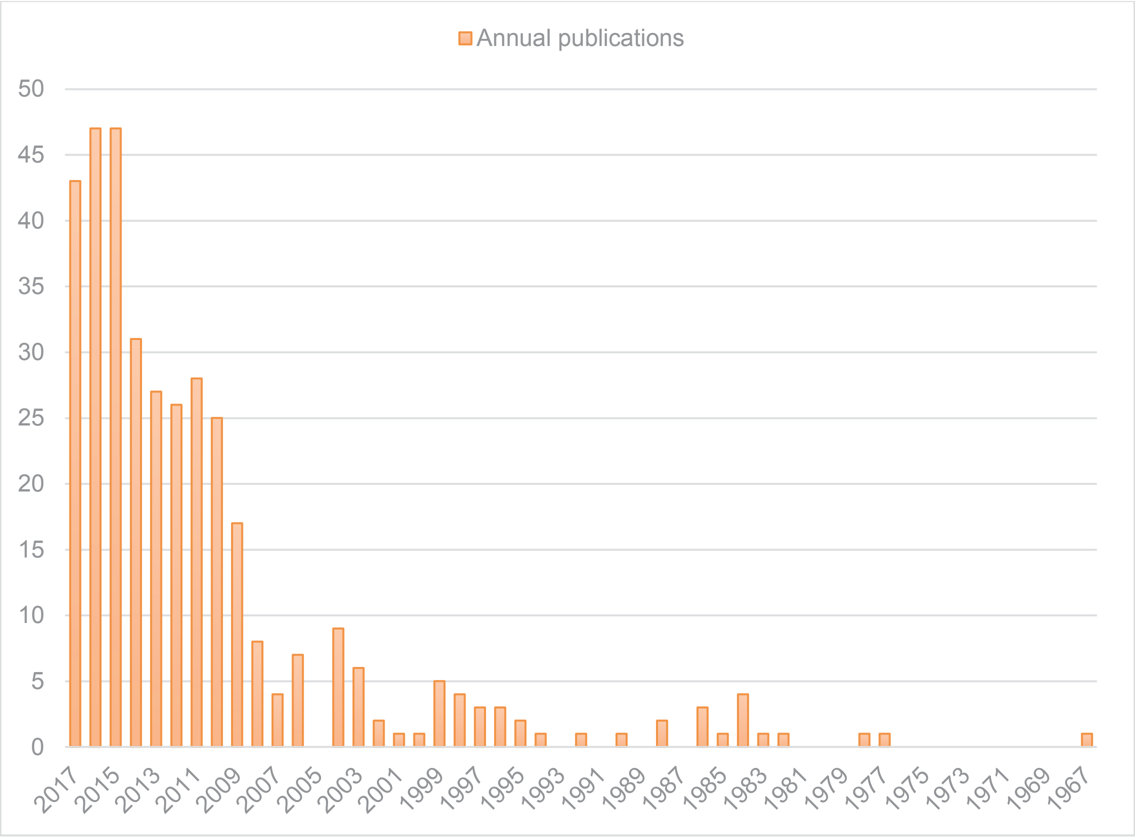

Figure 1: Trends of publications

The first publication occurred in 1967 with the work of Richardson (1967) dealing with the production of vinegar. During 1967-2007, there were few publications, followed by an increase in 2008. Publications in the last 10 years (299) represent more than 82 % of the total. It should be noted that the study in question did not cover all the publications made in the year 2017, since when the research was carried out, publication data for 2017 were not complete. Thus, the observation of the results allows inferring that the annual publications have presented an increasing and constant trend during the last decade. This result can be explained by growing global concern about environmental issues, aiming at sustainable production.

3.2 Areas of activity

The studies that contributed to this research theme involved 21 different academic areas, as shown in Figure 2.

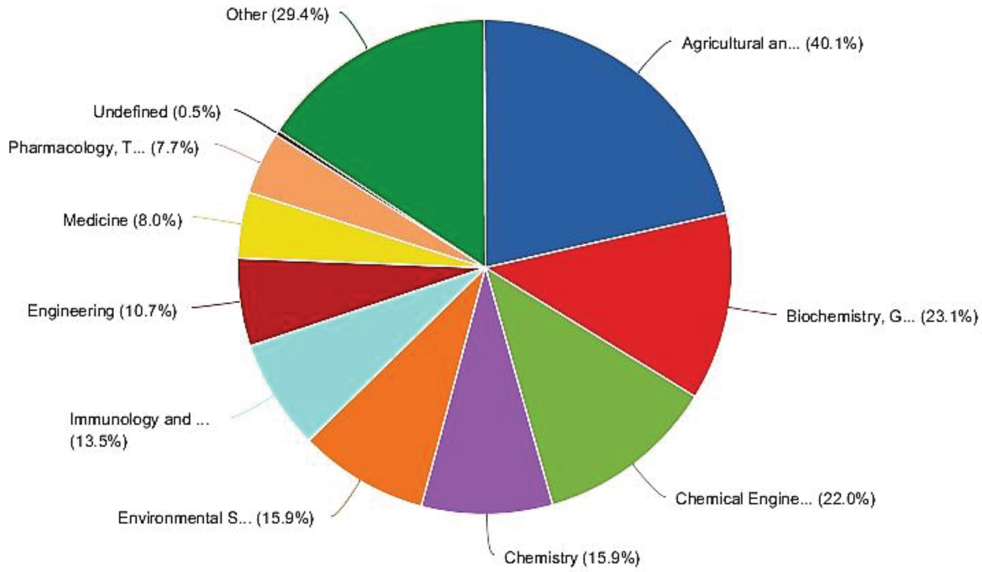

Figure 2: Areas involved in research

Among the areas (Figure 2), agricultural and biological sciences are highlighted as number 1, with 146 publications (40.1 %), followed by biochemistry and genetics (23.1 %) and chemical engineering (22.0 %). Publications involved in environmental sciences, chemistry, immunology, engineering, medicine and pharmacology also contributed to the development of the theme of agroindustrial residues of pineapple, while other areas contributed to the remaining 29.4 %.

3.3 Journals

The 364 articles selected were published in 158 different journals, but most of these journals (55.7 %) published only one article related to the use of agroindustrial residues of pineapples. Table 1 lists the top ten journals with the largest number of publications. In general, citation times for an article might reflect its influence, though the wrong citations might occur. Thus, total citations (TC) and the average number of citations per document of a journal (TC/P) in the period are also shown in Table 1.

Table 1: Periodicals with more publications

#	Periodic	Publications	%	TC[a]	TC/P[b]
1	Bioresource Technology	13	3.57	457	35.15
2	Chemical Engineering Transactions	8	2.19	15	1.87
3	Process Biochemistry	5	1.37	246	49.20
4	Journal of Food Science and Technology	5	1.37	14	2.80
5	International Food Research Journal	5	1.37	5	1.00
6	Carbohydrate Polymers	4	1.09	128	32.00
7	International Biodeterioration And Biodegradation	4	1.09	33	8.25
8	RSC Advances	4	1.09	29	7.25
9	International Journal of Chemtech Research	4	1.09	20	5.00
10	Revista Brasileira de Zootecnia	4	1.09	20	5.00

[a] Total Citations of the document.
[b] Average Citations per published document.

In terms of journals performance, Bioresource Technology was the most productive with 13 articles, followed by Chemical Engineering Transactions (8 articles). Bioresource Technology also presented the highest number of citations (457), followed by Process Biochemistry (246) and Carbohydrate Polymers (128). Process Biochemistry had the highest TC/P score, followed by Bioresource Technology and Carbohydrate Polymers. It is interesting to note that despite being in second place in the publications, Chemical Engineering Transactions presents TC/P well below the last three mentioned.

Figure 3 illustrates the performance of journals on the use of agroindustrial residues of pineapples.

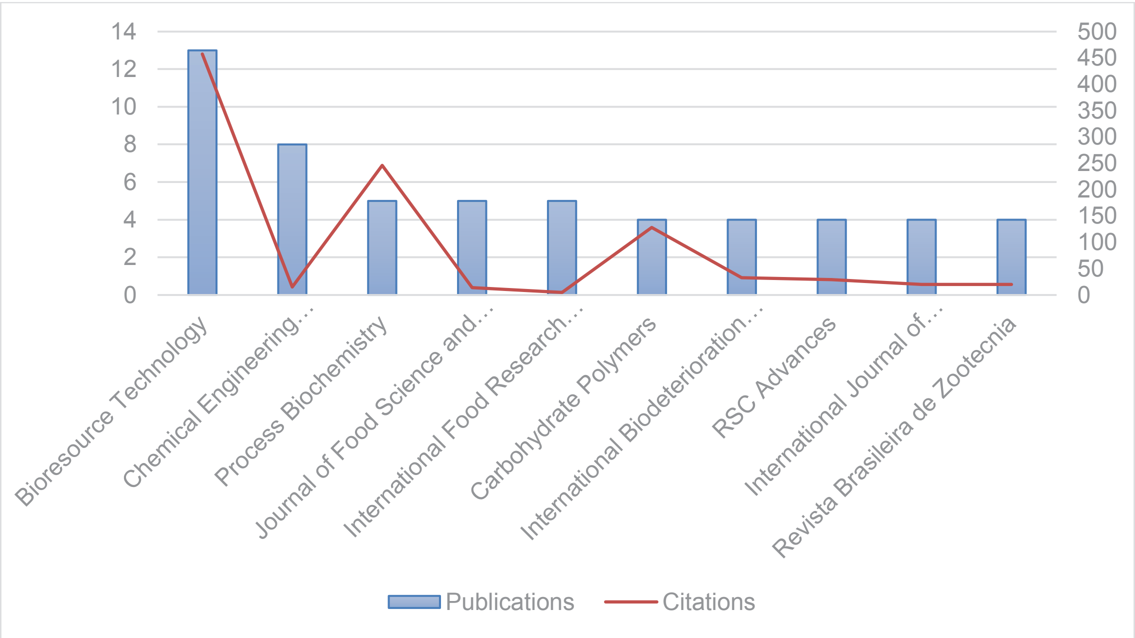

Figure 3: Number of publications and citations in periodicals

The results imply that Bioresource Technology, Process Biochemistry and Carbohydrate Polymers are the leading journals in this area. Other major journals include International Biodeterioration and Biodegradation, RSC Advances, International Journal of Chemtech Research and the Revista Brasileira de Zootecnia.

3.4 Country

From the point of view of the countries, institutions from 50 countries were responsible for the publication of the 364 articles related to the use of agroindustrial residues of pineapples. Of the total countries, 40 % contributed with only one document. Table 2 lists the ten most productive countries.

Table 2: Publications by country, and collaboration between countries

#	Country	n.p.[a]	%	coll[b]	Country 1	Country 2	n.p.[a]
1	India	78	19.38	9	Thailand	Japan	5
2	Brazil	59	16.28	4	India	South Korea	4
3	Malaysia	49	15.12	6	India	United States	3
4	Thailand	40	12.40	15	Malaysia	Australia	3
5	China	19	6.20	2	Thailand	South Korea	3
6	Nigeria	18	5.81	2	Thailand	United States	3
7	United States	17	5.43	20	Brazil	United States	2
8	Mexico	14	3.88	1	Thailand	France	2
9	Japan	13	3.49	8	United States	France	2
10	Australia	11	3.49	4	United States	South Korea	2

[a] Publications number
[b] Collaboration

From Table 2, it can be seen that the ten most productive are responsible for 87.6 % of all publications, with 63.18 % being distributed among the first four. India has more publications in this area, followed by Brazil, Malaysia and Thailand. In this way, it is understood that the main countries of the researchers related to the residues are also the main producers of the fruit. These countries are emerging economies with favorable climates for the cultivation of pineapples, and the use of waste can represent an important economic activity.

Table 2 also shows the main collaborations between countries. Although the United States (USA) is not ranked among the five most productive countries, it is the most active country for international collaboration in this field with 20 collaborations.

Countries collaborating with the USA include India, Thailand, Brazil, France and South Korea. The USA has collaborated with many countries. On the other hand, India, Brazil and Malaysia, although with more publications, have few collaborations with other countries. It has been found that thirteen nations (such as Portugal, Taiwan, Indonesia, among others) have not collaborated with other countries in their publications.

The United States has the largest cross-country cooperation, maintaining research relationships with 13 countries worldwide by sharing 20 articles, followed by Thailand (6 countries and 15 articles), South Korea (4 countries and 11 articles), India (4 countries and 9 articles), South Africa (9 countries and 9 articles) and Italy (8 countries and 8 articles), while South Africa and Italy, despite high collaboration, share only one article with other countries. Highlight the relationship between Japan and Thailand by cooperating five articles.

In relation to the most productive countries, Malaysia (6 articles) and Brazil (4 articles), although highly productive, maintains collaboration with only four and three countries, respectively, indicating that the research is little diversified and restricted to national researchers, while Nigeria, which despite being among the most productive, shared only two articles.

3.5 Authors

In total, 1,183 authors wrote contributions to this subject, although only 166 authors participated in more than one document. The authors with the highest number of publications are listed in Table 3, including the respective numbers of published documents, h-index, country of origin and collaboration articles. We see Tambourgi E. B. as the most productive author with 9 articles, followed by Huang H. and Vasiljevic T. with 8 and 7 articles, respectively.

Table 3: Main authors and main co-authors

#	Author	n.p.[a]	h-Index	Country	coll[b]	Author 1	Author 2	n.p.[a]
1	Tambourgi, E. B.	9	22	Brazil	39	Tambourgi, E. B.	Silveira, E.	6
2	Huang, H.	8	10	China	17	Carreira, R. L.	Silvestre, M.P.C	6
3	Vasiljevic, T.	7	24	Australia	21	Vasiljevic, T.	Donkor, O. N.	4
4	Silveira, E.	6	6	Brazil	33	Vasiljevic, T.	Sah, B. N. P.	4
5	Carreira, R. L.	6	5	Brazil	28	Vasiljevic, T.	McKechnie, S.	4
6	Silvestre, M.P.C.	6	17	Brazil	28	Huang, H.	Dai, H.	4
7	Zakaria, Z. A.	5	11	Brazil	21	Huang, H.	Hu, X.	4
8	Ahmad, W. A.	5	11	Malaysia	19	Zakaria, Z. A.	Ahmad, W. A.	3
9	Hu, X.	5	4	China	16	Jamal, P.	Alam, M. Z.	3
10	Jamal, P.	5	11	Malaysia	16	Jamal, P.	Saheed, O. K.	3

[a] Publications number
[b] Collaboration

The h-index of each author was obtained from Scopus, being a non-static indicator to measure the author's productivity and the impact of his research. In general, it implies that the total number of articles published by a person is quoted at least h times.

However, regarding the h-index, the number of publications are not proportional to the scores of this indicator. The reason for this can be attributed to the fact that studies related to the use of pineapple residues require the involvement of researchers from different fields, highlighting those that exert a greater influence in their area of research, as is the case of Vasiljevic T. (agricultural and biological sciences).

In order to analyze the collaboration between authors, Table 3 also shows the main authors and main co-authors in this field of research. Interestingly, the authors listed in main authors are also present in main co-authors, indicating knowledge integration of different authors.

Table 3 shows collaboration within a given country, such as Tambourgi, Silveira, Carreira and Silvestre in Brazil; Huang, Dai and Hu in China; Jamal and Alam in Malaysia, and Vasiljevic and Sah in Australia. Therefore, the collaborations among the most productive authors are among authors from the same country.

Looking at Table 3, it is possible to verify that the authors who most shared articles related to the use of agroindustrial residues of pineapples were Tambourgi E.B. and Silveira E., both from the Campinas State University, and Silvestre M.P.C. and Carreira R.L., both from the Federal University of Minas Gerais. This shows that despite the high collaboration observed, the Brazilian scientific production in this field is concentrated among national authors, since Brazil has low international collaboration.

3.6 Institutions

A total of 158 institutions published articles on the use of agroindustrial residues of pineapple, of which 57 (36.1 %) had only one publication. Table 4 lists the ten most productive organizations.

Table 4: Contribution of institutions

#	Institution	Country	Publications
1	Universiti Teknologi Malaysia	Malaysia	19
2	Universidade Estadual de Campinas	Brazil	14
3	Universidade de São Paulo – USP	Brazil	12
4	South China University of Technology	China	10
5	Kasetsart University	Thailand	9
6	Universidade Federal de Minas Gerais	Brazil	9
7	Central Food Technological Research Institute Índia	India	8
8	Victoria University Melbourne	Australia	7
9	Universiti Putra Malaysia	Malaysia	7
10	Universiti Sains Malaysia	Malaysia	6

From Table 4 it can be seen that Malaysia has three of the ten most productive institutions, including Universiti Teknologi Malaysia, which has the largest number of publications. India, although the most productive country, has only one institution (Central Food Technological Research Institute India), indicating a probable production distributed in that country.

Brazil also has three of the ten most productive institutions. The Campinas State University presents greater national production, followed by the University of São Paulo and the Federal University of Minas Gerais. As the second most productive country (section 3.4) and with more than thirty productive organizations, it is concluded that the rest of the Brazilian production is well distributed among the other institutions.

3.7 Keywords

In order to understand a subject during a specific period, it is possible to use keywords which can provide important information. In this sense, the present work extracted 1,061 keywords defined by the authors of the articles, some of them being similar. The most frequently adopted are listed in Table 5.

Table 5: Frequently used keywords

#	Keywords	Frequency
1	Bromelain	33
2	Pineapple	32
3	Pineapple waste	27
4	Pineapple peel	16
5	Fermentation	16
6	Adsorption	12
7	Enzimatic hidrolysis	9
8	Aspergilus niger	8
9	Bioethanol	8
10	Ananas comosus	8

From Table 5 it is concluded that the most used keywords are related to the words bromelain, pineapple and fermentation.

As mentioned in section 3.1, studies on the utilization of agroindustrial pineapple residues can be divided into periods according to annual publications. Thus, the keywords were identified in two periods (1967-2007 and 2008-2017) in order to verify the main research themes. Figures 4 and 5 show the most used keywords and their respective networks during the years 1967-2007 (165 words) and 2008-2017 (947 words), respectively.

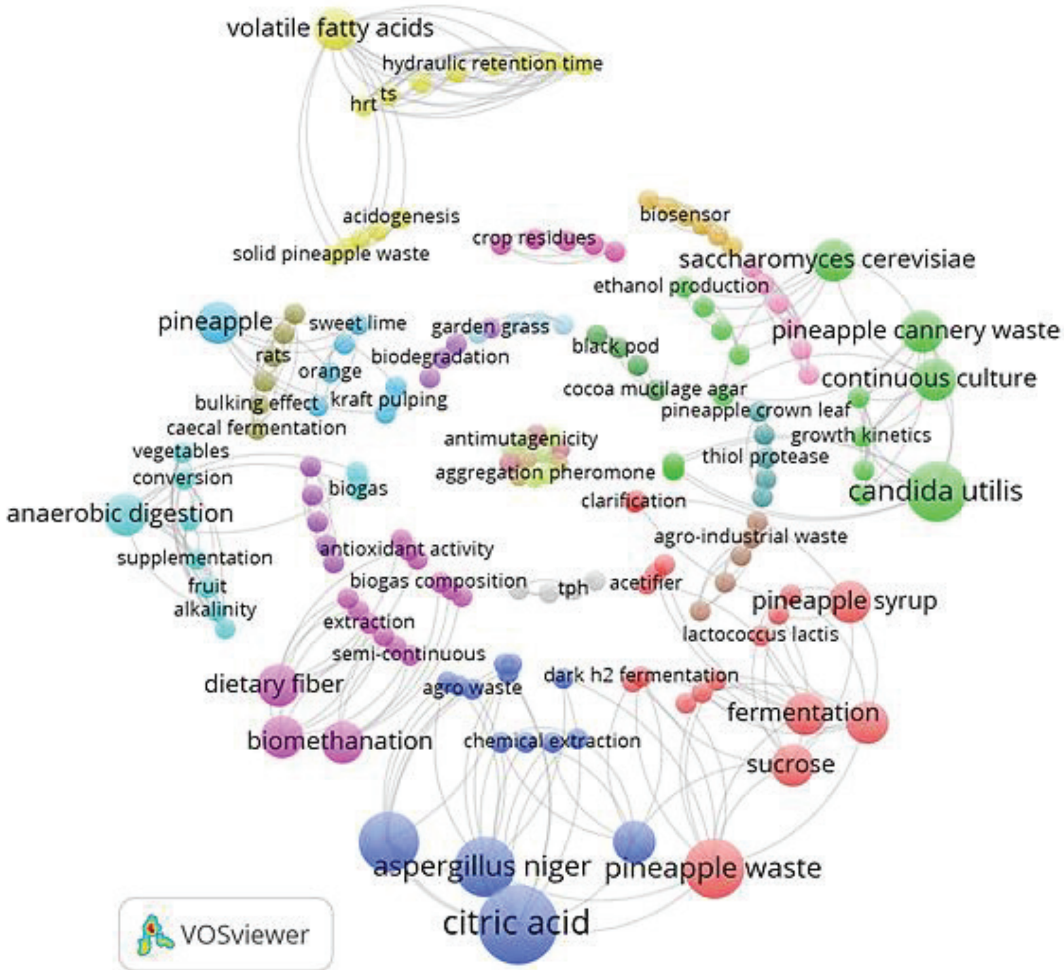

Figure 4: Keywords frequently used in the period 1967-2007

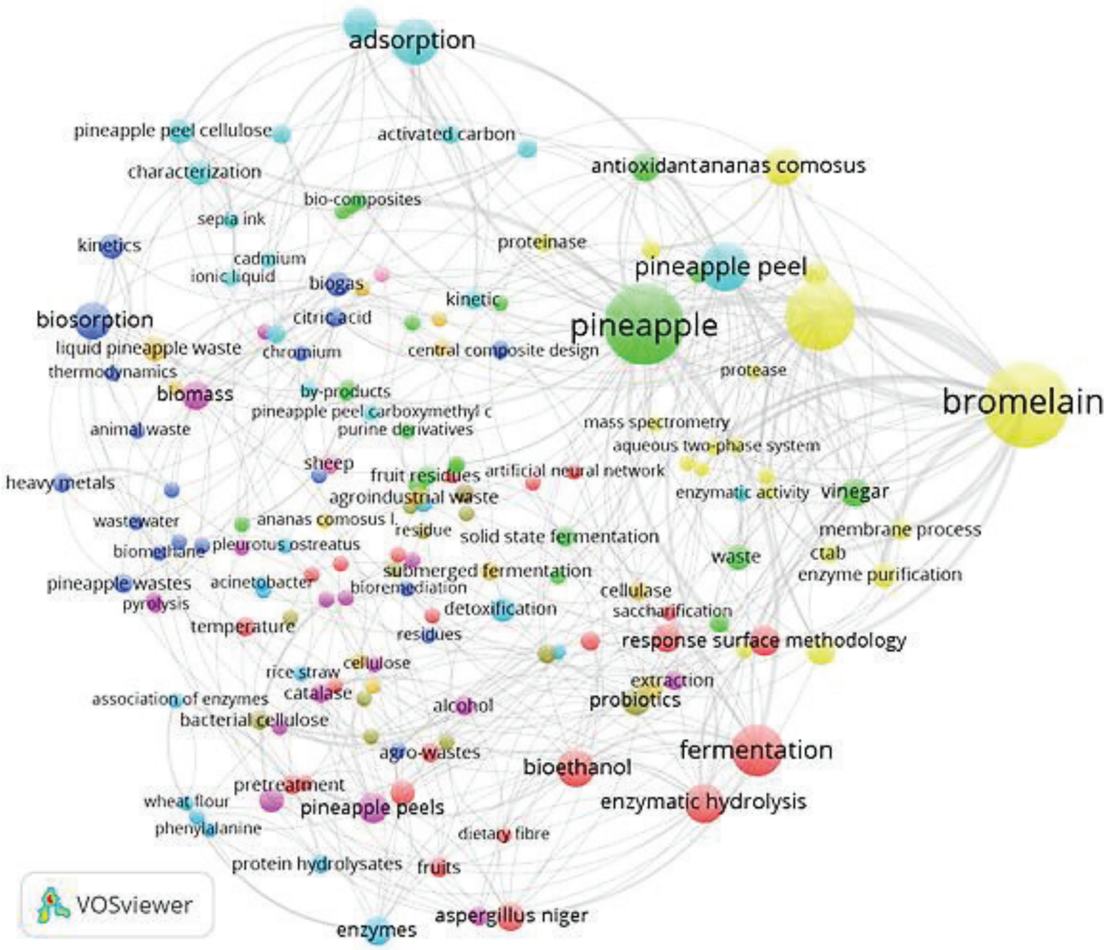

Figure 5: Keywords frequently used in the period 2008-2017

There are circles, in Figures 4 and 5, whose sizes serve to demonstrate the use of the keyword, i.e. the larger the circle size the more often the keyword was used. The lines are used to indicate the number of times that two keywords were used together. From Figure 4, it is concluded that during the first stage (1967-2007), the researchers focused mainly on the contents of pineapple residue, citric acid production and fermenting microorganisms, such as *Candida utilis* (Hennenberg) Lodder & Kreger, *Aspergillus niger* van Tieghem and *Saccharomyces cerevisiae* Meyen ex E.C. Hansen. In the second stage (2008-2017), the topic of residues of pineapple and fermentation remained. In addition, new topics related to bromelain and substance adsorption have become a frequent target of recent research.

In this study, the keywords most frequently used by the authors of the articles, without the use of synonym grouping, were presented, so that the keywords "*Ananas comosus*" and "pineapple" are presented as distinct terms in Figure 5. Nevertheless, these words are related and arranged nearby and connected through the lines by the software.

3.8 Subject Type

The use of agroindustrial residue of pineapple has strong economic appeal, having seen the amount of applications. When conducting an analysis of the summary of the 364 articles selected, it was verified that certain subjects were presented with more frequency. The five main themes studied in the research on the use of agroindustrial residues of pineapples were responsible for accounting for more than half of the publications (204 articles or 56.04 %).

Among the most studied (56.04 %), the highest use of the residue as a protein supplement (14.56 %), source of enzymes (11.54 %), biofuel (10.44 %), bromelain (10.16 %) and use as adsorbent (9.34 %). Other applications involve the production of acids, antioxidant phenols and vinegar / wine.

Protein supplement

The use of agroindustrial residues of pineapple as a supplement usually involves the use of microorganisms for protein enrichment. For example, Mensah and Twumasi (2017) used pineapple residues as a substrate for production of Single Cell Protein (SCP) with yeast *Saccharomyces cerevisiae* as inoculum. According to its results, it was concluded that residues from the food processing industry serve as good substrates for SCP production, which presents high benefit. In this way, protein-rich foods for animals and humans can be produced.

According to Damasceno et al. (2016), the pineapple peel flour is high in fiber and can be introduced into cereal bars as food. In their work, they evaluated the use of this flour in different concentrations in cereal bars, concluding that this material is a good alternative to be introduced in the human diet while minimalizing the environmental impact.

Other work on supplements involves the use of pineapple residues as cattle silage (Yang et al., 2016; Braga et al., 2016), piglet feeding (Ramos et al., 2016) and forage for dairy cows (Kraiprom et al., 2013), presenting an alternative food in periods of scarcity or as food of low cost.

Enzymes

In recent work, Selvakumar and Sivashanmugam (2017) produced lipase from pineapple skin. According to the authors, these enzymes are important because they can be used in the hydrolysis of the acylglycerols present in fatty acids and glycerol. They also investigated its use in the conversion of palm oil into biodiesel, obtaining yield 88.63 %.

Arun and Sivashanmugam (2015) produced different types of enzymes from pre-consumed pineapple residues. These enzymes were used in the treatment of activated sludge, solubilizing insoluble organic compounds in soluble compounds, which can later be treated to produce methane or hydrogen.

Silvestre et al. (2012) performed the extraction of protease from pineapple peels. When evaluating the stability, they verified the maintenance of the initial activity in 60.9 % and 53.7 % for the different pH and temperature conditions, respectively.

Other studies involved, for example, the production of amylase (Orlandelli et al., 2017), xylanase (Harris & Chidambaram, 2015) and cellulase (Kannahi & Elangeswari, 2015).

Biofuel

Shamsul et al. (2017) performed the production of biomethane and biometanol in batch bioreactor using pineapple peels as one of the substrates. They verified ideal temperature, concentration and retention times for production, obtaining 2.49 % methanol with 74.24 % methane.

Ogunleye et al. (2016) used pineapple residues in mixture with animal waste for the production of biomethane. They used 1.5 l anaerobic digesters and incubated for 10 weeks. When using the pineapple residues, they obtained increased yield and shorter start-up time for the generation of biomethane.

Other studies have involved biomethanization of pineapple peels (Aworanti et al., 2017) and bioethanol production (Conesa et al., 2016; Venkateswarulu et al., 2015), contributing as a biotechnological alternative to the use of fossil fuels.

Bromelain

Bromelain is a group of proteolytic enzymes found in pineapple tissues. Due to its high activity, it can potentially be used in the cosmetic, pharmaceutical and food industries (Spir et al., 2015).

Chaurasiya et al. (2015) used reverse micelle extraction (RME) for the separation and purification of bromelain from the pineapple nucleus. This bromelain was used in beef softening and compared to commercial bromelain. The results indicated a high recovery of activity (85 %) and a greater reduction of tenacity (52.1 %) compared to commercial bromelain (26.7 %).

Work on the separation, extraction and purification of bromelain from pineapple residues were carried out, for example, by Coêlho et al. (2015), Martins et al. (2014) and Novaes et al. (2013).

Adsorption/Absorption

Solidum (2013) evaluated the ability to remove heavy metals from water by pineapple peels. They analyzed the kinetic parameters of the removal of lead and cadmium in contaminated waters, varying pH, contact time and metal concentration. According to the authors, the use of these residues in the absorption of the metals presents considerable profitability.

Shifera et al. (2017) conducted studies on the removal of lead (II) and chromium (VI) present in water. They concluded that the adsorption on pineapple peels is viable, besides being spontaneous and exothermic.

Gandhi et al. (2012) analyzed the removal of fluoride from water from pineapple peel powder. The results showed that the use of residues as adsorbents are a viable alternative, because they have significant removal capacity and low cost.

Other examples include adsorption of methylene blue (Yamuna & Kamaraj, 2016), nickel (Dotto et al., 2016; Rao & Khan, 2017) and copper (Romero-Cano et al., 2017).

Other applications

Other applications involve the production of different compounds, such as: antioxidant phenols (Nor Halaliza & Zulkifly, 2017; Alias & Abbas, 2017), acids (Tang et al., 2014; Lun et al., 2014), wine/vinegar (Roda et al., 2017; Praveena & Estherlydia, 2014), bacterial cellulose.(Kumbhar et al., 2015), acetone-butanol-ethanol (ABE) using *Clostridium acetobutylicum* McCoy et al. Emend. Keis et al.(Khedkar et al., 2017), hydrogel (Dai & Huang, 2016), phenolic antioxidants using *Rhizopus oligosporus* Saito(Correia et al., 2004), yellowish orange pigment from *Chryseobacterium artocarpi* Venil et al. 2014(Aruldass et al., 2016).

3.9 Citation analysis

Citation analysis is a widely used method for assessing the academic performance of a researcher. When one article is quoted by another, it means that the search results of that article can provide useful information for others, the relevance of which is proportional to the number of quotes.

The simplest way in citation analysis is to count the number of citations received by a document. However, authors can cite their own articles, or some citations can be negative. Thus, total citations do not always indicate the true value of an article (Geng et al., 2017).

Thus, the citation analysis was based on the average number of citations per year after publication (TC/Y), since recently published articles are less likely to be cited. Table 6 shows the top ten articles based on this indicator.

Table 6: Most cited articles by year of publication

#	Author[a]	Periodic	TC/Y[b]	TC[c]
1	Hameed (2009)	Journal of Hazardous Materials	17.3	156
2	De Oliveira (2009)	Food Chemistry	13.4	121
3	Castro (2011)	Carbohydrate Polimers	13.1	92
4	Bansal (2012)	Waste Management	13.0	78
5	Foo (2012)	Microporous and Mesoporous Materials	9.7	58
6	Imandi (2008)	Bioresource Technology	9.4	94
7	Idris (2006)	Process Biochemistry	8.6	103
8	Umesh hebbar (2008)	Bioresource Technology	8.0	80
9	Nanda (2016)	Energy Conversion and Management	8.0	16
10	Mahamad (2015)	International Biodeterioration and Biodegradation	7.3	22

[a] Data referring to the first author.
[b] Annual average citations.
[c] Total citations.

In total, 94 articles (25.8 %) were not cited. Table 6 shows that the work done by Hameed et al. (2009) has a great popularity, being also the one with the highest TC/Y index. Soon this is the most credible study to be quoted. His research explored the use of the waste as a low cost adsorbent of methylene blue aqueous solution, studying adsorption kinetics and isotherms.

Also noteworthy for the studies carried out by Nanda et al. (2016), on supercritical water gasification of pineapple bark for bio-gas production, and by Mahamad et al. (2015), on dye worship, which, although recent and with few quotations, present a high TC/Y value.

4 CONCLUSIONS

Based on the data extracted from the Scopus database, this paper investigated the development characteristics of publications (from 1967 to 2017) on the use of agroindustrial residues of pineapples, using bibliometrics analysis. The studies that contributed to this research theme involve 21 different academic areas, being 40.1 % in the area of agriculture and biological sciences, 23.1 % biochemistry and genetics and 22.0 % in chemical engineering.

Although there have been publications on the subject in the last 40 years, 82 % of these publications have been published in the last decade, which shows a growing trend of research in this area in the last years. The growth can possibly be attributed to the growing environmental concern and high costs of agroindustrial waste disposal.

India, Brazil and Malaysia collaborate weakly with other countries despite producing large number of publications. The United States is the leader in international collaboration. However, Brazil has three of the ten most productive institutions, with Campinas State University being nationally the most prominent. Universiti Teknologi Malaysia is the most productive institution.

Finding ways to reuse waste brings benefit not only to the environment but also to industries. During this work, several studies on the reuse of pineapple residues were found, such as: protein supplement, source of enzymes, biofuel, bromelain, use of adsorbent and production of acids, antioxidant phenols, vinegar and wine. Thus, this study provides a framework and serve as a base for future studies into the identification of influential authors, journals, works, institutions and subjects in the field of agroindustrial residues of pineapple helping new researches and interactions in this area.

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Effects of mild and severe drought stress on the biomass, phenolic compounds production and photochemical activity of *Aloe vera* (L.) Burm.f.

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ABSTRACT

In this study, the biomass, compatible solutes, PSII functioning and phenolic profiles of *Aloe vera* (L.) Burm.f. leaves were investigated at different time intervals after drought stress (20, 40 and 80 % of the field capacity). While the impaired ability of leaves for synthesis of assimilates caused growth inhibition in *A. vera* under severe drought stress, we observed that the content of proline, soluble sugars, total phenolic and flavonoids tended to increase in plants treated with mild drought stress. Under mild drought stress, the increased leaf thickness correlated with the higher productivity in terms of leaf biomass and gel production. Also, mild drought stress enhanced photochemical activity in *Aloe* leaves, and changed the entire quantity of secondary metabolite of vanillic acid produced, which may be considered to obtain better growth and considerable secondary metabolite of the medicinal *Aloe* plants treated with mild drought stress.

Key words: *Aloe vera* leaves; carotenoids; leaf thickness; photosystem II performance index; vanillic acid; drought stress

IZVLEČEK

UČINEK BLAGEGA IN MOČNEGA SUŠNEGA STRESA NA BIOMASO, TVORBO FENOLNIH SNOVI IN FOTOKEMIČNO AKTIVNOST VRSTE *Aloe vera* (L.) Burm.f.

V raziskavi so bili preučevani biomasa, osmotiki, delovanje fotosistema II in profil fenolnih snovi v listih vrste *Aloe vera* v različnih intervalih po vzpostavitvi sušnega stresa (20, 40 in 80 % poljske kapacitete). Medtem, ko je bila rast zavrta zaradi zmanjšane fotosintetske sposobnosti listov, je bila pri blagem sušnem stresu povečana vsebnost prolina, topnih sladkorjev, celokupnih fenolov in flavonoidov. Pri blagem sušnem stresu je povečana debelina listov korelirala z večjo produktivnostjo glede na biomaso in tvorbo gela. Blag sušni stres je povečal fotokemično aktivnost listov in spremenil količino sekundarnega metabolita vanilijske kisline in njenih derivatov. Sklepamo lahko, da blag sušni stres poveča rast in tvorbo sekundarnih metabolitov pri tej zdravilni rastlini.

Ključne besede: *Aloe vera* listi; karotenoidi; debelina listov; indeks učinkovitosti fotosistema II; vanilijska kislina; sušni stres

1 INTRODUCTION

Environmental stresses, such as drought, low or high temperature and excessive salinity have negative influence on the plant, causing changes in its normal growth, development and metabolism (Bohenert et al., 1995; Kranner et al., 2010). Drought stress induces oxidative stress through enhancing the formation of reactive oxygen species (ROS). ROS can react with photosynthetic pigments, lipids, proteins and DNA (Ahmad et al., 2010), leading eventually to lipid peroxidation, membrane damage, inactivation of antioxidant enzymes and cell death (Gill and Tuteja, 2010). However, for the detoxification of excessively produced ROS, plants possess a developed antioxidative defense mechanism. ROS scavenging occurs by a large number of ROS detoxifying enzymes and by antioxidants (Gill and Tuteja, 2010; Mittler et al., 2011).

To survive at drought stress conditions and to achieve maximum drought resistance, plants have developed adaptation strategy, which is associated with physiological traits, e.g., increased osmoprotectant and accumulation of sugars (Kooyers, 2015). In addition, drought avoidance occurs when plants enhance water-use efficiency (WUE) by decreasing transpiration, or increasing root growth. Generally, CAM plants are the best accommodated to hot and dry climatic conditions,

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because they have higher water-use efficiency (WUE) than that of C_3 plants (Habibi, 2016). *A. vera* (L.) Burm. f. is a drought tolerant species that is considered as a constitutive CAM species (Silva et al., 2010). Nevertheless, drought stress can limit *A. vera* growth and production (Delatorre-herrera et al., 2010; Cousins and Witkowski, 2012). In *A.vera*, the synthesis of soluble sugars, proline and secondary metabolites are highly affected by water deficit stress (Lucini et al., 2013; Ray et al., 2013).

Phenolic compounds are the most widespread substantial groups of plant secondary metabolites that exhibit antioxidant properties (Quan et al., 2016). Several investigators have reported that the levels of phenolic compounds such as phenolic acids and flavonoids were influenced by drought stress in tobacco and wheat leaves (Ma et al., 2014).These compounds can scavenge ROS (Quan et al., 2016) and prevent lipid peroxidation, protein denaturation and DNA damage (Mittler, 2002; Król et al., 2014). Interestingly, the relationship between water availability and phenolic compounds synthesis is dependent on the plant species, treatments and/or experimental systems. Some studies have shown that environmental stress can cause a decline (Weidner et al., 2009). However, other studies have indicated that stress increases phenolic compounds accumulation (Weidner et al., 2009; Król et al., 2014). Thus, the exact mechanism of phenolic compounds accumulation in response to long and continuous stressor remains unknown.

Aloe vera as a CAM plant and one of the most important medicinal plants grows in warm and dry regions. Several studies have been conducted focusing on *A. vera* adaptation to water deficit stress; however information is lacking on the role of phenolic compounds, compatible solutes as well as photochemical reactions in *Aloe vera* plants exposed to mild and severe water stress, which may be one of the most important approaches to overcome with water deficit stress. In this study, changes in compatible solutes, PSII functioning and individual phenolic acids in response to mild and severe drought stress in *A. vera* were investigated. We also hypothesized that regulation of water availability may be a promising way to obtain the highest concentrations of secondary metabolite in the medicinal plant.

2 MATERIALS AND METHODS

2.1 Plant material and treatments

The 15–17 cm pups (small plants growing from the sides of the mother plant) of *Aloe vera* (L.) Burm.f. plants were chosen and planted in top of the cylindrical plastic pots (18 cm in diameter and 45 cm in depth) containing 10 kg sandy loam soil (pH 7.3) for five months, and irrigated with distilled water every 10 days to maintain at 80 % field capacity (FC). Plants were grown under day/night temperature of 30-35/18-22 ºC, relative humidity of 50-55 % and daily photon flux density of about 500-600 µmol m^{-2} s^{-1} in an environmentally controlled growth chamber throughout the pre-experimental period. The treatments of drought stress were composed of control (80 % FC), mild drought stress (40 % FC) and severe drought stress (20 % FC). Finally, plants were harvested and analyzed in a temporal (on different days after imposition of drought stress) manner.

2.2 Chlorophyll *a* fluorescence measurements

Chlorophyll *a* fluorescence transients (*OJIP* transients) were evaluated with a Packet-PEA chlorophyll fluorimeter (Plant Efficiency Analyser, Hansatech Instruments Ltd., King's Lynn, Norfolk, PE 32 1JL, England) in dark-adapted leaves for at least 20 min, using the *JIP*-test to analyse chlorophyll *a* fluorescence rises. Some groups of measured and calculated parameters using the *JIP*-test (Strasser et al., 2004) were described in the following section.

- F_v/F_m, the maximum PSII photochemical efficiency, namely the maximum quantum yield of primary photochemistry. Where F_m or F_{max} is maximal chlorophyll fluorescence intensity measured when all photosystem II (PSII) reaction centers are closed, F_v is variable chlorophyll fluorescence (F_m–F_o), F_o is minimal fluorescence (all PSII RCs are assumed to be open), respectively.
- PI_{abs}, the performance index that is calculated as: (RC/ABS) × ($\varphi_{Po}/(1 - \varphi_{Po})$) × ($\psi_o/(1 - \psi_o)$), where, RC is for reaction center; ABS is for absorption flux; φ_{Po} is for maximal quantum yield for primary photochemistry; and ψ_o is for the quantum yield for electron transport

2.3 Determination of total carotenoids, chlorophyll *a* and *b*

The leaf concentration of chlorophyll and carotenoids was analysed according to Lichtenthaler and Wellburn (1983). After centrifugation at 1000 rpm for one minute, supernatants were used for determination of photosynthetic pigments, and the absorbance was read at 400-700 nm on spectrophotometer. Leaf concentrations of chlorophylls and carotenoids were calculated as:

Chl a = 15.65 A666 - 7.340 A653
Chl b = 27.05 A653 - 11.21 A666
Total carotenoids = 1000 A470 - 2.860 Ca - 129.2 Cb/245

2.4 Estimation of total proline, soluble sugars and starch

Proline was determined by the method of Bates et al. (1973). Leaf samples from each group were homogenized in 3 % (w/v) sulphosalycylic acid and the homogenate was centrifuged at 3,000 g for 20 min. Mixture was boiled for 1 h in water bath after addition of acid ninhydrin and glacial acetic acid. Reaction was then stopped by ice bath, and then absorbance at 520 nm was determined. Proline (Sigma) was used for production of a standard curve. For determination of total soluble sugars and starch contents, fresh leaves were extracted in 20 ml of 80 % (v/v) ethanol at 95 ºC for 1 h. After centrifugation at 10,000 g for 10 min, starch was determined in the pellet according to Jarvis and Walker (1993). Total soluble sugars were analyzed using anthrone reagent according to Irigoyen et al (1992).

2.5 Assay of phenylalanine ammonia-lyase (PAL) activity and related metabolites

To determine PAL activity, formation of cinnamic acid was recorded by spectrophotometry at 290 nm according to modified method of Zucker (1965). One unit (U) of PAL activity was defined as the amount of the enzyme that produced 1 nmol cinnamic acid per h. Total phenolic content was quantified by the method of Velioglu et al. (1998). Gallic acid was used for constructing the standard curve. Results were expressed as mg gallic acid (GA) per gram of the fresh mass. Total flavonoid content was assessed using the method adapted by Meda et al. (2005). Briefly, 5 ml of 2 % aluminium chloride ($AlCl_3$) in methanol was mixed with the same volume of leaf extracts (0.02 mg ml^{-1}). Absorption readings at 415 nm were taken after 10 minutes against a blank sample without $AlCl_3$. The total flavonoid content was calculated using a standard curve of quercetin and expressed as mg quercetin equivalent (QE)/100 g extract. Anthocyanin content was estimated according to the method of Krizek et al. (1993) using HCl-methanol solvent (1: 99, v: v), and the amount of anthocyanin was ranked from the absorbance at 550 nm.

2.6 HPLC analysis

For the sample preparation and calibration curves, the powdered leaves (0.5 g) were extracted with methanol (5 ml) in a shaking incubator for 8 hours at room temperature. The supernatant was centrifuged at 3000 g for 3 minutes, and then filtered prior to HPLC analysis. For the calibration curve, the stock solutions of the identified phenolic compounds (chlorogenic acid, syrginic acid, gallic acid, rosmarinic acid, vanillic acid and luteolin) were equipped with methanol to obtain a 1 mg ml^{-1} concentration, and the calibration curves for standard samples were fabricated by plotting the peak area of the identified phenolic compounds against their concentrations through dilution of each stock solution in methanol to six concentrations (0.78 ppm, 1.58 ppm, 3.12 ppm, 6.25 ppm and 25 ppm). Vanillic acids were identified at 245 nm, gallic and syrginic acids at 275 nm, chlorogenic and rosmarinic acids at 320 nm and luteolin at 350 nm. The correlation coefficients (r^2) of all phenolic standards were higher than 0.993.

The HPLC analysis was done using a Knauer liquid chromatography apparatus Shimadzu HPLC instrument (a 1000 Smartline Pump, a 5000 Smartline Manager Solvent Organizer and a 2800 Smartline Photodiode Array Detector). Separation achieve on a 25 cm × 4.6 mm with a pre-column, Eurospher 100-5 C18 analytical column provided by Knauer (Berlin, Germany). Data acquisition and integration perform with EZchrom Elite software. A 20 µl sample of the methanol extract of *Aloe vera* leaves was injected into an HPLC column through a 3900 Smartline Auto-sampler injector equipped with a 100 µl loop. Separation was performed using 0.02 % trifluoroacetic acid in water (elution A) and methanol (elution D). The total running time was 55 minutes at a flow rate of 0.5 ml min^{-1}, and the oven temperature was 20 ^{o}C.

2.7 DPPH assay

The antiradical activity was estimated by using the method described by Yen and Chen (1995). Briefly, the mixture of methanolic extract and DPPH (2, 2-diphenyl-1-picrylhydrazyl) solution was left in the dark at room temperature for 20 min, and then absorbance was recorded at a wavelength of 517 mm.

2.8 Statistical analysis

Experiments were performed in complete randomized block design (RBD) with 4 replications. Statistical analysis was carried out using sigma stat (3.5) with Tukey test ($P < 0.05$). Correlation analysis using Spearman Rank Order Correlation in sigma stat (3.5) was applied to determine the relationship between parameters. Chlorophyll fluorescence data were analyzed and conducted using the PEA Plus V1.10 software.

3 RESULTS AND DISCUSSION

3.1 Biomass and gel production and water relations under mild and severe water stress

Drought is one of the major abiotic stresses affecting plant growth worldwide (Wu et al., 2018). However *Aloe vera* is a promising crop for arid zones, with high yield of leaf biomass and gel production under mild water stress conditions (Silva et al., 2010), but it has been demonstrated that severe drought stress decreases leaf yield and growth of *Aloe vera* (Silva et al., 2014; Hazrati et al., 2017). This is confirmed by the present study showing that the production of leaves biomass and gel was not influenced by mild drought stress and only the severe drought stress caused significant biomass reduction (Table 1). In plants treated with drought for 180 days, leaf thickness tended to increase under mild drought stress, while leaf thickness and relative water content (RWC) were reduced under severe drought stress. In these conditions, *A. vera* exhibited morphologic plasticity to adapt to water deficit. Indeed, succulent plants can exhibit plasticity in their photosynthesis depending on the presence of thick leaves (Habibi and Ajori, 2015, Habibi, 2016).

Table 1: Effects of mild and severe drought stress on the leaf biomass (g leaf^{-1}), relative water content (RWC, %) and leaf thickness (mm) of *Aloe vera*. Measurements were performed 180 d after drought treatments. Data of each row within each defined plant part indicated by the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 8).

	Control	Mild drought stress	Severe drought stress
Fresh leaf biomass	550±29 [a]	526±35 [a]	352±38 [b]
Photosynthetic tissue	185±21 [a]	197±24 [a]	145±28 [b]
Gel	365±17 [a]	329±29 [a]	207±23 [b]
Dry leaf biomass	45±6.2 [a]	44±3.7 [a]	32±3.2 [b]
Photosynthetic tissue	19±4.4 [a]	21±4.0 [a]	15±3.3 [a]
Gel	26±2.3 [a]	23±3.6 [a]	17±2.9 [b]
RWC	88±3.7 [a]	84±4.5 [a]	71±2.2 [b]
leaf thickness (mm)	13.2±1.14 [ab]	16.0±2.23 [a]	10.4±1.07 [b]

3.2 Possible importance of compatible solutes in the responses of *A. vera* plants to mild drought stress

After 120 and 180 days of exposure, the content of proline and soluble sugars was increased by mild drought stress. The content of starch, however, was not affected in plants treated for 60, 120 and 180 days with both mild and severe drought stress (Fig. 1). CAM plants ability to maintain growth under drought stress is based on the efficient synthesis of sugars, polysaccharides and other osmolytes, such as proline and glycine betaine (Delatorre-herrera et al., 2010; Salinas et al., 2016) in order to adaptation to water deficit. Similarly, our results showed that mild drought stress significantly increased proline and soluble sugars contents, which might have a scavenger function and act as an osmolyte (Sankar et al., 2007; Salinas et al., 2016). Nevertheless, under the severe drought stress, the content of proline and soluble sugars did not continue to increase probably due to source limitations. In this study, the maintenance of dry matter production under mild drought stress may have contributed to the enhanced levels of compatible solutes during the experiment.

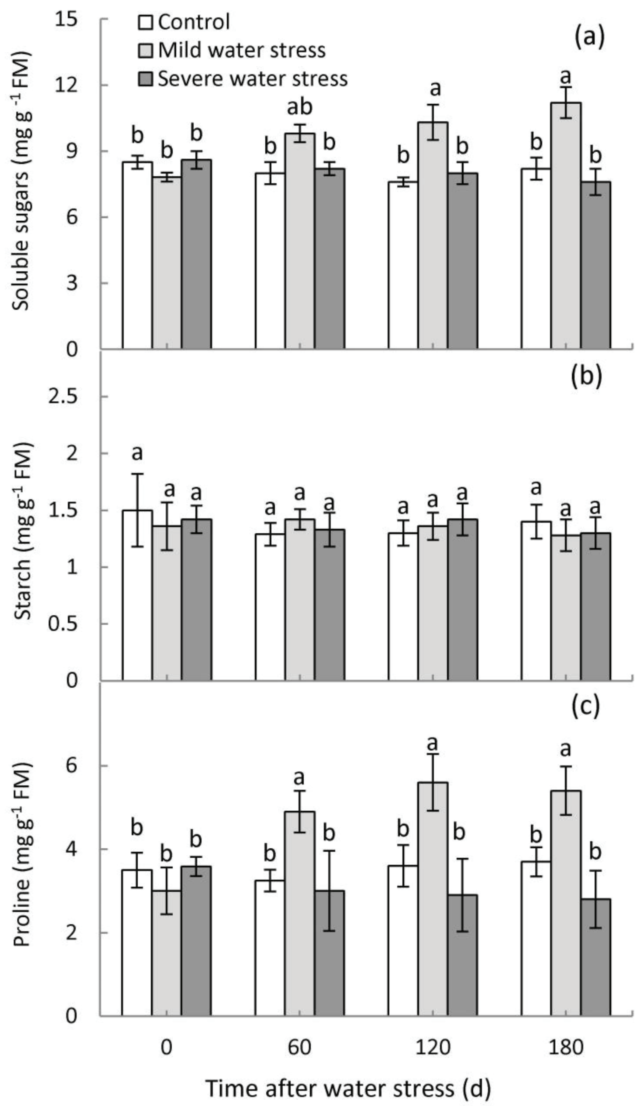

Figure 3: Effects of mild and severe drought stress on the concentration of soluble sugars, starch and proline in *Aloe vera* plants. Bars indicated with the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 4).

3.3 Treatment with mild drought stress enhances photoprotection activity in *Aloe* leaves ISTT

Chlorophyll *a* and b contents were not affected significantly by various drought levels in *Aloe vera* plants (Fig. 2). After 120 days treatment, however, carotenoid content of leaves was enhanced only under the mild drought stress (Fig. 2). Under mild drought stress, an increase in carotenoid content can perform an important role as a non-enzymatic antioxidant in the photoprotection of photosynthesis (Miura and Tada, 2014; Habibi and Ajory, 2015) as well as in the dissipation of absorbed light energy as thermal energy (qE) (Cazzonelli and Pogson, 2010). Indeed, when *Aloe vera* plants were exposed to mild drought stress, the accumulation of protective pigments such as carotenoids in leaves developed an effective photoprotection mechanism, as demonstrated by the maintenance of the maximum quantum yield of photosystem II (F_v/F_m) and photosystem performance index (PI_{abs}) in plants treated with mild drought stress (Fig. 3). In contrast, after 120 and 180 days of treatment, F_v/F_m and PI_{abs} exhibited a significant decrease only in plants subjected to severe drought stress, suggesting a photoinhibitory effect (Diao et al., 2014). Since the shape of the OJIP curve is very sensitive to environmental stress (Strasser et al., 2004), we measured chlorophyll fluorescence to determine the precise effects of drought stress on the photosynthetic apparatus (Su et al., 2015) using the JIP-test (the analysis of the fluorescence rise OJIP). Under mild drought stress conditions, the OJIP chlorophyll fluorescence curve obtained from the *Aloe vera* leaves revealed a normal fluorescence rise when compared to control plants (Fig. 4), indicating that reaction centers behave almost normally (Kalaji et al., 2011; Zhang et al., 2014). In this study, however, a quicker fluorescence rise in the *J* step was perceived in response to severe drought stress, which is related to deactivation of the reaction center leading to drastic reduction in photochemistry through a blockage of electron flow (Strasser et al., 2004; van Heerden et al., 2007; Kalaji et al., 2011).

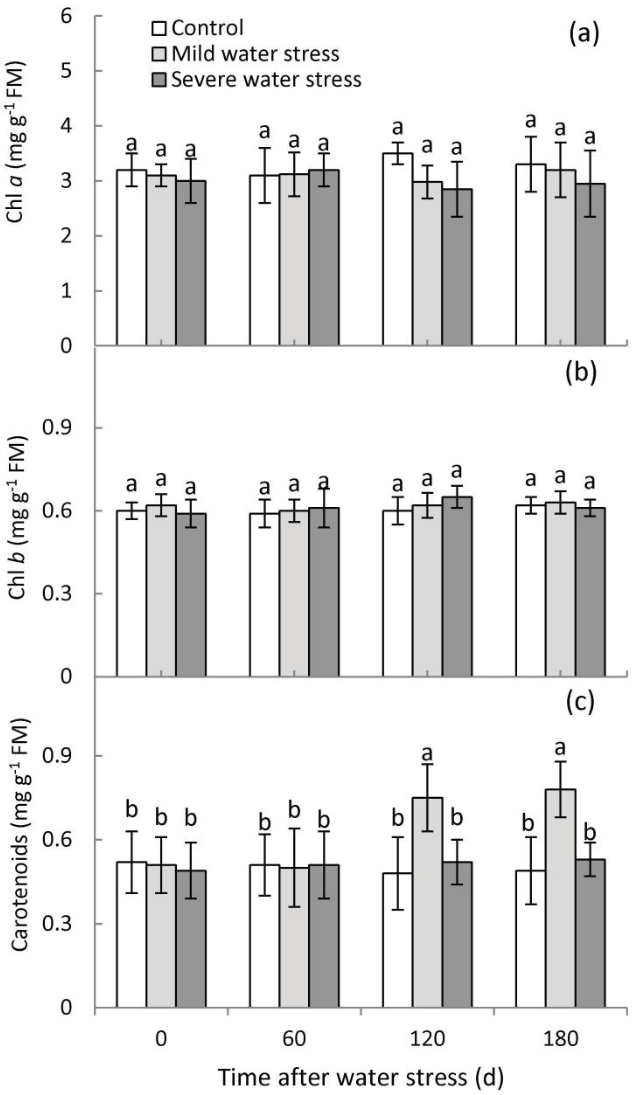

Figure 2: Effects of mild and severe drought stress on the content of chlorophyll *a*, *b* and total carotenoids in *Aloe vera* plants. Bars indicated with the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 4).

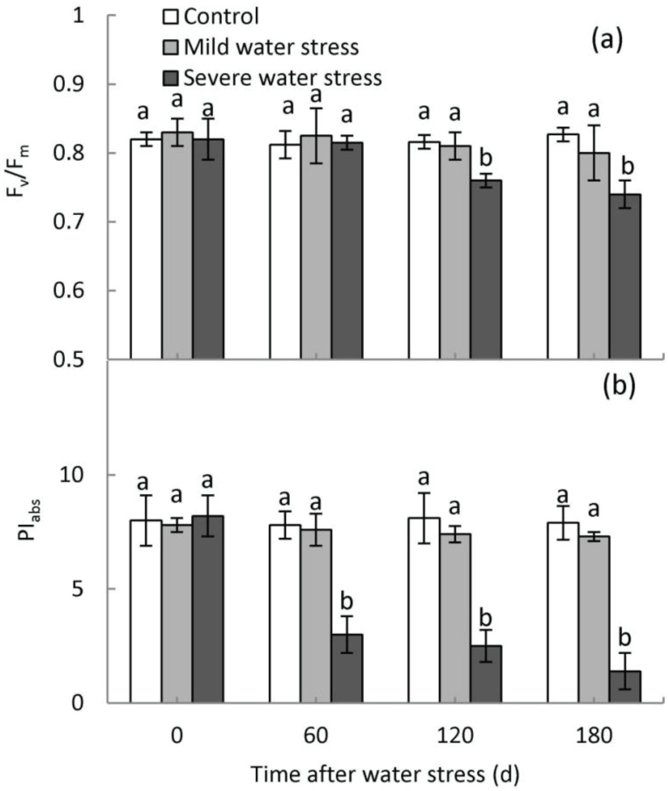

Figure 3: Effects of mild and severe drought stress on the maximum quantum yield of PSII (F_v/F_m) and the Performance Index (PI_{abs}) in *Aloe vera* plants. Bars indicated with the same letter are not significantly different (p < 0.05, Tukey test). Values are the mean ± SD (n = 4).

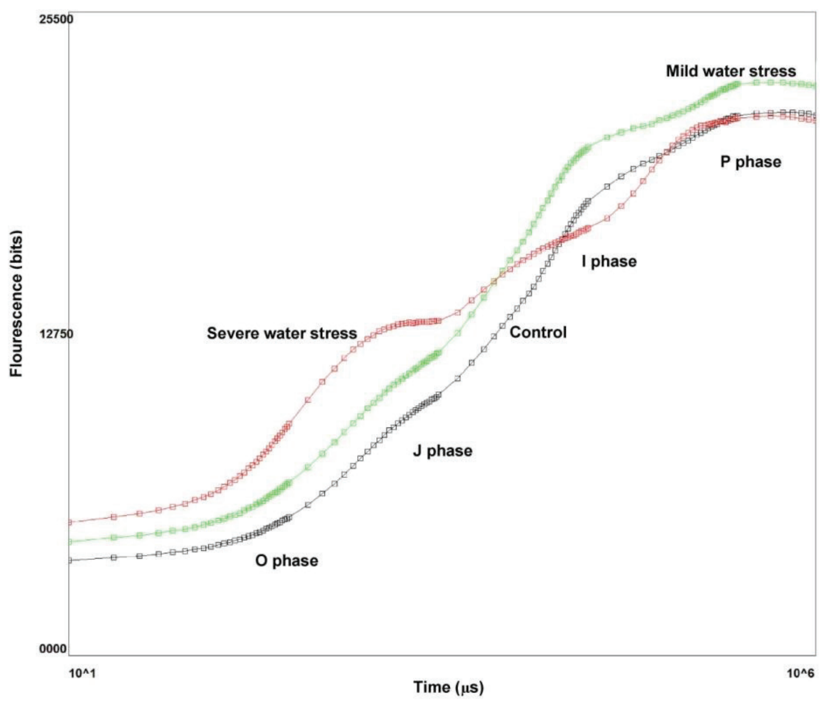

Figure 4: Effects of mild and severe drought stress on the chlorophyll *a* fluorescence induction curve of *Aloe vera* plants. Bars indicated with the same letter are not significantly different (p < 0.05, Tukey test). Values are the mean ± SD (n = 4).

3.4 Mild drought stress increases phenolic compounds production in *Aloe* leaves

Aloe leaves contain remarkably high amounts of phytochemical contents with therapeutic and preventive properties for human beneficial health effects. Phenolic compounds are the second major substances found in *Aloe vera*, which are used for medicinal purposes (Sathyaprabha et al., 2010; Lopez et al., 2013). Numerous studies have revealed that the quantity of phenolic acids in natural plants was affected by various environmental stresses (Lee et al., 2012; Zhang et al., 2015). In plants treated with drought stress for 180 days, total phenolic and flavonoids was increased under mild drought stress, while their contents remained unchanged under severe drought stress conditions (Fig. 5). After 120 and 180 days of exposure, however, an increase in anthocyanin levels was observed only in plants treated with severe drought stress. These result showed that long-term water loss may be necessary but not sufficient to induce anthocyanin compounds. In our experiments, accumulation of phenolic compounds in treated plants (Fig. 5) was correlated with PAL activation in the aloe leaves (Fig. 6). Under sever dfrought stress PAL activity suppressed, resulting in reduced phenolic compounds in the aloe leaves. Since phenolic compounds accumulation is associated with antioxidant activity (Aladedunye et al., 2008; Zhou et al., 2014; Lee et al., 2017), we investigated antioxidant properties including DPPH radicals in the present research. In more detail, the highest scavenging effect was observed under mild drought stress. As a result, the antioxidant activities of *Aloe* leaves against DPPH radicals were correlated with higher metabolite contents. In confirmation of this, there was a linear and positive correlation ($r = 0.77$, $P < 0.01$) between total phenol content and antioxidant activities against DPPH radicals in plants subjected to mild drought stress (Fig. 7). In addition, due to the protective effect of phenolic compounds in the screening of photoradiation (Takahashi and Badger, 2011) as well as their function as a non-enzymatic antioxidant during exposure to drought stress (Quan et al., 2016), this higher phenolic compounds accumulation in plants subjected to mild drought stress, may be an important protection mechanism for photosynthetic primary reactions of *Aloe* leaves under drought stress. This was corroborated by a linear and positive correlation ($r = 0.73$, $P < 0.01$) between total phenol content and PI_{abs} in plants subjected to mild drought stress (Fig. 7).

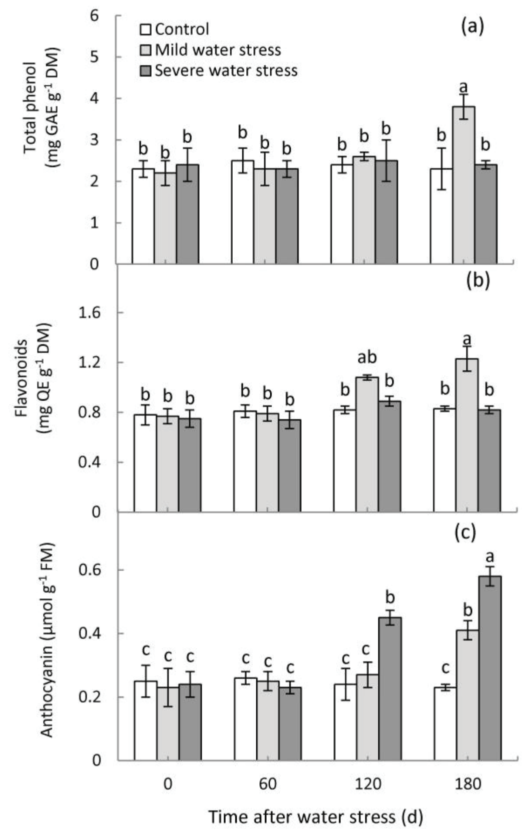

Figure 5: Effects of mild and severe drought stress on the total phenol, flavonoids and anthocyanin content in *Aloe vera* plants. Bars indicated with the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 4).

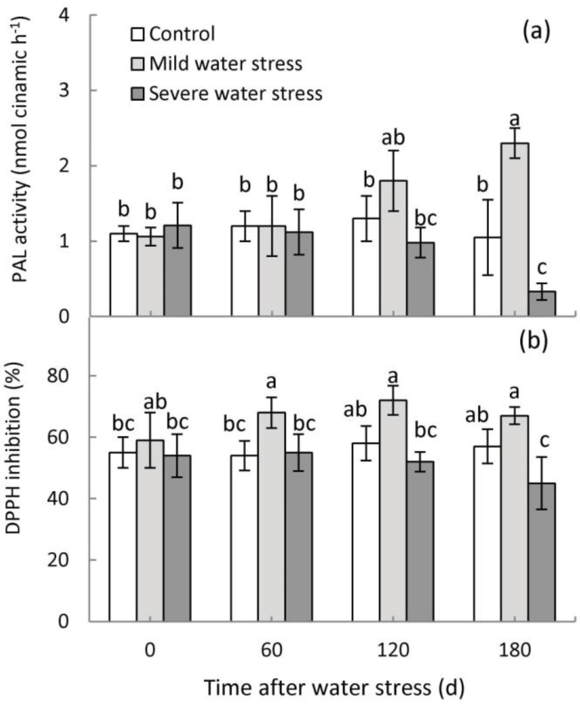

Figure 6. Effects of mild and severe drought stress on the activity of phenylalanine ammonia-lyase (PAL) and the antioxidant activities against DPPH radicals in *Aloe vera* plants. Bars indicated with the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 4).

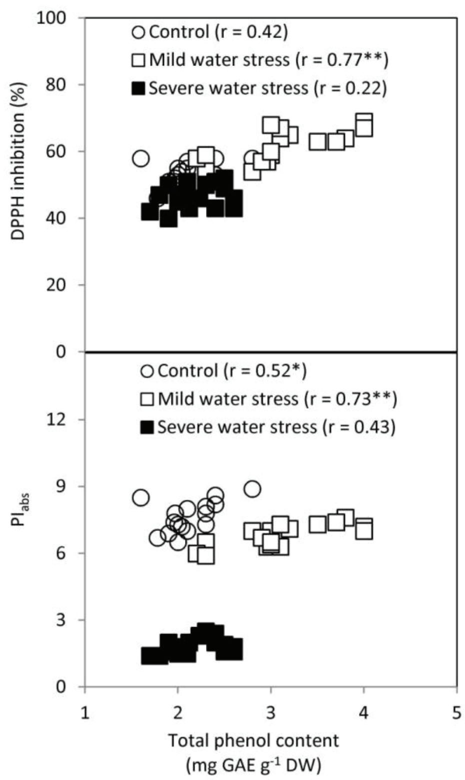

Figure 7: Effects of mild and severe drought stress on the correlation between values of performance index (PI_{abs}) or DPPH inhibition and total phenol content recorded in *Aloe vera* plants: ns, non-significant, * and **, significant at the 5 % and 1 % levels of probability, respectively.

Additionally, we assessed the main phenolic acids in the methanol extract of drought-stressed *Aloe* leaves using HPLC analysis. The typical chromatograms of *Aloe* leaves are presented in Fig. 8 and 9. The identification of each peak in the sample was performed using the retention time in the chromatogram. Since methanol is an appropriate solvent for the maximum extraction of phenolic compounds (Naczk and Shahidi, 2004), we used this solvent for extraction of phenolic acids in leaves. The quantification of each phenolic acids peak was done using the calibration curves in the range of 0.78-25 ppm, and their equations were measured as y = 130951x-143924 (chlorogenic acid, r^2 = 0.992), y = 97759x-97156 (vanillic acid, r^2 = 0.995) and y = 81796x-95603 (rosmarinic acid, r^2 = 0.998). This study revealed three major phenolic acid including rosmarinic acid, chlorogenic acid and vanillic acid in leaves. The metabolite profiles exhibited similar patterns except vanillic acid peak. Chlorogenic acid showed little differences with 500 µg g^{-1} (control) and 494 µg g^{-1} (mild drought stress), respectively. Moreover, no significant difference was observed in rosmarinic acid content of leaf among all treatments (control 547 µg g^{-1} and mild drought stress 551 µg g^{-1}). Under mild drought stress, vanillic acid content was significantly increased in drought-stressed leaves (845 µg g^{-1}) as compared with the control leaves (682 µg g^{-1}). Vanillic acid and chlorogenic acid are secondary metabolites, which have anti-inflammatory and anti-oxidative and anticancer properties (Chiang et al., 2003; Leal et al., 2011; Gengmao et al., 2015). For this reason, accumulation of vanillic and chlorogenic acid in the *Aloe* leaves under mild drought stress conditions was measured in this experiment. Here we showed that content of secondary metabolite of vanillic was highest at mild drought stress, suggesting that the level of vanillic acid like other plant secondary metabolites was influenced by the availability of water.

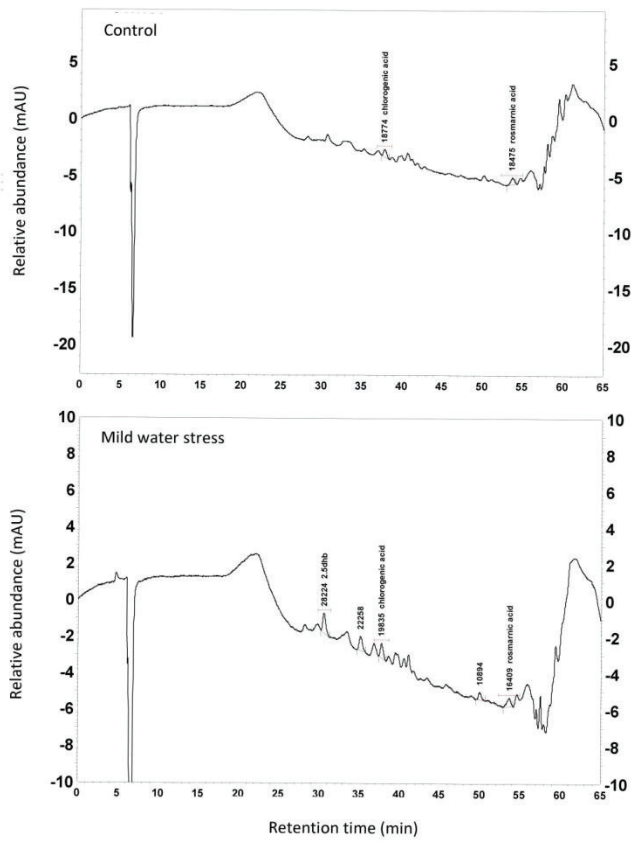

Figure 8: Effects of mild and severe drought stress on the representative chromatogram of prepared extract of *Aloe vera* leaves for chlorogenic acid and rosmarinic acid measurements.

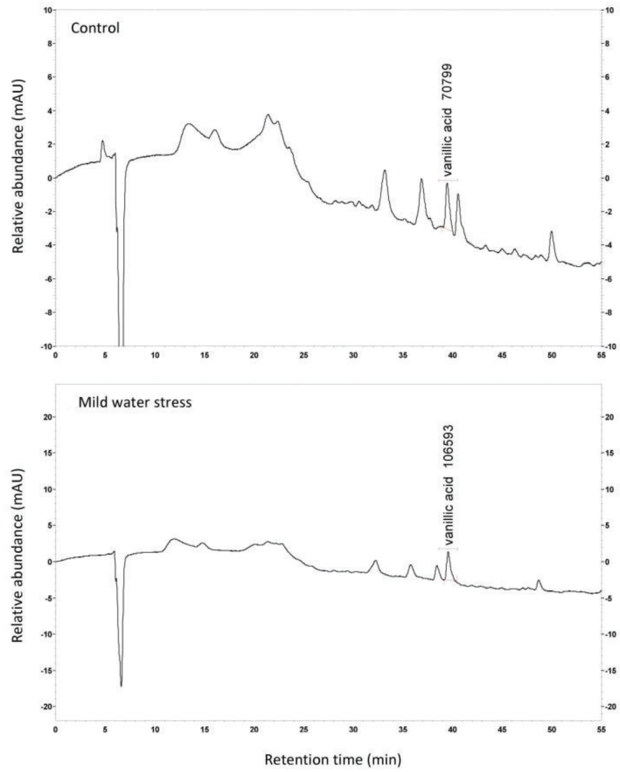

Figure 9: Effects of mild and severe drought stress on the representative chromatogram of prepared extract of *Aloe vera* leaves for vanillic acid measurement.

4 CONCLUSION

After 180 days of exposure, leaf thickness, biomass and gel production were reduced only under severe drfought stress. However, in line with physiological observations, medicinal *Aloe* plant adapted to mild drought stress during a long-term growth is mainly depended on compatible solutes adjustment, improvement of phenolic compounds production and presence of thick leaves. Mild drought stress improved growth by increasing photosynthesis via enhancing PI_{abs}. This improvement of PSII activity is coupled with the higher carotenoid and phenol production. In this study, the level of vanillic acid like other plant secondary metabolites was influenced by the availability of water, indicating that fluctuation in the content of vanillic acid was a plant response to environmental factors and part of an adaptative strategy leading to tolerance to abiotic stresses. However, considering conditions of natural habitat, biosynthesis and accumulation of vanillic acid by *Aloe* leaves in response to mild drought stress is not yet completely clear.

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Effects of exogenous proline on the physiological characteristics of *Triticum aestivum* L. and *Lens culinaris* Medik. under drought stress

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ABSTRACT

Proline, which is an indicator of stress, is often considered as a good parameter for the testing of plants with good drought tolerance capacity. Thus, exogenous application of proline is a possible technique to avoid the deleterious effects of the drought on plant growth. The objectives of this study are to investigate the impact exogenous proline on the physiological behavior of two plant species, bread wheat, a monocot, and lentil, a dicot, under drought stress conditions. After several preliminary tests, optimal concentrations of exogenous proline were determined (6 mM for bread wheat and 2 mM for lentil) and both species were treated in normal and drought conditions. The results showed that water deficit affected both species leading to a reduction in growth, chlorophyll content and relative water content. Likewise, 15 % PEG-6000, which is equivalent to osmotic potential of -0.31MPa, caused a high accumulation of proline. In almost of cases we also noted a remarkable decrease in catalase (Cat), ascorbate peroxidase (APX) and gaiacol peroxidase (GPX) activities which was probably due to the oxidative stress caused by drought stress. The application of proline in stressful conditions reduced the deleterious effects caused by the stress on both species, due, particularly, to the accumulation of free endogenous proline and the increase of Cat, APX and GPX activities.

Key words: *Triticum aestivum* L.; *Lens culinaris* Medik.; exogenous proline; drought tolerance

IZVLEČEK

UČINKI DODAJANJA PROLINA NA FIZIOLOŠKE LASTNOSTI KRUŠNE PŠENICE (*Triticum aestivum* L.) IN NAVADNE LEČE(*Lens culinaris* Medik.) V RAZMERAH SUŠNEGA STRESA

Proline je kot indikator stresa pogosto uporabljen za testiranje odpornosti rastlin na sušo. Z dodajanjem prolina se je mogoče izogniti škodljivim učinkom suše na rast rastlin. Predmet te raziskave je bil preučiti vpliv dodajanja prolina v rastni medij na fiziologijo dveh rastlinskih vrst, krušne pšenice kot enokaličnice in navadne leče kot dvokaličnice, gojenih v razmerah sušnega stresa. Po predhodnih poskusih sta bili določeni optimalni koncentraciji prolina, 6 mM za krušno pšenico in 2 mM za navadno lečo. Obe vrsti sta bili obravnavani s prolinom v normalnih in v sušnih razmerah. Rezultati so pokazali, da je pomanjkanje vode prizadelo obe vrsti, kar je vodilo v zmanšano rast, manjšo vsebnost klorofila in zmanjšano relativno vsebnost vode. Podobno je obravnavanje s 15 % PEG-6000, kar je enakovredno osmotskemu potencialu -0.31MPa, povzročilo veliko kopičenje prolina v obeh vrstah. Opažen je bil tudi znaten upad v aktivnosti katalaze (Cat) in askorbat perkosidaze (APX), kar je bilo verjetno posledica oksidacijskega stresa, povzročenega s sušo. Uporaba prolina je pri obeh vrstah zmanjšala škodljive učinke sušnega stresa z akumulacijo endogenega prolina, kar je tudi zmanšalo aktivnosti Cat, APX in GPX.

Ključne besede: *Triticum aestivum* L.; *Lens culinaris* Medik.; eksogeni prolin; odpornost na sušo

1 INTRODUCTION

Stress is defined as a set of disorders that adversely affect the plants development (Farooq et al., 2009). Abiotic stresses shall include drought, cold, salinity, etc. These stresses would lead to reducing field crop yield by 70 % via morphological and physiological alterations. As a consequence, a better understanding of the tolerance mechanisms for these stresses is in itself a major economic challenge (Passioura, 2007). The tolerance of plants to drought stress is a complex phenomenon. When plants are exposed to drought

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stress, they synthesize the osmoregulatory compounds which play the role of a regulator of osmotic pressure and a stabilizer of enzymes and membranes (Farooq et al., 2009). Consequently, the synthesized compounds play a major role to overcome the negative impact of drought stress. The proline is considered to be as one of the most widely distributed and accumulated osmolyte under various drought stress conditions. It is a proteinogenic amino acid and its cyclic form provides an exceptional conformational rigidity (Szabados & Savouré, 2009; Lehmann et al., 2010). Free proline is involved in the osmotic potential adjustment in a variety of plants that are subjected to hyper osmotic conditions (Verbruggen & Hermans, 2008). Additionally, it has been proven that the proline functions as a chaperone molecule that preserves the integrity of proteins, prevents their aggregation (Rajendrakumar et al., 1994), maintains membrane integrity (Ashraf & Foolad, 2007) and acts as an antioxidant which eliminates free radicals (Sharma & Dietz, 2006; Liang et al., 2013). The involvement of proline in plant tolerance to stress has attracted researchers since it has an efficient and influential role to maintain good plant production under stress conditions. For example, overproduction of proline would play a role of cryoprotectant under chilling conditions (Gleeson et al., 2004). Proline applied exogenously ameliorated photosynthetic pigments content, stomatal conductance and CO_2 assimilation under drought and salinity conditions (Ben Hassine et al., 2008; Ben Ahmed et al., 2010). The results carried by Gleeson et al. (2004) showed that over-accumulation proline may have a role in protection of forest species from environmental stresses. However, a high concentration of proline can inhibit growth and causes other deleterious effects on cellular metabolism, according to Mani et al. (2002). Ashraf & Foolad (2007) suggest that the protective effect of proline depends on several parameters: the type of plant species, developmental stage, and the optimal concentration. On the basis of these parameters, we intended to investigate the consequences of the exogenous proline on the growth and the physiology for wheat and lentil subjected to drought stress. Since, osmotic stress can result in oxidative stress, we proposed to study antioxidant enzymes activities of catalase (Cat), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) which are the key enzymes of the adaptive response by maintaining the redox balance during oxidative stress. *Triticum aestivum* L. and *Lens culinaris* Medik have been choosen for this study due to their economic importance and because these two species belong to different plant classes (monocot and dicot).

2 MATERIALS AND METHODS

2.1 Plant material

The present study was conducted on two species, bread wheat (***Triticum aestivum*** 'Arz') and lentil (***Lens culinaris*****'**Dahra'). The seeds were obtained from Technical Institute of Field Crops (Algeria). These species have been chosen due to their great economic importance and to their belonging to different plant classes (monocot and dicot). Wheat var. Arz, harvested in the region of Tlemcen (West Algeria), is adapted to semi-arid region. Lentil var. Dahra, harvested in the region of Tiaret (West Algeria), presents an adaptation to drought and high temperatures. After germination, the seedlings of both species were transposed in potting soil-filled pots (65 g). The characteristics of the soil are: medium structure, pH 6, electrical conductivity (EC) of 450 $\mu S.cm^{-1}$, N 270 of mg l^{-1}, P_2O_5 of 150 mg l^{-1}, and K_2O of 300 mg l^{-1}. The pots were kept under ambient conditions (photoperiod 16 h and day/night temperatures 24/18 °C).

2.2 Treatments imposed on bread wheat and lentil seedlings

Treatments consisted in watering with 6 mM proline solution for bread wheat and 2 mM for lentil (C+P), with 15 % PEG-6000 solution (S) or 15 % PEG-6000 solution plus 6 mM proline solution for bread wheat and 2 mM for lentil (S+P). Control was watered with distilled water (C). The first application of treatments (50 ml) was applied after a week of growth, and the second application was applied in the third week. The leaves of control and stressed seedlings have been sampled after one month of growth (in the third stage for the bread wheat and in the seventh stage for the lentil leaves).

2.3 Growth measurement

For each treatment, growth measurement was taken on one month old plant. Five plants taken for each treatment, were used to calculate the mean of each parameter. The measurements taken were: height of shoot, fresh mass of leaves and roots, dry mass of leaves and roots (by oven drying at 80 °C for 48 h).

2.4 Determination of relative water content (RWC)

Leaves' turgor was estimated by the determination of RWC according to Barrs (1968). The leaf discs mass of lentil and leaf segments of wheat were measured immediately after sampling (fresh mass) and then, after

24 h of incubation in distilled water (mass at full turgor). Subsequently, the leaf discs were dried in oven at 80 °C for 24 h to obtain their dry mass. Relative water content was calculated from the following formula:

RWC = (fresh mass - dry mass) X 100/ (fresh mass of full turgor - dry mass)

2.5 Cell membrane integrity evaluation

Cell membrane integrity was estimated by measuring the relative leakage of electrolytes from foliar discs of the lentil and foliar segments of the wheat. The leakage of electrolytes was measured according to the method described by Bajji et al. (2002). Electrical conductivity was measured before (E1) and after the sample was boiled at 100 °C for 1 h (E2). The percentage of electrolytes leakage was calculated according to the following relation: Electrolyte leakage (%) = (E1/E2) X 100

2.6 Determination of photosynthetic pigments content

The contents of chlorophylls (a,b) and carotenoids were determined by extraction in 80 % acetone. Measured absorption values were used for chlorophyll and carotenoids contents calculation according to Lichtenthaler (1987):

$\text{Chl a } (\mu g\ ml^{-1}) = 12{,}25 \times OD_{663} - 2{,}79 \times OD_{647}$
$\text{Chl b } (\mu g\ ml^{-1}) = 21{,}5 \times OD_{647} - 5{,}10 \times OD_{663}$
$\text{Chl a+ Chl b } (\mu g\ ml^{-1}) = 7{,}15 \times OD_{663} + 18{,}71 \times OD_{647}$
$\text{Carotenoids } (\mu g\ ml^{-1}) = (1000 * D.O_{470} - 1{,}82) \times (\text{Chl a} - 85{,}02)/198$

2.7 Proline assay

The proline amount in the leaves was determined according to the method describbed by Troll & Lindsley (1955) and modified by Magné & Larher (1992). Sample of 50 mg dry mass of leaves was homogenized with 1 ml of distilled water at 90 °C during 30 minutes. After centrifugation at 12000 rpm for 10 min, 500 µl aliquot of the supernatant was mixed with 1 ml of the reagent mixture (60 ml glacial acetic acid, 40 ml distilled water and 1 g ninhydrin) and heated in sealed test tubes at 95 °C for 30 minutes. After cooling down, 3 ml toluene was added to each sample. Proline content was read on a spectrophotometer at 520 nm and expressed as $mg.g^{-1}$ dry mass.

2.8 Total soluble protein quantification

An amount of 100 mg of samples were grinded with 1ml of 100 mM Tris-HCl buffer (pH 8.1) containing 10 % sucrose, 10 mM Na-EDTA and 0.05 % β-mercaptoéthanol. After centrifugation at 15000 rpm for 20 minutes, the supernatant was used to estimate soluble protein contents and antioxidant enzyme activity. Protein concentration was determined by measuring the absorbance at 595 nm. The protein content of each sample was determined by spectrophotometer according to the method of Bradford (1976) using Bovine Serum Albumin (BSA) as a protein standard.

2.8 Oxidative stress assessment

2.8.1 Measurement of lipid peroxidation

Lipid peroxydation was determined in terms of malondialdehyde (MDA) content by measuring the concentration of MDA, based on the method described by Alia et al. (1995). The leaf samples (100 mg) were weighed and homogenized in 2 ml of 0.1 % trichloroacetic acid (TCA) solution. The mixture was centrifuged at 12000 rpm for 20 minutes at 4 °C. Then, 0.5 ml of the supernatant was mixed with 0.5 ml of 0.5 % thiobarbituric acid (TBA) in 20 % TCA. The reaction mixture was heated in water bath at 95 °C for 30 minutes. cooled to room temperature and then centrifuged at 1000 rpm for 10 minutes. The absorbance of supernatant at 532 nm was determined and nonspecific absorbance of supernatant at 600 nm was subtracted from it. The MDA content was calculated by using the extinction coefficient of $\varepsilon = 155\ mM^{-1}cm^{-1}$ and expressed as nmol of MDA g^{-1} fresh mass.

2.8.2 Catalase activity

Catalase (Cat) activity was assayed as described by Dorey et al. (1998). The reaction mixture in a total volume of 2 ml contained 25 mM sodium phosphate buffer (pH 7.0) and 10 mM H_2O_2. The reaction was initiated by the addition of 100 µl of the protein exact containing enzyme, and Cat activity was assayed by monitoring the disappearance of H_2O_2 at 240 nm for 1 minute ($\varepsilon_{240} = 36\ mM^{-1}\ cm^{-1}$).

2.8.3 APX activity

Ascorbate peroxidase activity activity was determined according to Nakano and Asada (1981). The reaction mixture in a total volume of 1.5 ml contained 50 mM sodium phosphate buffer pH 7.0, 0.1 mM Na_4EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2, and 100 µl of the enzyme extract. H_2O_2-dependant oxidation of ascorbate was followed by decrease in the absorbance at 290 nm for 1 minute ($\varepsilon_{290} = 2.8\ mM^{-1}cm^{-1}$).

2.8.4 Guaiacol peroxidase (GPX)

Guaiacol peroxidase (GPX) activity was estimated according to MacAdam et al. (1992). For this assay, 100 mg of fresh leaves were homogenized in 1 ml of 50 mM (KH_2PO_4/K_2HPO_4) buffer (pH 6.5). The homogenate was centrifuged at 12000 g for 20 minutes at 4 °C. The supernatant was used for enzyme activity and protein content assays. All steps during enzyme extract preparation were carried out at 4 °C. The reaction mixture contained 100 µl enzyme extract, 18 mM guaiacol and 50 µl H_2O_2. The enzyme activity was calculated using absorption coefficient for tetraguaiacol (26.6 mM^{-1} cm^{-1}) at 470 nm and was expressed as µmoles tetratgaicol.min^{-1}.mg^{-1} protein.

2.9 Statistical analysis

The results were depicted in histogram and table forms, representing the averages of the measured five values as well as their standard error (mean ± standard error). The results were evaluated statistically using the Student t-test at $P \leq 0.05$.

3 RESULTS

3.1 Shoot height

Osmotic stress led to an important reduction in shoot heigth, especially for wheat with decreases from 20.3±0.33 to 13.8±0.30 cm (-32.26 %). However, under normal conditions, the exogenous proline provoked a positive effect on wheat seedlings heigth (+28.33 %) but not significant effect on lentil seedlings. Under stressful conditions, applied proline increased highly bread shoot heigth (+48.87 %) versus a low increase for lentil (+8.46 %) (Figure 1).

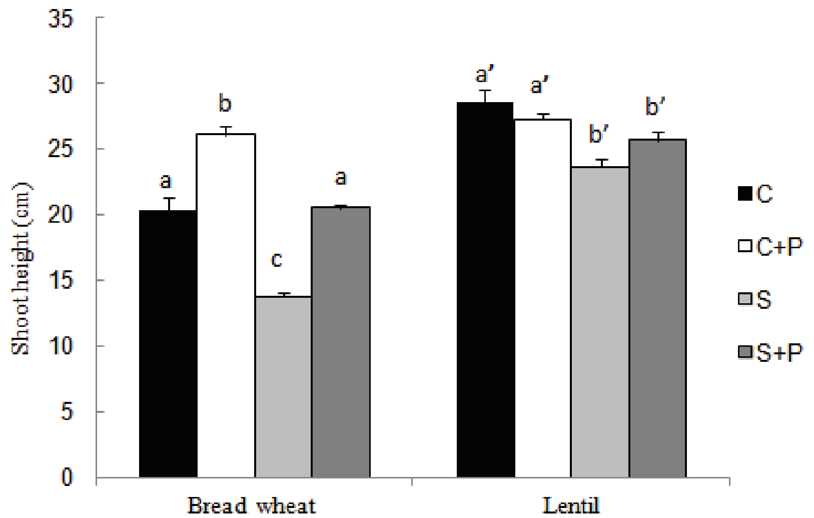

Figure 1: Effect of exogenous proline on shoot height of seedlings of *Triticum aestivum* and *Lens culinaris* under normal and drought stress conditions. Error bars represent the standard errors of the means and in some cases, the error bars are too small to be visible. For each species, different letters show significant differences ($P \leq 0.05$).

3.2 Fresh and dry leaves and roots mass

Osmotic stress induced a significant reduction ($P \leq 0.05$) in fresh and dry mass of leaves and roots of bread wheat and lentil seedlings. Under PEG exposure, exogenous proline induced a significant increase in fresh mass of wheat compared to stressed seedlings without exogenous proline. Thes increases reached +24.29 % and +24.71 % for leaves of bread wheat and lentil respectively, and +78.72 % and +47.12 % for roots of wheat and lentil respectively (Table 1).

Table 1: Effect of exogenous proline on fresh and dry mass of leaves and roots of bread wheat and lentil seedlings under normal and drought stress conditions

Treatments	Fresh mass of leaves (mg) Bread Wheat	Fresh mass of leaves (mg) Lentil	Dry mass of leaves (mg) Bread wheat	Dry mass of leaves (mg) Lentil
C	290.32±33.62 **a**	534±13.13 **a'**	28.57±3.36 **a**	59.85±1.60 **a'**
C+P	319.47±6.32 **a**	461.93±12.31**b'**	37.15±3.5 **b**	51.37±1.08 **a'**
S	152.40±16.55 **b**	267.40±14.54 **c'**	20.22±2.88 **c**	34.03±1.91**b'**
S+P	267.35±14.42 **a**	332.37±8.17 **d'**	36.02±4.86 **b**	42.43±2.17 **c'**
	Fresh mass of roots (mg) Bread Wheat	Fresh mass of roots (mg) Lentil	Dry mass of roots (mg) Bread wheat	Dry mass of roots (mg) Lentil
C	183.67±9.42 **a**	325.48±17.25 **a'**	16.3±1.85 **a**	20.37±2.28 **a'**
C+P	177.35±12.76 **a**	310.07±10.57 **a'**	17.35±4.65 **a**	20.5±20 **a'**
S	84.92±40.44 **b**	133.93±4.62 **b'**	10.77±1.17 **b**	10.93±2.47 **b'**
S+P	199.125±4.53**a**	239.35±20.97 **c'**	17.25±1.05 **a**	16.07±2.38 **a'**

Data represent mean ± standard error (SE). For each species and each organ, means in the same column, followed by ifferent letters are statistically different ($p < 0.05$).

3.3 Relative water content

Water stress induced a reduction of the relative water content (RWC) in leaves of wheat and lentil by 17.51 % and 17.45 %, respectively in comparison to the control plants. Under stressed conditions, the application of exogenous proline improved the relative water content of the leaves of both species. In the case of bread wheat, the RWC value was equal to untreated control (Figure 2).

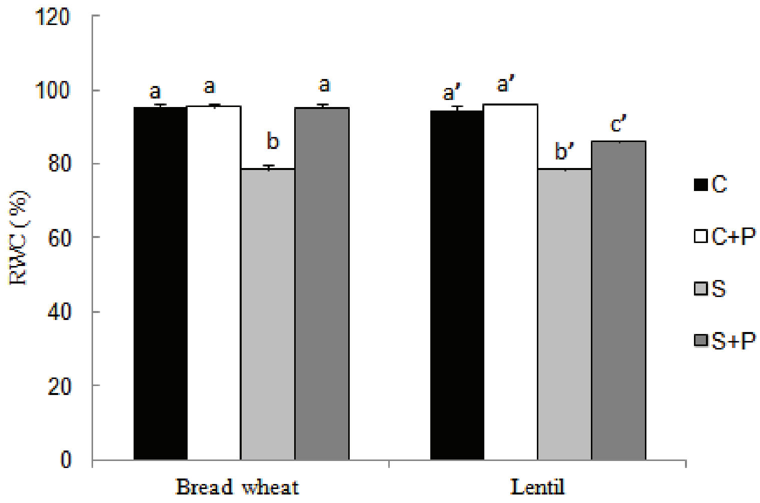

Figure 2: Effect of exogenous proline on relative water content of seedlings leaves of *Triticum aestivum* and *Lens culinaris* under under normal and drought stress conditions. Error bars represent the standard errors of the means. For each species, different letters show significant differences ($P \leq 0.05$).

3.4 Cell membrane integrity

Cell membrane integrity was assessed by electrolyte leakage. PEG treatment caused an important increase of electrolyte leakage in the leaves of wheat and lentil (52.03 % and 30.48 %, respectively) compared to the control plants. Under stress conditions, this electrolyte leakage has been reduced in the presence of exogenous proline in comparison to the stressed seedlings in the same rate for both species but with a lower absolute value for bread wheat (Figure 3).

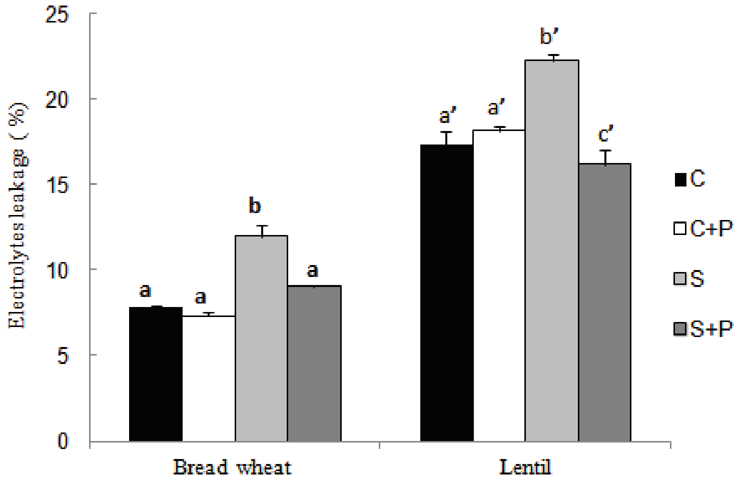

Figure 3: Effect of exogenous proline on electrolytes leakage of seedlings leaves of *Triticum aestivum* and *Lens culinaris* under under normal and drought stress conditions. Error bars represent the standard errors of the means. For each species, different letters show significant differences (P ≤ 0.05).

3.5 Chlorophyll content

The total chlorophyll and carotenoids contents were significantly (P ≤ 0.05) decreased under drought stress in both species. Under stress conditions, the addition of proline resulted in a significant increase (P ≤ 0.05) in leaf chlorophyll contents of the wheat seedlings (+43.87 %) and carotenoids (+54.42 %) in comparison to the stressed without proline. In contrast, for the lentil seedlings, no positive effect has been observed. Under normal conditions and with exogenous proline, Chl a:Chl b ratio did not vary significantly for both species (P ≤ 0.05). However, under water stress conditions, proline application on bread wheat increased this ratio but it had no effect on lentil (Table 2).

Table 2: Effect of exogenous proline on chlorophylls and carotenoids contents of leaves of bread wheat and lentil seedlings under normal and drought stress conditions

Treatments	Chlorophyll (a+b) (mg.g^{-1} fresh mass)		Chlorophyll a/ b		Carotenoids (mg.g^{-1} fresh mass)	
	Bread wheat	Lentil	Bread wheat	lentil	Bread wheat	lentil
C	0.061±0.004 **a**	0.23±0.04 **a'**	1.98±0.06 **a**	2.1±0.06 **a'**	2.31±0.18 **a**	0.042±0.005 **a'**
C+P	0.054±0.002 **a**	0.26±0.03 **a'**	1.95±0.07 **a**	2.23±0.01 **a'**	1.84±0.17 **a**	0.045±0.001 **a'**
S	0.038±0.001 **b**	0.18±0.044 **b'**	1.12±0.20 **b**	2.17±0.03 **a'**	1.21±0.10 **c**	0.033±0.007 **b'**
S+P	0.055±0.002 **a**	0.18±0.08 **b'**	1.93±0.40 **a**	2.16±0.11 **a'**	1.86±0.10**a**	0.034±0.001 **b'**

Data represent mean ± standard error (SE). For each species and each organ, means in the same column, followed by different letters are statistically different (p < 0.05).

3.6 Free proline content

The accumulation of proline was significantly higher in stressed plants (**S**) for both species in comparison to the control (+133.33 % for wheat and +114.58 % for lentil). In the absence of stress, application of proline induced significant differences ($P \leq 0.05$) in proline content with +44.44 % for wheat and +41.66 % for lentil. The presented results revealed that the highest level of proline content in leaves was recorded by supplying proline under both conditions (Figure 4).

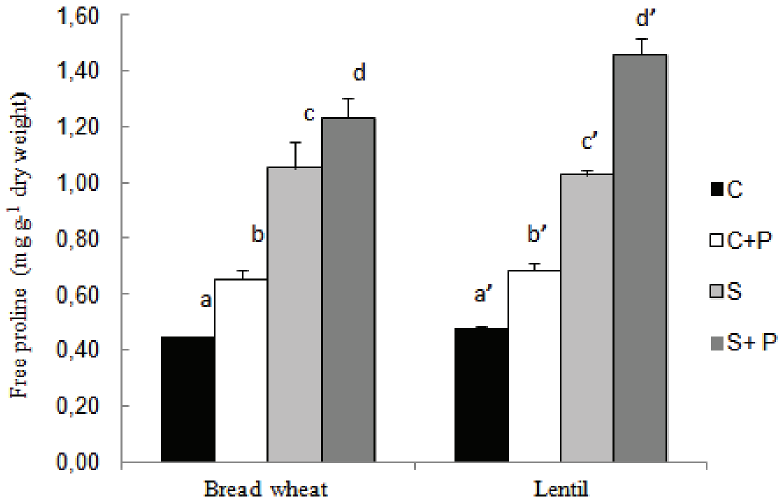

Figure 4: Effect of exogenous proline on free proline content of seedlings leaves of *Triticum aestivum* and *Lens culinaris* under under normal and drought stress conditions. Error bars represent the standard errors of the means. For each species, different letters show significant differences ($P \leq 0.05$).

3.7 Total soluble protein content

Osmotic stress induced a significant reduction ($P \leq 0.05$) of the total soluble protein content of lentil seedlings leaves (-48.48 % compared to the control). In contrast, for the wheat seedlings, no effect has been observed. Under PEG treatment, exogenous proline induced a significant increase in protein content of wheat (+27.08 %) and in lentil seedlings (+54.01 %) compared to stressed seedlings (S) (Figure 5).

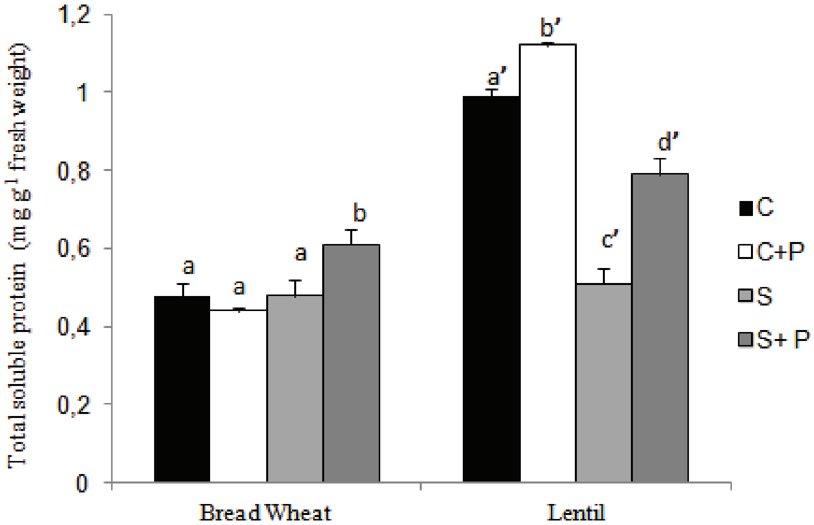

Figure 5: Effect of exogenous proline on total soluble protein content of seedlings leaves of *Triticum aestivum* and *Lens culinaris* under under normal and drought stress conditions. Error bars represent the standard errors of the means. For each species, different letters show significant differences ($P \leq 0.05$).

3.8 MDA content

PEG resulted in increased accumulation of MDA content by +89.26 % and +95.45 % in wheat and lentil seedlings, respectively, compared to control seedlings (Figure 6). Proline in combination with PEG reduced MDA content in bread (-87.72 %) and lentil (-53.48 %) compared to stressed without proline (S).

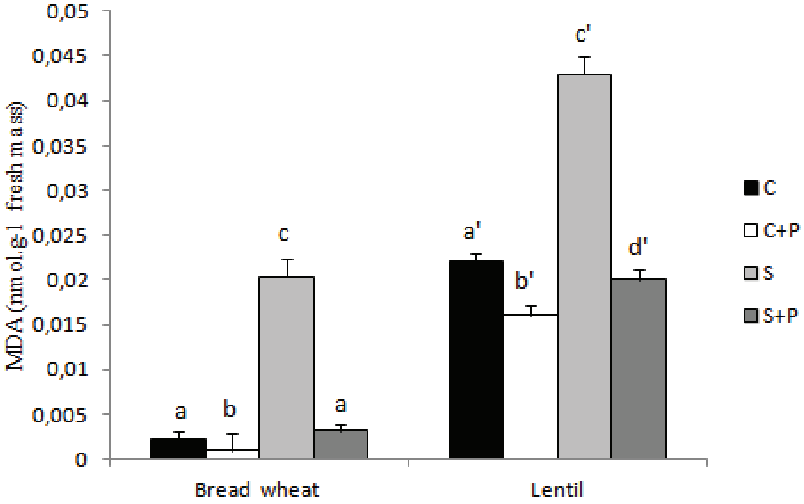

Figure 6: Effect of exogenous proline on the MDA content of seedlings of *Triticum aestivum* and *Lens culinaris* under under normal and drought stress conditions. Error bars represent the standard errors of the means. For each species, different letters show significant differences (P ≤ 0.05).

3.9 Catalase activity

Under stressful conditions, there was no significant change in the catalase activity in lentil leaves, but this activity decreased significantly (P ≤ 0.05) in the case of wheat (-45.35 %) compared to the control plants. Under these stressful conditions, the addition of proline amplified the catalase activity especially in wheat seedlings. The catalase activity increased for +197.02 % in the case of wheat and for +85.98 % in lentil compared to the stressed seedlings (S). Under unstressed conditions, the exogenous proline resulted in a significant decrease in catalase activity in the leaves of both species (-37.13 % for wheat and -30.44 % for lentil) in comparison to control seedlings (C) (Figure 7).

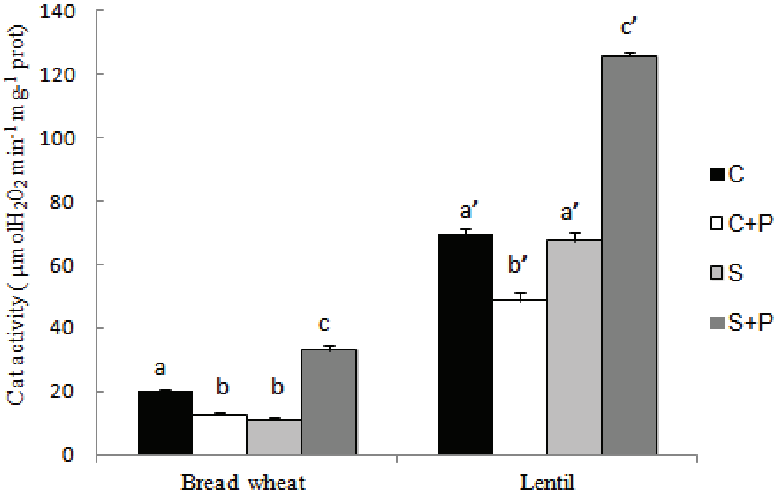

Figure 7: Effect of exogenous proline on the catalase activity of seedlings of *Triticum aestivum* and *Lens culinaris* under under normal and drought stress conditions. Error bars represent the standard errors of the means. For each species, different letters show significant differences (P ≤ 0.05).

3.10 Ascorbate peroxidase activity (APX)

PEG caused a significant ($P \leq 0.05$) decrease in APX activity in the leaves of wheat and lentil (-67.50 % and -40.74 %, respectively) compared to the control (Figure 8). Also, the addition of proline under stress conditions increased APX activity in lentil (+56.25 %) in comparison to the stressed (S). However, exogenous proline reduced APX activity in wheat (-11.50 %).

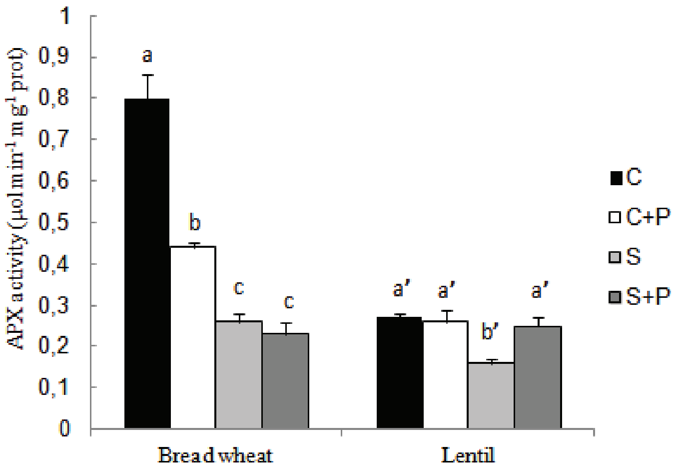

Figure 8: Effect of exogenous proline on the APX activity of seedlings of *Triticum aestivum* and *Lens culinaris* under under normal and drought stress conditions. Error bars represent the standard errors of the means. For each species, different show significant differences ($P \leq 0.05$).

3.11 Guaiacol peroxidase (GPX)

Drought stress caused a significant reduction ($P \leq 0.05$) in activity of GPX for both species**.** We noted -46.15 % for bread wheat and -20 % for lentil in comparison to the control plants.

Under normal and drought stress conditions, supply of proline significantly increased activity, especially for bread wheat (Figure 9).

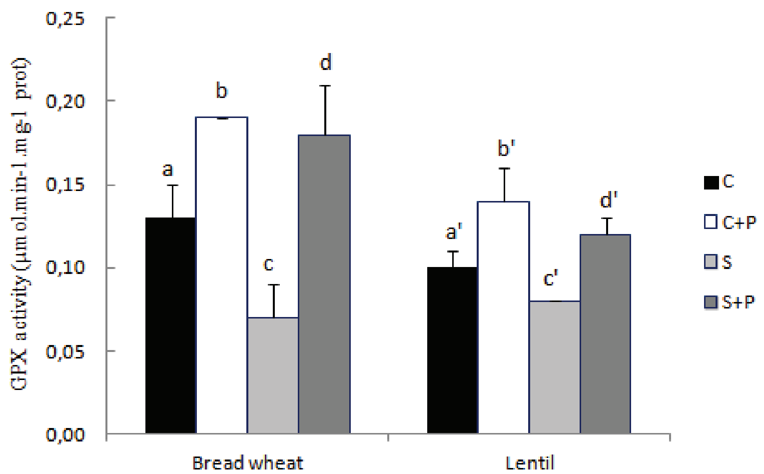

Figure 9: Effect of exogenous proline on the GPX activity of seedlings of *Triticum aestivum* and *Lens culinaris* under under normal and drought stress conditions. Error bars represent the standard errors of the means. For each species, different show significant differences ($P \leq 0.05$).

4 DISCUSSION

4.1 Effect of drought stress

In the presence of polyethylene glycol (PEG-6000), the results showed a decrease in growth of wheat and lentil seedlings. This can be explained by a increase of the osmotic pressure in the medium which prevented the absorption of water by the root system. Osmotic stress decreased the relative water content in both studied species, indicating that drought stress induced a decrease of turgor in wheat and lentil leaves.

A decrease in chlorophylls and carotenoids contents were also recorded. This consequently led to a reduction in growth resulting in a decrease in cell turgor and photosynthesis (Akcay et al., 2011). Exposure to drought stress leads to a significant effect in chlorophyll a and b contents (Ranjbarfordoei et al., 2000). The reduction in chlorophylls content under drought stress may be because of inhibition of chlorophyll synthesis or an acceleration of its degradation or membrane deterioration (Smirnof, 1995). Osmotic stress induced by PEG-6000 can lead to lipid peroxidation and, consequently, chlorophyll destruction also, with decreasing chlorophylls and carotenoids contents.

The effect of increased osmotic pressure caused by drought stress resulted in a significant increase in endogenous proline level in both species. It has been reported that the proline is an important compatible solute accumulated in higher plants under conditions of abiotic stress (Delauney & Verma, 1993). Proline plays a crucial role in osmoregulation and osmotolerance (Szabados & Savouré, 2009). It is considered as biomarker of stress. An increase in free proline content during dought stress conditions due to PEG has also been shown in different crops as maize (Meeta et al., 2013) and bread wheat (Ji et al., 2014). Handa et al. (1986) suggested that the level of proline accumulation depends not only on the plant osmotic potential or loss of turgor, but also on its level of stress adaptation of the plant. During stress, the expression of *P5CS* (pyrroline-5-carboxylate synthetase), but not of *P5CR* (pyrroline-5-carboxylate reductase) gene, is well correlated with proline content (Savouré et al., 1995).

Osmotic stress did not affect leaf total soluble protein content of wheat seedlings, but it reduced this content in the lentil. Changes in protein expression, accumulation, and synthesis have been observed in many plant species as a result of plant exposure to drought stress (Akhzari & Pessarakli, 2015; Li et al., 2018).The reduction in protein content in plants under drought stress is due to proteins synthesis inhibition, acceleration of the proteolysis process or reduction in the amino acids content (Dubey & Rani, 1990). Nitrate reductase, implied in protein synthesis, is the most altered enzyme by drought (Sepehr et al., 2012).

Drought stress led to oxidative stress illustrated for both species by enhanced electrolyte leakage. Electrolyte leakage is considered as an indicator of loss in membrane integrity as a result of lipid peroxidation (Bajji et al., 2002). Similar results were obtained by Bandurska et al. (2001). They reported that a high PEG concentration increased the membrane permeability of barley varieties. As consequence of this peroxidation, both species exhibited an obvious increase of MDA content which was directly related to the oxidative stress induced by PEG. However, plants possess a battery of antioxidant mechanisms, both enzymatic and non-enzymatic, by which ROS are removed from the cell (Noctor & Foyer, 1998).

Among non-enzymatic systems, proline provides a protective role in drought induced oxidative stress by reducing H_2O_2 levels and by increasing the antioxidant defense system (Molla et al., 2014). Catalase is the most responsive of enzymes mechanisms to H_2O_2 (Gondim et al., 2012). However, our results showed that this enzyme activity did not vary for lentil and has been significantly decreased in wheat seedlings. This decrease was reported by Alam et al. (2014). Contradictory results, where the catalase activity is increased, have been reported by Yuan et al. (2014) and Antonić et al. (2016). In fact, the behavior of antioxidant enzyme not only depends on the severity and duration of the stress treatment, but they also depends on the species and age of the plant (Carvalho, 2008). Our results showed that activities of APX and GPX decreased in response to osmotic stress. Indeed, Lokhande et al. (2010) and Jisha & Puthur (2015) observed a decrease in APX and GPX activities due to application of PEG.

4.2 Application of exogenous proline

Exogenous application of proline mitigated stress-induced inhibitory effects on the growth of both species. In the present study, proline supplementation increased free proline content in wheat and lentil leaves. However, this amino-acid was more accumulated in lentil leaves. Similar responses were observed in *Lepidium sativum* (Khalil & El-Noemani, 2012) and in rice (Hasanuzzaman et al., 2014; Samota et al., 2017). These facts allow us to suppose that exogenous proline was absorbed by the roots, transported and distributed to the leaves. Indeed, Bar-Nun and Poljakoff-Mayber (1977), showed that radioactive exogenous proline was incorporated in roots of *Pisum sativum* L. and *Tamarix tetragyna* Ehrenb.. Schobert et al. (1988) have reported the same observation for castor bean roots. Thereafter, Rentsch et al. (1996) have showed the existence of proline transporters in plant. Moreover, Okuma et al. (2000) have demonstrated by non accumulation of glutamate that proline accumulation in *Nicotiana tabacum* L. cultured cells was due to exogenous proline. Free proline accumulation in both species due to stimulation by drought stress was, thus, increased by exogenous supplementation. The positive effects of proline on plant growth would be explained by its role as a nutrient, as well as its role as an osmoprotectant and its implication in osmoadjustment (Dawood et al., 2014). Our results showed that exogenous proline increased relative water content in both species despite stressful conditions allowing the plant to stabilize leaf water balance. Additionally, proline reduces transpiration via its regulatory effect of the opening/closing of the stomata (Yancey, 2005). Futhermore, proline supply showed that degradation in glutamate was increased (Mani et al., 2002). These results suggest that proline can be a good source of energy during stress too, and that the second step of the oxidation pathway is not rate limiting.

Exogenous proline increased the chlorophyll content, especially in bread wheat seedlings. These results are in accord with Rasheed et al. (2014). These authors noted that the addition of 20 mM proline under oxidative stress causes an increase in the chllorophylls and carotenoids contents in leaves of bread wheat. Shahid et al. (2014) showed that exogenous proline improved the growth by increasing photosynthesis. The effect of exogenous proline on chlorophyll contents may also have been due to stabilizing photosynthetic reactions (Abdelhamid et al., 2013).

Exogenous proline increased protein leaves content, especially in lentil seedlings. Similar results have been reported by Demir & Kocacaliskan (2002). These authors noted that the addition of 10 mM exogenous proline under salt stress causes an increase in the protein content in the bean leaf. Khedr et al. (2003) showed that 10 mM exogenous proline improves the salt-tolerance of *Pancratium maritimum* L. by protecting the protein turnover machinery against stress-damage and up-regulating stress protective proteins. Proline has been shown to act as a chemical protein chaperone and to prevent protein aggregation and thermodenaturation (Ignatova & Gierasch, 2006).

The prevention of oxidative stress-induced damages was resulted in a lesser membrane peroxidation thanks to enhanced catalase activity, especially for bread wheat. The same response was observed by Rady & Hemida (2016**)** in maize seedlings and Singh et al. (2016) in *Solanum melongena* L.

PEG had a strong effect on MDA production in both species. On the other side, significant reduction in MDA content was observed when proline was applied to wheat and lentil seedling. These results suggested positive correlation between decreased production MDA and increased in APX and Cat activities under stressful conditions.

Our study showed that under stressed conditions, the addition of proline enhanced APX activity in leaves of lentil. An increase in APX activity has been observed in sugarcane by application of 20 mM proline (Medeiros et al., 2015). In contrast, Kibria et al. (2016) showed that exogenous application of 25 and 50 mM proline, reduced APX activity in *Triticum aestivum*. Additionally, exogenous proline enhanced the activity of GPX. An increase in GPX activity was due to proline pretreatment.

5 CONCLUSION

The main aim of this study was to show the major role of exogenous proline in improving the plant stress tolerance. We conclude that for bread wheat and lentil, the addition of exogenous proline minimized the damages caused by water stress simulated by PEG-6000. Our results showed that the contribution of exogenous proline improved the growth of the aerial part, reduced membrane damages, and improved cell turgor. Additionally, the exogenous proline enhanced the activity of an antioxidant enzymes (Cat, APX and GPX), and increased the level of endogenous proline content. Interestingly, tolerance of bread wheat (a monocot) to water stress was more improved compared to lentil (a dicot). All these results suggest that proline is absorbed by the roots of the studied plants.Thus, under water stress, the presence of compatible solutes such as proline is a promising approach in the amelioration of vegetable production. However further investigations are needed to determine the most effective concentrations and number of applications as well as the most responsive growth stage(s) of each species. Furthermore, it remains to be understood the mechanisms of action of exogenous proline.

6 ABREVIATIONS

C:Control; C+P: Control+Proline; PEG-6000: Polyethelene glycol 6000; S: Stressed; S+P: Stressed+Proline**.**

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Plenilske pršice (Acari: Phytoseiidae), prvič najdene na gojenih rastlinah v Sloveniji v obdobju 2012-2017

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IZVLEČEK

V obdobju 2012-2017 smo na različnih gostiteljskih rastlinah v Sloveniji preučevali zastopanost domorodnih vrst plenilskih pršic. V obsežni raziskavi smo potrdili zastopanost vrst *Amblyseius andersoni* (Chant, 1957), *Euseius finlandicus* (Oudemans, 1915), *Euseius gallicus* (Kreiter & Tixier, 2010), *Euseius stipulatus* (Athias-Henriot, 1960), *Kampimodromus abberans* (Oudemans, 1930), *Neoseiulus californicus* (McGregor, 1954), *Paraseiulus triporus* (Chant & Shaul, 1982) in *Phytoseius horridus* (Ribaga, 1904). V prispevku predstavljamo vseh sedem vrst, za področje biotičnega varstva rastlin pa so najzanimivejše vrste *A. andersoni*, *N. californicus* in *E. gallicus*. Medtem ko sta prvi dve vrsti že vpisani na Seznam domorodnih vrst organizmov za namen biotičnega varstva rastlin, pa bo pršica *E. gallicus* na ta seznam uvrščena v bližnji prihodnosti. Vse tri vrste plenilskih pršic imajo velik aplikativni pomen in jih najdemo na velikem številu vrst gostiteljskih rastlin.

Ključne besede: plenilske pršice; Phytoseiidae; prve najdbe; biotično varstvo rastlin; Slovenija; *Euseius gallicus*

ABSTRACT

PREDATORY MITES (ACARI: PHYTOSEIIDAE) FIRST RECORDED ON CULTIVATED PLANTS IN SLOVENIA IN THE PERIOD 2012-2017

In the period 2012-2017 we investigated the occurrence of indigenous species of predatory mites in different cultivated plants in Slovenia. In a comprehensive study we confirmed the occurrence of the following predatory mites: *Amblyseius andersoni* (Chant, 1957), *Euseius finlandicus* (Oudemans, 1915), *Euseius gallicus* (Kreiter & Tixier, 2010), *Euseius stipulatus* (Athias-Henriot, 1960), *Kampimodromus abberans* (Oudemans, 1930), *Neoseiulus californicus* (McGregor, 1954), *Paraseiulus triporus* (Chant & Shaul, 1982) and *Phytoseius horridus* (Ribaga, 1904). In the paper all seven species are presented, but for the field of biological control *A. andersoni*, *E. gallicus* and *N. californicus* are the most interesting species. The first two of them are already on the List of indigenous organisms for the purpose of biological control, while the third will be placed into it in the near future. All three of them have a great potential in biological control, since they can be found in different host plants.

Key words: predatory mites; Phytoseiidae; first record; biological control; Slovenia; *Euseius gallicus*

1 UVOD

Plenilske pršice iz družine Phytoseiidae spadajo med pomembne biotične agense, ki lahko uspešno sodelujejo pri zatiranju človeškim očem težko vidnih škodljivih organizmov iz taksonov Tetranychoidea, Eriophyoidea, Thysanoptera in Homoptera (Stojnić et al., 2002) v sadovnjakih, vinogradih in na zelenjadnicah, gojenih na prostem in v zavarovanem prostoru (Palevsky et al., 1999), prehranjujejo pa se tudi z rastlinskim sokom in pelodom (Blackwood et al., 2001).

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Namen našega prispevka je predstaviti rezultate vzorčenja domorodnih naravnih sovražnikov v obdobju 2012-2017, z namenom povečanja Seznama domorodnih vrst organizmov za namen biotičnega varstva rastlin v Sloveniji. V prispevku se osredotočamo na pomen pri nas prvič najdenih vrst plenilskih pršic v varstvu rastlin.

2 MATERIALI IN METODE

2.1 Vzorčenje plenilskih pršic

S spremljanjem zastopanosti plenilskih pršic smo začeli v letu 2012. Od junija do oktobra smo na različnih vrtninah in sadnih vrstah nabirali vzorce listov. Vzorčenje je junija potekalo na Zgornji Lipnici (46.322427; 14.187794) v travniškem sadovnjaku na navadni slivi. V avgustu smo v Slovenski Istri na različnih lokacijah nabirali vzorce vrtnin, v ekstenzivnem sadovnjaku na Laboratorijskem polju Biotehniške fakultete (46.049403;14.473137) pa smo vzorčili liste jablan, na katerih so bile predhodne vidne poškodbe rdeče sadne pršice (*Panonychus ulmi* [Koch]).

V letu 2014 smo od maja do oktobra v sklopu načrtnega vzorčenja škodljivih žuželk v dveh različnih travniških rastlinjakih na območju Ribnice in Kočevja nabirali liste jablan, kjer smo opazili poškodbe pršic prelk (Tetranychidae). Prvo vzorčenje je potekalo v 200 m^2 velikem sadovnjaku v Rakitnici (45.69121, 14.756171), drugo pa v 150 m^2 velikem sadovnjaku v Kočevski Reki (45.571227;14.809602).

V letu 2016 smo med aprilom in oktobrom v sklopu načrtovanega vzorčenja škodljivih žuželk na različnih lokacijah v Slovenski Istri nabirali vzorce gojenih in samoniklih rastlin. V septembru smo na večjem številu listov fige (*Ficus carica* L.) ugotovili poškodbe pršic prelk. Prvo vzorčenje je potekalo na figah v Vinjolah (45.501990; 13.620267), drugo pa na posestvu Purissima (45.572714; 13.775552) v bližini Ankarana. Vzorčili smo fige, ki so rastle v bližini rastlinjakov.

S spremljanjem škodljivih žuželk smo nadaljevali v letu 2017. V jugozahodni Sloveniji smo na različnih lokacijah in gostiteljskih rastlinah nabrali poškodovane liste zaradi pršic prelk. 21. julija smo v Bukovici (45.89.5899; 13.665020) nabrali vzorce listov kumar in paprike, ki so jih gojili v rastlinjaku. Na isti dan smo v Šempetru pri Gorici (45.93217; 13.64504) in v Novi Gorici (45.95657; 13.64259) nabrali liste malin oziroma vrtnic. V 21. avgusta smo v rastlinjaku na posestvu Purissima nabrali poškodovane liste paprik, na smokvi v Serminu (Bertokih) pa smo nabrali poškodovane liste smokve. 27. julija smo v Zgornji Pohanci (45.93848; 15.54116) na jagodah nabrali tiste liste, ki so kazali poškodbe zaradi fitofagnih pršic.

V vseh primerih smo nabrane liste shranili v plastične vrečke in jih v hladilni torbi odpeljali v Laboratorij za fitomedicino na Biotehniški fakulteti v Ljubljani, kjer smo pod stereomikropskopom vzorce pregledali. Najdene plenilske pršice smo shranili v 70 % etanol.

2.2 Determinacija plenilskih pršic

Determinacija šestih vrst plenilskih pršic je bila izvedena na Montpellier SupAgro, UMR CBGP, SupAgro/CIRAD/INRA/IRD v Montpellieru v Franciji, vrste *Euseius gallicus* pa na National Reference Centre (NRC), National Plant Protection Organization (NPPO), Netherlands Food and Consumer Product Safety Authority (NVWA), Ministry of Agriculture, Nature and Food Quality v Wageningenu na Nizozemskem.

Morfološka determinacija vrst je bila izvedena na način, ki ga nazorno opisujejo Kreiter et al. (2018ab).

3 REZULTATI IN DISKUSIJA

3.1 Vrste iz rodu *Euseius*

3.1.1 *Euseius stipulatus* (Athias-Henriot, 1960)

Pršico *Euseius stipulatus* (Athias-Henriot) smo v Sloveniji prvič našli 27. julija 2017 v plastenjaku z jagodami (*Fragaria* x *ananassa* Duchesne) v vasi Zgornja Pohanca. Najdena vrsta plenilske pršice spada v rod *Euseius*, kamor je bilo doslej uvrščenih 212 vrst (McMurtry in sod., 2013). Rod *Euseius* spada v četrto skupino plenilskih pršic, kamor spadajo plenilske pršice, ki za svoj razvoj potrebujejo pelod. Poleg peloda pa se prehranjujejo tudi s fitofagnimi pršicami. Med sadnimi vrstami ji najbolj ustrezajo pelod mandljevca, slive, češnje in marelice (Bouras in Papadoulis, 2005). To pršico najdemo tudi na vinski trti (Global Biodiversity Information Facility, 2018).

Zastopanost plenilskih pršic iz rodu *Euseius* v nasadih citrusov je na območju Sredozemlja zelo velika (Abad-Moyano in sod., 2010). Vrsta *E. stipulatus* je značilni predstavnik južnega območja zahodnega Palearktika (Gbif, 2018). Pojavlja se v Grčiji, Turčiji, Italiji, Španiji

in Alžiriji (Bouras and Papadoulis, 2005), pa tudi v Turčiji, Franciji, Tuniziji, Siriji, na Portugalskem, Črni Gori, Madeiri, na Madžarskem in na Kanarskih otokih. Poleg z različnimi vrstami peloda se vrsta *E. stipulatus* največrat prehranjuje s fitofagno pršico *Panonychus citri* (McGregor) (Abad-Moyano in sod., 2010).
Dosedanje raziskave kažejo, da se ta plenilska pršica lahko prehranjuje tudi z navadno pršico (*Tetranychus urticae* Koch), vendar pri njenem zatiranju ni tako učinkovita kot nekatere druge plenilske pršice, na primer *Neoseiulus californicus* (McGregor) in *Phytoseiulus persimilis* Athias-Henriot. Pomembna neželena lastnost pršice *E. stipulatus* je, da pleni jajčeca ostalih vrst plenilskih pršic, s katerimi tekmuje za hrano (Abad-Moyano in sod., 2010). McMurtry in sod. (1992) navajajo, da so »splošni plenilci«, kamor spadajo predstavniku rodu *Euseius*, pri zmanjševanju populacij fitofagnih pršic učinkoviti predvsem takrat, kadar je njihova populacija nizka.

3.1.2 *Euseius finlandicus* (Oudemans, 1915)

Ta pršica se najpogosteje pojavlja na listih sadnega drevja, kot so jablane, breskve in češnje (Broufas in Koveos, 2001). Najdemo pa jo tudi na listih drugih drevesnih vrst (*Cercis* sp., *Crataegus* sp., *Ligustrum* sp., *Pittosporum* sp., *Juglans* sp., *Morus alba* L., *Rubus ulmifolius* Schott idr.), tudi takšnih, ki rastejo v gozdu. Omenjena pršica je ena od redkih vrst iz rodu *Euseius*, ki se pojavljajo v območjih z zmernih podnebjem (Broufas in Koveos, 2001), pri nas pa smo jo prvič našli 21. junija 2013 na listih sliv na Zgornji Lipnici.

Ta plenilska vrsta velja za enega od pomembnejših naravnih sovražnikov fitofagnih pršic v sadovnjakih (Abdallah in sod., 2001). Glede na način prehranjevanja jo uvrščamo med splošne plenilce fitofagnih pršic in specialiste na pelodu (Schausberger in Croft, 1999, Kasap, 2009), saj se lahko prehranjuje (in svoj razvojni krog zaključi) na pršicah prelkah (Tetranychidae), pršicah šiškaricah (Eriophyidae), mehkokožnatih pršicah (Tarsonemidae) in pršicah iz družine Acaridae-Tyroglyphidae. Prehranjuje pa se lahko tudi s pelodom različnih rastlinskih vrst (Kasap, 2009), celičnim sokom, sporami gliv, z medeno roso in z jajčeci in ličinkami manjših žuželk (Abdallah in sod., 2010). Razvojni krog te pršice je glede na ugotovitve Abdallah et al. (2010) najkrajši, ko pleni pršice šiškarice, najdaljši pa tedaj, ko se pršica prehranjuje s pelodom. Omenjena plenilska vrsta velja za pomembnega naravnega sovražnika fitofagnih pršic, kot sta vrsti *Panonychus ulmi* in *Aculus schlectendali* (Nalepa) (Kasap, 2009).

3.1.3 *Euseius gallicus* (Kreiter & Tixier, 2009)

Vrsta je bila v Evropi prvič najdena na jugu Francije leta 2010 (Tixier et al., 2010), v Sloveniji pa 21. julija 2017 v Šempetru pri Gorici na listih robide. Dőker in sod. (2014) navajajo njeno zastopanost še v Belgiji, Turčiji, Nemčiji in na Nizozemskem. Za splošne plenilce iz rodu *Euseius* velja, da se lahko prehranjujejo tudi na pelodu. Pršica *Euseius gallicus* zelo hitro zaključi razvojni krog ob prisotnosti peloda širokolistnega rogoza (*Typha latifolia* L.) (Van Houten et al., 2016).

Dosedanje raziskave omenjeno to plenilsko vrsto kot učinkovitega naravnega sovražnika rastlinjakovega ščitkarja na vrtnicah (Van Houten in sod., 2016). Ko se samice vrste *E. gallicus* prehranjujejo z jajčeci rastlinjakovega ščitkarja, navadno odložijo več jajčec (3,6 jajčec na dan), kot pri hranjenju z ličinkami cvetličnega resarja (1,5 jajčeca/dan). Preferenca plenilca do različne hrane se kaže v situacijah, ko ima na razpolago več vrst hrane, saj ob razpoložljivosti peloda širokolistnega rogoza in ličink cvetličnega resarja raje izbere pelod (Van Houten in sod., 2016). Krajšanje dneva ne vpliva na vstop samic v diapavzo. Znano je tudi, da razvoj mlajših stadijev pršic poteka tudi pri 13 °C (Van Houten in sod., 2016).

Podjetje Biobest Belgium N.V. skrbi, da je omenjena pršica tržno dostopna v pripravku Dyna-Mite® G-System. Glede na navedbe v tehničnem listu pripravka (Technical sheet…, 2018) lahko plenilska pršica svoj razvojni krog razvije že pri 10 °C, pa vse do 32 °C. Njen razvoj je možen že pri 50 % relativni zračni vlagi. Idealne razmere za razvoj plenilca so sicer pri 25 °C in 70-80 % relativni zračni vlagi.

3.2 *Kampimodromus abberans* (Oudemans, 1930)

To pršico uvrščamo v poddružino Amblyseiinae Muma, kamor poleg plemena Kampimodromini Kolodochka uvrščamo še 8 plemen. Doslej so v pleme Kampimodromini uvrstili 90 vrst plenilskih pršic iz različnih rodov (Chant in McMurtry, 2006).

V rodu *Kampimodromus* Nesbitt, 1951 je trenutno 15 vrst. Vrsto *K. abberans* smo našli na dveh različnih lokacijah v Slovenski Istri, in sicer 7. septembra 2016 v Vinjolah in na posestvu Purissima na listih fig, 21. julija 2017 pa na navedenem posestvu na listih kumar v rastlinjaku, v Šempetru pri Gorici na listih malin na prostem in v Novi Gorici na listih vrtnic na prostem. Prva najdba te vrste pri nas sicer datira v leto 2006, ko je bila najdena na listih jablane (Miklavc, 2006; Bohinc and Trdan, 2013).

Pršico *K. abberans* so med drugim našli na jablani, leski in vinski trti. Pojavlja se v Evropi in v Severni Ameriki. Velja za pomembnega naravnega sovražnika pršice prelke *Eotetranychus carpini* (Oudemans) in pršic šiškaric. Ob odsotnosti pršic se lahko prehranjuje na ličinkah ameriškega kaparja (*Diaspidiotus perniciosus* [Comstock]) in pelodu različnih rastlinskih vrst

(Broufas in sod., 2007). Duso in sod. (2009) navajajo, da je ta plenilec zelo občutljiv na fitofarmacevtska sredstva, zato je v intenzivni pridelavi jabolk praktično ne zasledimo. Zaradi njene učinkovitosti pa je veliko raziskav usmerjenih v iskanje odpornih populacij na fitofarmacevtska sredstva.

3.3 *Neoseiulus californicus* (McGregor, 1954)

Prve najdbe tega plenilca v Sloveniji datirajo v leto 2012 (Trdan in sod., 2013), ko je bil 29. avgusta v Kortah najden na jajčevcu na prostem, 30. avgusta pa v Ljubljani na melonah v rastlinjaku in na jablani. 21. julija 2017 je bila pršica najdena tudi v Bukovici pri Novi Gorici, in sicer na papriki v rastlinjaku.

Vrsta *N. californicus* spada med plenilske pršice, katere so že izvorno zastopane na območju Sredozemlja in na območjih z njemu podobnim podnebjem po celem svetu. Glede na način prehranjevanja jo uvrščamo v skupino pršic tipa II-III, kar pomeni, da za svoj plen izbira specifične skupine fitofagnih organizmov. Omenjena plenilska vrsta se omenja kot uspešen biotični agens pri zatiranju pršic prelk (Tetranychidae), predvsem navadne pršice (*Tetranychus urticae*) (Kim sod., 2012) in rdeče sadne pršice (*Panonychus ulmi*) (El Taj in Jung, 2012). Prehranjuje se lahko tudi s pelodom in drugimi malimi žuželkami (Ferragut Peréz in sod., 2010) ter z mehkokožnimi pršicami.

Omenjena pršica dobro prenaša tudi nizko zračno vlago (Weintraub in Palevsky, 2008) in visoke temperature (Ferragut Peréz in sod., 2010). Pri zanjo idealnih razmerah (15-35°C) in dostopnostjo vrste *Tetranychus urticae* (kot plena) ima lahko omenjena plenilska pršica do 28 rodov na leto (Weintraub in Palevsky, 2008).

Najdba vrste *N. californicus*, ki je znana tudi pod starim imenom *Amblyseius californicus*, je bila prvič omenjena v Kaliforniji (Zhang, 2003). Danes jo najdemo tudi v Srednji in Južni Ameriki in v Južni Evropi (Zhang, 2003; Walzer in sod., 2007), kar kaže, da ji bolj ugajajo aridna območja. Pojavlja se na sadnih vrstah (marelice, breskve, jablane, hruške, kivi, citrusi, vinska trta), poljščinah (soja, koruza, bombaž,…) in na zelenjadnicah (jajčevec, bučke, paprika, solata,…) (Ferragut Peréz in sod., 2010). Nekatere rase so zelo odporne na fitofarmacevtska sredstva, kar omogoča lažjo uporabo tega biotičnega agensa v integriranem varstvu rastlin (Walzer in sod., 2007).

Vrsta *N. californicus* velja med pršicami iz družine Phytoseiidae za tržno najbolj dostopno (Walzer in sod., 2007). Glede na podatke EPPO je uporaba omenjene vrste kot komercialnega biotičnega pripravka že dovoljena v Belgiji, Nemčiji, Franciji, Grčiji, Italiji, Španiji, Švici, Tuniziji in na Češkem, Danskem, Finskem, Nizozemskem (List of biological control agents …, 2018).

3.4 *Paraseiulus triporus* (Chant & Shaul, 1982), *Phytoseius horridus* (Ribaga, 1904) *in Amblyseius andersoni* (Chant, 1957)

Plenilska pršica *Paraseiulus triporus* je bila v Sloveniji prvič najdena leta 2006 na jablanah (Miklavc, 2006, Bohinc and Trdan, 2013). V letu 2017 smo jo 21. julija našli v Bukovici na kumarah v rastlinjaku. Rod *Paraseiulus* uvrščamo v prvo skupino plenilskih pršic, kamor spadajo vrste, ki se prehranjujejo samo na prelkah (McMurtry in sod., 2013).

Dosedanje raziskave omenjajo njeno zastopanost v Srbiji (Mladenović in sod., 2013), Franciji (van der Linden, 2008), Grčiji (Papadoulis and Emmanouel, 1997), na Češkem (Kabicek, 2010; Kabicek 2017), Slovaškem (Praslicka in sod., 2009) in Madžarskem (Szabo in sod., 2013).

Pršico *Phytoseius horridus* smo našli v dveh različnih travniških sadovnjakih, in sicer v Rakitnici in Kočevski Reki, v juniju 2014, kar je omenjeno že v Bohinc and Trdan (2015). Gre za prvo najdbo te vrste v Sloveniji, ki sicer nima večjega gospodarskega pomena.

Plenilska pršica *Amblyseius andersoni* je bila v Sloveniji prvič ugotovljena že v letu 2006 (Miklavc, 2006; Bohinc and Trdan, 2013). V letu 2017 smo jo našli na listih fige 21. avgusta v Slovenski Istri (Sermin, Bertoki). Omenjena pršica je v naravi zastopana na območju Palearktika in Nearktika. Je pomemben naravni sovražnik pršic *Aculops lycopersici* (Tryon), *Aculus schlechtendali*, *Panonychus ulmi*, *Phytonemus pallidus* (Banks), *Polyphagotarsonemus latus* (Banks), *Tetranychus urticae* in *Tetranychus cinnabarinus* (Boisduval). V varstvu rastlin v tržni pridelavi se ta plenilska pršica uporablja v Franciji, Italiji, Španiji, Sloveniji in na Poljskem (List of biological control agents …, 2018).

Pršica *Amblyseius andersoni* spada v skupino III, kamor uvrščamo plenilske pršice, ki so splošni plenilci. Kot koristni organizem je najbolj učinkovita na listih trte, ki niso poraščeni s trihomi (McMurtry et al., 2013).

4 SKLEPI

Naša raziskava je temeljila na preučevanju zastopanosti plenilskih pršic na različnih gojenih rastlinah. Ugotovili smo, da je na Seznam tržno dostopnih koristnih organizmov v Evropi uvrščenih 16 vrst plenilskih pršic (List of biological control agents …, 2018). Na podlagi rezultatov naše raziskave sta bili na Seznam domorodnih vrst organizmov za namen biotičnega varstva rastlin v Sloveniji uvrščeni plenilski pršici *Amblyseius andersoni* in *Neoseiulus californicus.*

Poleg omenjenih dveh pa bo v bližnji prihodnosti na ta seznam uvrščena tudi pršica *Euseius gallicus*, za katero sicer skromno število raziskav potrjuje, da je lahko učinkovit plenilec ličink rastlinjakovega ščitkarja (*Trialeurodes vaporariorum*). Ta pršica je uporabna predvsem v okrasnem vrtnarstvu, saj je zelo učinkovita pri varstvu vrtnic pred rastlinjakovim ščitkarjem. Kljub temu, da ima rastlinjakov ščitkar tudi druge naravne sovražnike, pa je njihov razvoj otežen, saj odlagajo jajčeca na liste in cvetove vrtnic, ki so podvrženi hitrejšemu odstranjevanju (Van Heuten in sod., 2016).

Pri uporabi plenilskih pršic v integriranem varstvu rastlin je pomembnih več dejavnikov. Med drugim je potrebno paziti moramo na sočasno uporabo s fitofarmacevtskimi sredstvi. Medtem, ko je le nekaj fungicidov škodljivih za plenilske pršice, velja omenjeno za skoraj vse insekticide in akaricide. O delovanju herbicidov na plenilske pršice je bilo do sedaj zelo malo raziskav (Fountain and Medd, 2015). Pomemben vpliv pri razmnoževanju plenilskih pršic imajo tudi dostopnost hrane, oblika lista in pelod gostiteljske rastline, kot tudi semiokemikalije, ki jih rastline oddajajo (Gerson, 2014).

Po doslej znanih podatkih zastopanosti plenilskih pršic v Sloveniji lahko ugotovimo, da je zastopanost plenilskih pršic v Sloveniji velika, a vse vrste niso ustrezne za implementacijo v okoljsko sprejemljive načine rastlinske pridelave. Zato bo potrebno nadaljevati z njihovim načrtnim spremljanjem, da bi našli morebitne nove vrste, ki se v drugih evropskih državah že uporabljajo v biotičnem varstvu rastlin.

5 ZAHVALA

Rezultati, predstavljeni v tem prispevku, so bili pridobljeni v okviru Programa strokovnih nalog s področja zdravstvenega varstva rastlin, ki ga financira Ministrstvo za kmetijsko, gozdarstvo in prehrano RS – Uprava RS za varno hrano, veterinarstvo in varstvo rastlin.

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Uporabnost izbranih invazivnih tujerodnih rastlin pri zatiranju škodljivih organizmov gojenih rastlin

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IZVLEČEK

Invazivne tujerodne rastline s hitrim razširjanjem povzročajo nemalo težav ne le domorodnim sortam rastlin, ker jih spodrivajo, temveč tudi ljudem, saj so pogosto vzrok za številne zdravstvene težave, kot so alergije, pripomorejo pa tudi k večji gospodarski škodi. Varstvo rastlin je dejavnost, ki na podlagi ustreznih znanstvenih dognanj z različnimi metodami in na gospodaren način varuje gojene rastline pred škodljivimi organizmi. Pri nas in drugod po svetu je zaradi cene in drugih prednosti najbolj razširjena uporaba fitofarmacevtskih sredstev (FFS) v varstvu rastlin pred škodljivimi organizmi. Zaradi negativnih vplivov na okolje, njihovega neciljnega delovanja, pojava rezistence škodljivih organizmov na FFS in vse strožje okoljske politike raziskovalci iščejo nove, okoljsko bolj sprejemljive načine varstva rastlin pred škodljivimi organizmi. Eden od tovrstnih ukrepov je tudi preučevanje rastlinskih izvlečkov pri zatiranju gospodarsko pomembnih škodljivih organizmov. V preglednem članku smo se osredotočili na pregled literature vezane na uporabnost rastlinskih izvlečkov izbranih tujerodnih rastlinskih vrst v varstvu rastlin; japonskega dresnika (*Fallopia japonica* [Houtt.] Ronse Decr.), češkega dresnika (*Fallopia* x *bohemica* [Chrtek & Chrtková] Bailey), velikega pajesena (*Ailanthus altissima* [Mill.] Swingle), kanadske zlate rozge (*Solidago canadensis* L.), orjaške zlate rozge (*Solidago gigantea* Aiton), octovca (*Rhus typhina* L.), navadne amorfe (*Amorpha fruticosa* L.) in smrdljivo ditrihovko (*Dittrichia graveolans* [L.] Greuter), ki jih preučujemo v sklopu projekta ApPLAuSE.

Ključne besede: invazivne rastlinske vrste; *Fallopia japonica; Fallopia* x *bohemica; Ailanthus altissima; Solidago canadensis; Solidago gigantea; Rhus typhina; Amorpha fruticosa;* rastlinski izvlečki; varstvo rastlin

ABSTRACT

APPLICABILITY OF INVASIVE ALIEN PLANTS IN CONTROLLING HARMFUL ORGANISMS OF CULTIVATED PLANTS

With a rapid growth invasive alien plants cause a lot of problems not only to indigenous varieties of plants, but also for people, as they are often the cause of many health problems, such as allergies, and they also contribute to greater economic loss. Plant protection is an activity which, on the basis of appropriate scientific knowledge by means of various methods and an economical manner, protects cultivated plants from harmful organisms. In Slovenia, as well as worldwide, the use of pesticides in plant protection programmes is the most widespread method due to price and other benefits. Researchers are looking for new, environmentally more acceptable ways of protecting plants against harmful organisms due to adverse environmental effects of pesticides, their non-target activity, the occurence of resistance to pesticides, and increasingly stringent environmental policies. One such measure is also the study of plant extracts in the suppresing of economically important harmful organisms. In a review article, we focused on the literature review of the usefeulness of plant extracts of several invasive alien plant species in plant protection: knotweeds (*Fallopia japonica* [Houtt.] Ronse Decr)*, F. x bohemica* (Chrtek & Chrtková) Bailey), goldenrods (*Solidago canadensis* L., *S. gigantea* Aiton), stag's-horn sumac (*Rhus typhina* L.), tree of heaven (*Ailanthus altissima* [Mill.] Swingle)*,* false indigo (*Amorpha fruticosa* L.) and stinkwort (*Dittrichia graveolens* [L.] Greuter), which we are studying within the project ApPLAuSE.

Key words: invasive alien plant species; *Fallopia japonica; Fallopia* x *bohemica; Ailanthus altissima; Solidago canadensis; Solidago gigantea; Rhus typhina; Amorpha fruticosa;* plant protection

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1 UVOD

Tujerodna rastlinska vrsta je tista, ki se na nekem območju brez posredovanja človeka ne bi mogla pojaviti. Ko te rastline na nekem novem območju vzpostavijo svoje nove populacije, se začnejo širiti v skladu s svojimi načini širjenja na primarnih območjih uspevanja (Jogan in sod., 2012). Poznamo arheofite. To so tujerodne rastlinske vrste, katerih pojav v naših krajih je povezan z delovanjem človeka in so se pojavile po koncu pleistocena. Omenjene vrste so k nam prihajale iz območja jugovzhodne Evrope in iz Bližnjega Vzhoda. Poznamo tudi t.i. neofite. Gre za rastlinske vrste, ki so se v njim tujih krajih s pomočjo človeka pojavile v zadnjih 500 letih (Jogan in sod., 2012).

Večina tujerodnih rastlinskih vrst v novem okolju ni invazivnih. O invazivni vrsti govorimo tedaj, ko se neka tuja vrsta nezadržno širi v predelih s podobnim podnebjem. Gre za neke vrste »invazijski potencial«, ki ga te rastline razvijejo, ko se pojavijo v konkurenčno šibkejšem tujem okolju (Jogan in sod., 2012). Jogan in sod. (2012) navajajo, da se pri nas vsaj občasno v naravi pojavlja okoli 750 tujerodnih vrst, od katerih se jih je 330 že udomačilo.

Stroški zatiranja invazivnih vrst in odprave škode zaradi njihovega delovanja so v letu 2012 v EU znašali med 10,6 do 14 milijard evrov (Sladonja in sod., 2015). Od leta 1992 je bilo v EU porabljenih več kot 38 milijonov evrov za 180 projektov, tako v mreži zavarovanih območij Natura 2000 kot zunaj nje (Shaw in sod., 2009). V ZDA ocenjujejo, da na leto porabijo približno 80 milijard evrov za zatiranje invazivnih vrst (Blackburn in sod., 2004). V popisu invazivnih vrst DAISIE je bilo v Evropi ugotovljenih 10.822 tujerodnih vrst. Vse med njimi niso invazivne, a se ocenjuje, da jih približno od 10 do 15 % lahko ogroža evropsko biotsko raznovrstnost (Sladonja in sod., 2015).

Varstvo rastlin (fitomedicina) je dejavnost, ki na podlagi ustreznih znanstvenih dognanj z različnimi metodami in na gospodaren način varuje gojene rastline pred škodljivimi organizmi za ohranitev in povečanje pridelka ter njihove kakovosti. V varstvu rastlin uporabljamo različne metode: agrotehnične, fizikalne, kemične, biotične in biotehnične (Milevoj, 2011). Pri nas in drugod po svetu je zaradi cene in drugih prednosti najbolj razširjena uporaba fitofarmacevtskih sredstev (FFS) v varstvu rastlin pred škodljivimi organizmi. Zaradi negativnih vplivov na okolje (ostanki FFS v rastlinah in v tleh), njihovega neciljnega delovanja, pojava rezistence škodljivih organizmov na FFS in vse strožje okoljske politike (umik številnih FFS iz trgovskih polic) raziskovalci iščejo nove, okoljsko bolj sprejemljive načine varstva rastlin pred škodljivimi organizmi. Eden od tovrstnih ukrepov je tudi preučevanje rastlinskih izvlečkov pri zatiranju gospodarsko pomembnih škodljivih organizmov (Pavela in sod., 2008).

V preglednem članku se osredotočamo na pregled literature vezane na uporabnost rastlinskih izvlečkov nekaterih tujerodnih rastlinskih vrst v varstvu rastlin; japonskega dresnika (*Fallopia japonica* [Houtt.] Ronse Decr.), češkega dresnika (*Fallopia* x *bohemica* [Chrtek & Chrtková] Bailey), velikega pajesena (*Ailanthus altissima* [Mill.] Swingle), kanadske zlate rozge (*Solidago canadensis* L.), orjaške zlate rozge (*Solidago gigantea* Aiton), octovca (*Rhus typhina* L.), navadne amorfe (*Amorpha fruticosa* L.) in lepljive ditrihovke (*Dittrichia viscosa* [L.] Greuter), ki jih preučujemo v sklopu projekta ApPLAuSE.

2 TUJERODNE RASTLINSKE VRSTE

2.1 Dresniki iz rodu *Fallopia*

Znanstveniki so preučevali vpliv rastlinskih izvlečkov različnih vrst dresnikov pri zatiranju nekaterih gospodarsko pomembnih škodljivih organizmov. Možno uporabo listnih izvlečkov sahalinskega dresnika so kot prvi omenili Herger in sod. (1988), ko so preučevali učinkovitost izvlečkov različnih rastlinskih vrst pri zatiranju kumarne pepelovke (*Erysiphe polyphaga* Hamm.). V njihovi raziskavi se je širjenje te glivične bolezni najbolj ustavilo (med 60 in 80 % zmanjšanje okužbe) ravno pri uporabi vodnega in alkoholnega izvlečka sahalinskega dresnika. V nadaljnjih raziskavah so rastlinski izvleček sahalinskega dresnika uporabili tudi drugi raziskovalci pri zatiranju nekaterih vrst pepelovk, ki so se pojavile na paradižniku, begoniji in jablani (Herger in Klingauf, 1990; Neuhaus in Pallut, 1992; Konstantinidou-Doltsinis in Schmitt, 1998; Konstantinidou-Doltsinis in sod., 2006). Na podlagi uspešnih rezultatov so raziskovalci v povezavi s podjetjem BASF razvili proizvod Milsana®, katerega formulacija temelji na rastlinskem izvlečku sahalinskega dresnika. Proizvod je registriran kot sredstvo za dvig odpornosti rastlin in ga tržijo v Evropi, v obeh Amerikah, na Japonskem ter v Južnoafriški Republiki (Konstantinidou-Doltsinis in sod., 2006). Nekatere poznejše raziskave so pokazale, da lahko pripravek Milsana® sočasno uporabljamo z nekaterimi drugimi sredstvi za varstvo rastlin

(Mycotal®, a.s. *Lecanicillium muscarium*, ciljni organizem: tobakov ščitkar [*Bemisia tabaci* (Gennadius)]) in tako pri enkratnem nanosu uspešno zatiramo več škodljivih organizmov hkrati (Bardin in sod., 2008). Francoski raziskovalci omenjeno kombinacijo priporočajo pri zatiranju pepelovke in ščitkarja na paradižniku, gojenem v rastlinjaku. Za uspešno kombinacijo se je tudi pokazala sočasna uporaba pripravkov Milsana® ter Sporodex L (a.s. *Pseudozyma flocculosa*) pri zatiranju pepelovke *Uncinula necator* (Schwein.) Burrill (Konstantinidou-Doltsinis in sod., 2007). Lalancette in sod. (2013) so v dvoletnem poskusu na prostem preizkušali učinkovitost devetih rastlinskih bioloških fungicidov pri zatiranju jablanove pepelovke (*Podosphaera leucotricha* [Ellis & Everh.] E.S. Salmon) na breskvah (sorta 'Jerseyglo'). Rastlinski izvleček sahalinskega dresnika v omenjeni raziskavi ni signifikantno vplival na zmanjšanje okužbe z omenjeno glivično boleznijo na breskvah. Rastlinski izvleček sahalinskega dresnika je bil uporabljen pri zatiranju navadne pršice (*Tetranychus urticae* C. L. Koch) na kumarah, gojenih v rastlinjaku (Tomczyk, 2006). Razmnoževanje in širjenje pršice je bilo zmanjšano za 34 %. V laboratorijski raziskavi so raziskovalci preučevali vpliv metanolnega izvlečka sahalinskega, japonskega in češkega dresnika na razvoj gosenic vrste *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) (Pavela in sod., 2008). Rezultati njihove raziskave so pokazali, da rastlinski izvlečki niso vplivali na smrtnost gosenic, so se pa te manj hranile.

Znano je, da zastopanost japonskega dresnika vpliva na zmanjšano pestrost drugih rastlinskih vrst (Aguilera in sod., 2010; Moravcova in sod., 2011; Murrell in sod., 2011; Stoll in sod., 2012). Znanstveniki so v raziskavah potrdili, da korenike japonskega dresnika (*Fallopia japonica*) izločajo kemične spojine, ki delujejo alelopatsko (Vrchotová in Šerá, 2008; Tucker Serniak, 2016). Tucker Serniak (2016) je v raziskavi preučeval alelopatski vpliv kemičnih snovi resveratrol, emodin, polidatin ter epikatehin na rast sadik redkve (*Raphanus sativus* L.). Gre za kemične snovi, ki se nahajajo v koreninskih izvlečkih japonskega dresnika (Fan in sod., 2010). Rezultati raziskave so pokazali, da kemične snovi resveratrol, emodin in epikatehin vplivajo na slabši razvoj korenin redkve. Do podobnih ugotovitev so prišli tudi drugi raziskovalci (Vrchotová in Šerá, 2008; Murrell in sod., 2011), ki so potrdili alelopatski učinek rastlinskih izvlečkov, pridobljenih iz različnih rastlinskih delov japonskega, češkega in sahalinskega dresnika, na razvoj nekaterih vrst metuljnic in zeli.

2.2 Rastlinske vrste iz rodu *Dittrichia* Greuter

Tako tuji kot tudi domači raziskovalci ugotavljajo negativen učinek rastlinskih izločkov ditrihovk na kalitev in razvoj korenin ter poganjkov drugih rastlinskih vrst (Levizou in sod., 2004; Omezzine in sod., 2011; Dor in Hershenhorn, 2012; Andolfi in sod., 2013; Grašič in sod., 2016; Tucker Sernik, 2016). Koncentracija izločkov iz listov ditrihovk se poveča ob suhih, vročih in sončnih poletjih (Stephanou in Manetas, 1995). Jesenska deževja te snovi sperejo v tla ravno v času kalitev večine sredozemskih vrst in tako ditrihovkam omogočijo kompeticijsko prednost pred drugimi vrstami (Levizou in sod., 2004; Grašič in sod., 2016).

Stamatakis in Konstantopoulou (2001) sta preučevala vpliv listnega izvlečka lepljive ditrihovke na N_2-fiksirajoče cianobakterije. Ugotovila sta, da se z večanjem koncentracije listnega izvlečka lepljive ditrihovke zmanjša delitev celic cianobakterij. Omezzine in sod. (2011) so ugotovili, da poleg listov, alelopatski potencial kažejo tudi drugi rastlinski organi lepljive ditrihovke. Najboljši alelopatski učinek kažejo izvlečki, pripravljeni iz listov. Znatno manjši učinek kaže izvleček pridobljen iz stebla, najšibkejši učinek pa imajo izvlečki iz korenin. Dor in Hershenhorn (2012) navajata, da so pleveli bolj občutljivi na izvlečke lepljive ditrihovke kot gojene rastline. Andolfi in sod. (2013) so iz desetih izbranih sredozemskih vrst skušali pridobiti izvleček, ki bi najbolj učinkovito zaviral kalitev pojalnikov (*Orobanche* spp.) in predenic (*Cuscuta* spp.). Gre za parazitske plevelne vrste, ki lahko vplivajo na manjši pridelek nekaterih gospodarsko pomembnih gojenih vrst rastlin (Parker, 2009). Največji herbicidni potencial je pokazal izvleček nadzemskih delov lepljive ditrihovke (Andolfi in sod., 2013). Rezultati njihove raziskave so pokazali, da so nekatere izolirane učinkovine iz omenjenega izvlečka v celoti zavrle kalitev testnih rastlinskih vrst. Listni izvleček lepljive ditrihovke bi lahko uporabljali kot selektivni herbicid za bolj trajnostno zatiranje plevelov (Stavrianakou in sod., 2004; Dor in Hershenhorn, 2012; Andolfi in sod., 2013). Slovenski raziskovalci so preučevali kalivost smrdljive in lepljive ditrihovke v odvisnosti od slanosti podlage (Grašič in sod., 2016a) ter učinek njunih izvlečkov na kalitev izbranih rastlinskih vrst (pelinolistna ambrozija (žvrklja), pšenica in solata) (Grašič in sod., 2016b). Ugotovili so, da obe vrsti ditrihovk uspevata pri vseh koncentracijah soli, a najbolje pri najmanjših. Kalivosti in razvitost kalic je z večanjem koncentracije NaCl upadala. Vrsti za uspevanje očitno ne zahtevata slane podlage, sta pa zelo tolerantni glede količine soli v tleh. Avtorji zaključujejo, da je najverjetnejši razlog za njun vzorec širjenja (predvsem ob avtocestah) njuna nezahtevnost ter zmožnost uspevanja na rastiščih z dokaj neugodnimi razmerami, kjer konkurence ni veliko. V poskusu, kjer so slovenski raziskovalci preučevali učinek izvlečkov lepljive in smrdljive ditrihovke na kalitev izbranih rastlinskih vrst, so rezultati pokazali, da vodna izvlečka obeh vrst ditrihovk zavirata kalitev in nadaljnji razvoj testnih vrst, predvsem pelinolistne žvrklje (Grašič in sod., 2016b). Znanstveniki poročajo tudi o akaricidnem delovanju izvlečkov lepljive ditrihovke. Sofou in sod. (2017) so iz rastlinskega izvlečka lepljive ditrihovke izolirali beta-selinensko kislino in preučevali njeno akaricidno delovanje na parazitsko pršico (*Varroa destructor* [Anderson & Trueman]) čebel. Laboratorijska raziskava je pokazala, da ima beta-selinenska kislina potencialno akaricidno delovanje. Prav tako so raziskovalci tudi potrdili, da omenjena kislina nima neciljnega delovanja na druge organizme.

2.3 Rastlinske vrste iz rodu *Solidago*

Najpomembnejše biološko aktivne snovi, ki jih najdemo v rastlinah iz zlate rozge so flavonoidi, saponini in terpeni (Apáti, 2003; Starks in sod., 2010). Nekateri terpeni služijo kot zaščita pred žuželkami, herbivori in patogeni (Anžlovar in Dolenc Koce, 2014). Terpeni in saponini imajo prav tako tudi baktericidno in fungicidno delovanje. Starks in sod. (2010) ter Demir in sod. (2009) poročajo o protibakterijski aktivnosti vrste *Solidago virgaurea* L. proti bakterijam *Staphylococcus aureus* Rosenbach 1884, *Enterococcus faecalis* (Andrewes & Horder, 1906) Schleifer & Kilpper-Bälz, 1984), *Bacillus cereus* Frankland & Frankland 1887 ter *Escherichia coli* (Migula 1895) Castellani and Chalmers 1919 . V sorodni raziskavi so protibakterijsko delovanje vrste *Solidago microglossa* DC. proti bakterijam *Staphylococcus epidermalis* (Winslow & Winslow) Evans 1916, *Staphylococcus aureus*, *Escherichia coli* in *Bacillus subtilis* (Ehrenberg) Cohn potrdili Morel in sod. (2006). Protibakterijska aktivnost vrste *Solidago canadensis* je bila potrjena proti bakterijam *Escherichia coli*, *Pseudomonas aureginosa* (Schröter) Migula ter *Bacillus subtilis* Ehrenberg 1835) Cohn 1872 (Mishra in sod., 2010). Zhang in sod. (2009) so potrdili delovanje ekstraktov vrste *Solidago canadensis* proti glivam *Rhizoctonia solani* J.G. Kühn, *Botrytis cinerea* Pers., *Alternaria solani* (Ellis & G. Martin) L.R. Jones & Grout, *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara, *Pythium ultimum* Trow ter *Rhizoctonia solani*. Ugotovljeno je tudi bilo, da rastlinski ekstrakti vrste *Solidago canadensis* inhibirajo kalitev semen različnih rastlinskih vrst (Butcko in Jensen, 2002; Bing Yao in sod., 2006; Fang in sod., 2007; Abhilasha in sod., 2008; Wang in sod., 2016). Alelopatsko delovanje vrste *Solidago canadensis* so potrdili Zhang in sod. (2011). Ugotovili so, da se flavoni, saponini in fenoli, ki jih rastlina izloča, akumulirajo v tleh. Te snovi nato alelopatsko delujejo na talne mikrobe. Zhang in sod. (2009) poročajo, da se vrste iz rodu *Solidago* v novih habitatih uspešno širijo zaradi alelopatskih snovi, ki jih izločajo in z njimi zavirajo rast okoliških rastlin. V sorodni raziskavi so Sekutowski in sod. (2012) preučevali vpliv vodnih ekstraktov orjaške zlate rozge (*Solidago gigantea*) na kalivost semen ajde in sončnice. Pri 50 % koncentraciji vodnega ekstrakta (500 g svežega zelinja je bilo namočenega v 100 cm^3 vode) je bila kalivost semen ajde najmanjša. Najmanjša kalivost semen sončnice je bila ugotovljena pri 12,5 % koncentraciji vodnega izvlečka orjaške zlate rozge. Baličević in sod. (2015) poročajo o alelopatskem delovanju vodnih ekstraktov orjaške zlate rozge na kalitev semen korenja, ječmena, koriandra in plevelov, navadnega ščira (*Amaranthus retroflexus* L.) ter baržunastega osleza (*Abutilon theophrasti* Med.). Ugotovili so inhibiranje kalitve semen pri manjših koncentracijah vodnega ektrakta (1, 5 in 10 %). Stephan in sod. (2005) poročajo o zmanjšanju okužb listov krompirja, ki so bili tretirani z vodnim ekstraktom kanadske zlate rozga, s krompirjevo plesnijo (*Phytophthora infestans* [Mont.] de Bary). Avtorji zaključujejo, da je bilo delovanje vodnega ekstrakta kanadske rozge sicer manj učinkovito kot uporaba fungicidov na podlagi bakra. Vodni ekstrakt rozge *Solidago altissima* L. var. scabra ima tudi nematicidno delovanje (Cox in sod., 2006). Avtorji navajajo 40 % smrtnost ogorčic vrste *Belonolaimus longicaudatus* Rau (1958) v poskusu, ki je potekal v rastlinjaku. Prathibha in sod. (2014) so preučevali larvicidno in ovicidno učinkovitost etilno-acetatnega ekstrakta kanadske zlate rozge pri zatiranju treh vrst komarjev. Sočasno so ugotavljali tudi učinkovitost ekstraktov kot odvračal za odlaganje komarjevih jajčec. V raziskavi so potrdili, da ima ekstrakt kanadske zlate rozge velik potencial pri omejevanju širjenja komarjev.

2.4 Rastlinske vrste iz rodu *Rhus*

Klingauf in sod. (1988) so preučevali insekticidno delovanje etanolnega izvlečka, pripravljenega iz listov navadnega octovca. Etanolni izvleček so preizkušali proti različnim škodljivim žuželčjim vrstam; sivi breskovi uši (*Myzus persicae* [Sulz.]), svetli žitni uši (*Metopolophium dirhodum* [Walk.]), črni fižolovi uši (*Aphis fabae* Scop.), kapusovemu molju (*Plutella xylostella* [L.]) in hrenarju (*Phaedon cochleariae* [F.]). Raziskovalci poročajo o visoki smrtnosti listnih uši (med 62 in 76 %), medtem ko izvleček ni vplival na smrtnost preostalih dveh žuželčjih vrst. S kemično analizo so identificirali prek 70 hlapljivih organskih snovi iz listov navadnega octovca. Kot močno repelentna (odvračalna) snov se je izkazal heksa-hidro-farnesil-aceton (Klingauf in sod., 1988). V sorodni raziskavi Mosch in sod. (1989) so preučevali antibakterijsko učinkovitost 131 različnih rastlinskih izvlečkov proti hruševemu ožigu (*Erwinia amylovora* [Burrill] Winslow *et al.*). Izmed učinkovitih se je izkazal prav rastlinski izvleček navadnega octovca, ki je v laboratorijskih testih pokazal zadovoljivo antibakterijsko delovanje proti omenjeni bakteriji. Rayne in Mazza (2007) poročata o antiglivnem delovanju izvlečkov rastlin iz rodu *Rhus* proti vrstam

Aspergillus flavus Link, *Candida albicans* (C.P.Robin) Berkhout, *Fusarium tricinctum* (Corda) Sacc., *Saccharomyces cerevisiae* Meyen ex E.C. Hansen in *Trichoderma viridae* Pers.

2.5 Rastlinske vrste iz rodu *Amorpha*

Liang in sod. (2015) so preučevali insekticidno delovanje rastlinskih izvlečkov navadne amorfe na ličinke komarja *Culex pipiens* L. Najboljše delovanje je pokazal etanolni izvleček (LC_{50}=22.69 mg/L). V nadaljnji kemični analizi so v izvlečku ugotovili prisotnost snovi amorfigenin (8-hidroksirotenon), ki je pokazal larvicidno delovanje (LC_{50}=4.69 mg/L; LC_{90}=11.27 mg/L) na omenjeno vrsto komarja. V sorodni raziskavi sta Gombos in Gasko (1977) preučevala vpliv rastlinskega izvlečka navadne amorfe na različne žuželčje vrste. Njuna raziskava je pokazala, da je rastlinski izvleček vplival na zmanjšano hranjenje (kot antifidant) koloradskega hrošča (*Leptinotarsa decemlineata* [Say]) in gosenic kapusovega belina (*Pieris brassicae* [L.]), medtem ko na navadno pršico (*Tetranychus urticae*) ni imel vpliva. Kutas in Nadasy (2005) rezultatov, ki sta jih ugotovila Gombos in Gasko (1977) nista potrdila, saj rastlinski izvleček navadne amorfe ni vplival na zmanjšano hranjenje ličink koloradskega hrošča. Polyakov in sod. (1977) so preučevali repelentni vpliv rastlinskih izvlečkov 607 rastlinskih vrst na različne vrste pršic (*Rhipicephalus bursa* Canestrini in Fanzago, *Dermacentor pictus* Herm., *Boophilus calcaratus* Lahille in *Hyalomma detritum* [Schulze]). Rastlinski izvleček navadne amorfe se je izkazal kot zelo odvračalen za različne vrste pršic. Csiszár (2009) so preučevali alelopatski vpliv različnih invazivnih lesnih vrst, ki rastejo na Madžarskem, na razvoj bele gorjušice (*Sinapis alba* L.). Največji alelopatski učinek je bil ugotovljen prav pri uporabi rastlinskega ekstrakta navadne amorfe ter velikega pajesna (*Ailanthus altissima*).

2.6 Rastlinske vrste iz rodu *Ailanthus*

Balassi in Meszaros (1999) sta preučevala insekticidno delovanje 3,7 % etanolnega izvlečka velikega pajesna na ličinke koloradskega hrošča. Rastlinski izvleček je bil narejen iz listov, vejic in korenin. Z izvlečki so tretirali liste krompirja. Rastlinski izvleček je pokazal učinkovito delovanje le na prvostopenjske ličinke (L1), medtem ko na starejše razvojne stopnje ni imel vpliva. Za vse razvojne stadije pa so potrdili zmanjšano stopnjo prehranjevanja z listi, ki so bili tretirani z rastlinskim izvlečkom velikega pajesna. Za primerjavo so uporabili tudi vodni izvleček, vendar tukaj niso potrdili nikakršnega insekticidnega/antifidantnega delovanja na ličinke koloradskega hrošča. V sorodni raziskavi so kitajski raziskovalci (Bing in sod., 2004) preučevali vpliv rastlinskih izvlečkov velikega pajesna na azijskega kozlička (*Anoplophora glabripennis* [Motschulsky]). Pri uporabi vodnega izvlečka niso potrdili nobenih razlik s kontrolnim (netretiranim) obravnavanjem v deležu smrtnosti hrošča in njegovo zmožnostjo prehranjevanja. Pri uporabi eternega izvlečka pa so potrdili zmanjšano stopnjo prehranjevanja. Balkan in sod. (2014) so preučevali vpliv rastlinskih izvlečkov iz listov navadnega oreha (*Juglans regia* L.) ter navadnega pajesna na razvoj nekaterih vrst gliv iz rodov *Aspergillus*, *Penicillium*, *Fusarium*, *Giberella* in *Cladosporium*. Rezultati raziskave so pokazali popolno inhibicijo germinacije glive *Cladosporium cladosporioides* (Fresen.) G.A. de Vries pri uporabi rastlinskega izvlečka velikega pajesna pri koncentraciji 10 mg/ml. Caboni in sod. (2012) so preučevali nematicidno delovanje snovi, ki so jih pridobili iz metanolnega rastlinskega izvlečka velikega pajesna. Snovi (E,E)-2,4-dekadienal ($LC_{50/24h}$=11.7 mg/L) in (E)-2-dekanal ($LC_{50/24h}$=20.43 mg/L) sta pokazali visoko stopnjo nematicidnega delovanja proti ogorčici *Meloidogyne javanica* (Treub). Alelopatsko delovanje rastlinskih izvlečkov velikega pajesna so potrdili v številnih raziskavah (Heisey, 1990; Moradshahi in sod., 2002; De Feo in sod., 2003). Moradashi in sod. (2002) so potrdili alelopatsko delovanje rastlinskega izvlečka velikega pajesna na vrtno krešo (*Lepidium sativum* L.), njihove rezultate pa so potrdili tudi De Feo in sod. (2003), ki so poročali o alelopatskem delovanju rastlinskih izvlečkov velikega pajesna na vrtno krešo, redkvico (*Raphanus sativus* L.) in navadni tolščak (*Portulaca oleracea* L.).

3 ZAKLJUČEK

V članku predstavljamo uporabno vrednost izbranih invazivnih rastlinskih vrst, ki se pojavljajo pri nas in jih je možno uporabljati v varstvu rastlin pred škodljivimi organizmi. Agresivne tujerodne rastline s hitrim razširjanjem povzročajo nemalo težav ne le domorodnim vrstam rastlin, ker jih izpodrivajo, temveč tudi ljudem, saj so pogosto vzrok za številne zdravstvene težave, kot so alergije, pripomorejo pa tudi k večji gospodarski škodi. Zatiranje večine tujerodnih invazivnih rastlinskih vrst je zelo težavno (Shaw in sod., 2009; Laznik in Trdan, 2017). V novem okolju namreč največkrat ni naravnih sovražnikov, ki bi vplivali omejevali rast in razvoj teh rastlin.

Organski rastlinski izvlečki so pridobljeni z ekstrakcijo rastlin. Rastline imajo različne aktivne sestavine, ki jih je mogoče pridobiti z različnimi metodami. Ekstrakcija z vodno ali oljno maceracijo omogoča pridobitev aktivnih sestavin rastlin brez njihovega spreminjanja, to so rastlinski izvlečki.

V Sloveniji je danes pridelava mnogih gojenih rastlin ob zadovoljivi kakovosti mogoča le z uporabo fitofarmacevtskih sredstev. Trenutni trendi v Sloveniji kažejo na zmanjševanje količine porabljenih fitofarmacevtskih sredstev in potrebo po razvoju, optimizaciji in implementaciji novih, nekemičnih načinov zatiranja škodljivih organizmov. Mednje sodijo tudi rastlinski izvlečki invazivnih tujerodnih rastlinskih vrst, ki imajo sodeč po rezultatih dosedanjih raziskav določen potencial v okoljsko sprejemljivem varstvu rastlin.

4 ZAHVALA

Prispevek je nastal v okviru projekta ApPLAuSE (Alien PLAnt SpEcies) - from harmful to useful with citizens' led activities (od škodljivih do uporabnih tujerodnih rastlin z aktivnim vključevanjem prebivalcev), ki ga financira (2017-2020) Evropski sklad za regionalni razvoj v okviru pobude Urban Innovative Actions (UIA).

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Production, uses and cultivars of common buckwheat in Japan: An overview

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ABSTRACT

Common buckwheat (*Fagopyrum esculentum* Moench) has attracted much attention due to its high nutritional value and medicinal properties. The crop has a long history of cultivation in Japan, and today, it is used mostly for manufacturing soba noodles which are quite popular in Japanese cuisine. Cultivation of common buckwheat in the country decreased gradually until the 1970's, but has started to increase again in recent years. In this paper, we provide an overview of common buckwheat production in Japan with emphasis on the agronomic characteristics of representative Japanese cultivars and landraces.

Key words: agronomic characteristics; breeding; common buckwheat; cultivar; genetic diversity; landrace; soba noodles

IZVLEČEK

PRIDELAVA, UPORABA IN SORTE NAVADNE AJDE NA JAPONSKEM: PREGLED

Navadna ajda (*Fagopyrum esculentum* Moench) je pritegnila veliko pozornosti zaradi svoje velike hranilne vrednosti in zdravilnih lastnosti. Poljščina ima dolgo zgodovino gojenja na Japonskem in se danes največ uporablja za izdelavo "soba" rezancev, ki so zelo popularni v japonski kuhinji. Gojenje navadne ajde je v državi postopoma upadalo do sedemdesetih let prejšnjega stoletja, a je začelo v zadnjih letih spet naraščati. V prispevku je podan pregled pridelave navadne ajde na Japonskem s poudarkom na agronomskih lastnostih reprezentativnih japonskih sort in lokalnih zvrsti.

Ključne besede: navadna ajda; agronomske lastnosti; žlahtnenje; sorta; genetska raznolikost; lokalne zvrsti; soba rezanci

1 INTRODUCTION

Common buckwheat (*Fagopyrum esculentum* Moench), a member of the Polygonaceae family, has been widely grown for human consumption in Japan (Mazza, 1988; Ohnishi, 1988; Kishima et al., 1995; Murai & Ohnishi, 1996). The crop is not a cereal, but its fruits are expediently classified among the cereal grains because of their similar usage. In fact, buckwheat flour is commonly employed in combination with wheat flour to prepare buckwheat noodles (soba noodles), a popular Japanese dish.

The history of buckwheat cultivation goes back to very ancient times in Asia (Murai & Ohnishi, 1996; Jacquemart et al., 2012). It is now broadly accepted that common buckwheat was initially domesticated in the northwest part of the Yunnan province in China (Murai & Ohnishi, 1996). This crop subsequently spread to Asian countries through two main routes (Murai & Ohnishi, 1996). The first route crossed the Himalayan region and Tibet, and the second ended up in Japan through Northern China. Available evidence suggests that common buckwheat was introduced into Japan via the Korean peninsula from China (Nagatomo, 1984; Ohnishi, 1995; Murai & Ohnishi, 1996). The crop became popular primarily due to its ability to grow well on marginal, infertile land as well as its rapid growth habit. When buckwheat first appeared in records in Japan in the 8th century, it had already been cultivated extensively as a catch crop (Shinoda, 1978; Murai & Ohnishi, 1996).

Although numerous local buckwheat landraces were grown in Japan at one time, common buckwheat culture in this country is currently dominated by only a small number of cultivars (http://www.tokusanshubyo.or.jp/jouhoushi/tokusanshubyo-26.pdf). In this paper, we review literature to provide an overview of common buckwheat production

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in Japan. Emphasis is placed on the agronomic characteristics of main Japanese cultivars and landraces. The expectation is that the synthesized information from this review will be useful for researchers and other stakeholders interested in the common buckwheat crop in Japan.

2 COMMERCIAL PRODUCTION

According to FAO statistics, the world buckwheat production in 2016 was approximately 2,396,000 metric tonnes (FAO, 2017). Russia and China were the largest producers, collectively accounting for ca. 65 % of global production that year. Japan ranked tenth in total buckwheat production (FAO, 2017).

In Japan, buckwheat production reached a maximum (139,000 tonnes from 165,000 hectares) in 1907 (Suzuki, 2003). Thereafter, it continuously declined until the 1970's (e.g., 18,000 tonnes from 18,000 hectares in 1975), because a number of farmers shifted their acreage from lower-yielding buckwheat to higher-yielding crops such as rice (Suzuki, 2003). In recent years, however, buckwheat acreage and yield have started to increase again. As shown in Table 1, average production per year, from 2012 to 2016 inclusive, was ca. 34,500 tonnes (MAFF, 2017). This recovery tendency is undoubtedly due to the Japanese government subsidies to farmers who grow buckwheat, with the aim of decreasing the amount of excessively produced rice. It should also be added that Japan imports 65-75 % of its domestic demand, mostly from China and the United States (MAFF, 2017).

Table 1: Production and cultivation area of buckwheat in Japan. Source: MAFF. (2017)

	2012	2013	2014	2015	2016
Cultivation area (ha)	61,000	61,400	59,900	58,200	60,600
Total harvest (t)	44,600	33,400	31,100	34,800	28,500

3 USES

Although the small leaves and shoots are also edible, common buckwheat fruits, generally considered as seeds are by far the most important for Japanese consumers. Harvested seeds are dehulled after drying, and the remaining part, called groats, is ground into flour. As mentioned above, buckwheat flour has traditionally been used in the preparation of soba noodles. The noodles play a major role in Japanese cuisine and are easily available in dried form in supermarkets throughout the country. Soba noodle dishes are served either cold with dipping sauce, or in hot broth as noodle soup.

Common buckwheat is also processed to various value-added products such as cakes, tea, beer and other local alcoholic beverages. The crop produces good quality of honey, whereas several buckwheat extracts are utilized for pharmacological and dietetics purposes (Bavec et al., 2002; Jacquemart et al., 2012; Kreft et al., 2016). In particular, flavonoids (mainly rutin and quercetin), D-chiro-inositol and proteins derived from buckwheat are in increasing demand, due to their biological and physiological activities including anti-oxidant, anti-inflammation and anti-hypertension properties (Jacquemart et al., 2012; Suzuki et al., 2012a; Giménez-Bastida & Zieliński, 2015; Kreft et al., 2016).

4 JAPANESE CULTIVARS

Common buckwheat exhibits a floral dimorphism known as distyly: each individual plant in a given cultivated population bears either pin (long pistil and short stamens) or thrum (short pistil and long stamens) flowers (Campbell, 1997; Woo et al., 2010). Both flower morphs are self-incompatible, and interfertile. Seed production thus depends upon insects and wind that mediate cross-pollination between pin and thrum plants. Owing to obligatory outcrossing characteristics, each cultivated population is expected to maintain a large amount of plant-to-plant variation. Almost all of the Japanese common buckwheat cultivars released to date have been developed from selection within locally grown landraces, and are not the products of controlled crosses.

Japanese common buckwheat cultivars are generally classified into three agroecotypes: summer, autumn (late-summer), and intermediate types (Matano & Ujihara, 1979; Namai, 1990; Hara et al., 2011; Hara &

Ohsawa, 2013). Summer-type cultivars are mainly grown in high-latitude regions, and seem non-sensitive to photoperiod. Autumn-type cultivars are grown in low-latitude regions and behave as facultative short-day plants. Intermediate-type cultivars show moderate photoperiodic sensitivity. The agroecotype and principal characteristics of representative Japanese buckwheat cultivars are presented in Table 2.

The important problem in common buckwheat cultivation is low and unstable yield. Breeding efforts have been made to improve seed yields. Most of the Japanese cultivars listed in Table 2 are actually larger-seeded genotypes (a grain mass of 32-38 g/1,000 seeds, http://www.maff.go.jp/tohoku/seisan/soba/pdf/25soba_manual.) with better grain yields. Additionally, common buckwheat reaches lower plant height and has been considered as more resistant to lodging. The cultivar ‘Kitawasesoba’ is an example of such a plant habit (Inuyama et al., 1994, see Table 2). In this crop, indeterminate seed setting and remarkable seed shattering make it difficult to determine the appropriate time for harvesting. It goes without saying that seed shattering causes serious yield losses. To overcome the problems there has been increasing emphasis upon the improvement of seed shattering and uniformity in ripening among seeds (Funatsuki et al., 2000; Matsui et al., 2003, 2004; Suzuki et al., 2012b).

Table 2: List of representative common buckwheat cultivars grown in Japan

Cultivar (Agroecotype)	Parentage and agronomic characteristics
Kitawasesoba (Summer)	Selected from a landrace ‘Botansoba’ and released in 1989. High-yielding and early maturing cultivar with lodging resistance and good market acceptability.
Hashikamiwase (Intermediate)	Selected from a locally grown buckwheat population and released in 1933. Large-seeded, nice flavor when cooked, but easy seed-shattering at maturity.
Dewakaori (Intermediate)	Developed in 1995 using a locally grown landrace ‘Mogamiwase’ for increased grain mass and improved taste. Moderately resistant to lodging.
Hitachi-akisoba (Intermediate)	Produced in 1985 using a local landrace as breeding material. Large-seeded and high-yielding cultivar with good taste.
Shinano No.1 (Intermediate)	Selected from a locally grown landrace and released in 1944. High-yielding genotype with good taste, but having the problem with lodging.
Oonozairai (Autumn)	High-yielding landrace having desirable flavor and sweetness upon cooking, and good market acceptability.
Miyazakiootsubu (Autumn)	Tetraploid cultivar bred using a locally grown landrace and released in 1982, having increased lodging resistance, but the problem with late maturity.

5 LANDRACE ‘BOTANSOBA’

Over the past two to three decades, Japanese buckwheat landraces have been replaced with improved cultivars that give better groats yields and are more or less genetically uniform (Campbell, 1997, 2003; Woo et al., 2010). Nevertheless, it is quite interesting to observe that a small number of landraces are still being cultivated to a limited extent. The landrace ‘Botansoba’ is a case in point (Figure 1). ‘Botansoba’ was selected by the National Agricultural Research Center for Hokkaido Region, Japan from a heterogeneous population grown in Date town (latitude: 42º28´N, longitude: 140º51´E) in Hokkaido, and released for cultivation in 1930 (Campbell, 2003). This landrace exhibited the high adaptability to local agroclimatic conditions in northern Japan, and was the most cultivated in the Hokkaido district until the release of an advanced cultivar ‘Kitawasesoba (see Table 2)’ in 1989 (Inuyama et al., 1994; Campbell, 1997).

‘Botansoba’ has problems such as higher plant height, and low and unstable yield (as seen in Table 3, ‘Botansoba’ had apparently higher plant height and lower grain yields than ‘Kitawasesoba’), but has high market acceptability; soba noodles made from ‘Botansoba’ flour actually gain a good reputation for their delicate, appetizing flavor as well as for their characteristic sweetness. The content of rutin in the groats of ‘Botansoba’ was found to range from 15.5 to 18.6 mg 100 g^{-1} (S. Motonishi, unpublished), comparable to that of Japanese common buckwheat cultivars such as ‘Kitawasesoba’ and ‘Shinano No.1’ (Kitabayashi et al., 1995).

The seeds of ‘Botansoba’ have been multiplied not by seed certifying agencies but by farmers for a long time. It is thus reasonable to assume that the farmers in question have not always used the seed-parent plant population large enough to represent a true sampling of the genetic variation present in the source population of

'Botansoba'. If the landrace has experienced the genetic bottleneck because of a too small population size, the genetic variation contained in the source population is perhaps fragmented and distributed over the several germplasm resources holdings today. This surmise was supported by morphological characterization of 'Botansoba' stocks preserved in four different seed sources: Breeder's stock, NCSS stock, Urausu-town stock and S-town stock (Honda et al., 2004). As shown in Table 3, S-town stock (source #4) obviously differed from Breeder's stock (source #1) in terms of plant height and days to maturity, whereas no apparent differences were observed among NCSS stock (source #2), Urausu-town stock (source #3) and Breeder's stock for these two characteristics. Moreover, the grain yield of S-town stock was revealed to be higher than that of Breeder's stock. Meanwhile, we cannot rule out the possibility that such differences may be attributable to inter-varietal cross pollination (Adhikari & Campbell, 1998). In order to preserve the purity of genetic resources such as 'Botansoba', the seeds need to be multiplied using effective population size of seed-parent plants, under a suitable isolation procedure (Adhikari & Campbell, 1998).

Figure 2: Landrace 'Botansoba'. Flowering on a farm (left panel) and inflorescence (right panel)

Table 3: Agronomic characteristics of landrace 'Botansoba' preserved in four different seed sources and an advanced cultivar 'Kitawasesoba'. Source: Y. Honda et al. (2004)

Genotype	Days to maturity	Plant height (cm)	Number of branches	Grain yields kg a^{-1}	1,000-seed mass (g)
Botansoba					
Seed source #1: Breeder's stock*	100	142	2.7	11.1	27.0
Seed source #2: NCSS**	100	146	3.0	9.5	27.2
Seed source #3: Urausu town	100	144	3.1	11.7	25.8
Seed source #4: S town	107	161	3.0	13.8	25.7
Kitawasesoba					
Seed source: NARO***	90	123	2.3	15.5	28.3

The study was carried out in 2004 at the experimental field located in Memuro town (latitude: 42º53´N, longitude: 143º03´E), Hokkaido. Seeds were sown on 3 June.
* Hokkaido Prefectural Plant Genetic Resources Center, Japan
** National Center for Seeds and Seedlings, Japan
*** National Agriculture and Food Research Organization, Hokkaido Agricultural Research Center, Japan

6 CONCLUDING REMARKS

In conclusion, two additional points merit comment. First, Tartary buckwheat (*Fagopyrum tatacicum* (L.) Gaertn.), a close relative of common buckwheat, is also cultivated in many areas of the world (Campbell, 1997; Senthilkumaran et al., 2008; Li et al., 2012). This crop, comparing to common buckwheat, is less widespread because of its bitter taste; in Japan, Tartary buckwheat was harvested from only 95 hectares with a total yield of 114 tonnes in 2015 (http://www.tokusanshubyo.or.jp/jouhoushi/tokusanshubyo-26.pdf). However, increased interest has been shown for human consumption of Tartary buckwheat due to some of its components that are very beneficial to human health. For instance, recent evidence indicates that the seed rutin content is approximately 100 times higher in Tartary buckwheat than in common buckwheat (Ikeda et al., 2012; Suzuki et al., 2014). Attempts have been made to improve Tartary buckwheat in Japan, resulting in the development of a promising Tartary buckwheat cultivar 'Manten-Kirari', whose flour is rutin-rich and lacks bitterness (Suzuki et al., 2014).

Secondly, buckwheat breeding is a recent endeavor when compared with breeding efforts devoted to major cereals. The use of molecular markers that are tightly linked to commercially important traits is now a widely accepted approach to help expedite the development of improved cultivars (Woo et al., 2010). In common buckwheat, studies have been performed to develop such markers as amplified fragment length polymorphism (AFLP) markers (Yasui et al., 2004), simple sequence repeat (SSR) markers (Konishi & Ohnishi, 2006), expressed sequence tag (EST) markers (Hara et al., 2011), and array-based markers (Yabe et al., 2014). As far as we know, however, the marker-assisted selection approach of the buckwheat crop is still in its infancy. Most recently, Yasui et al. (2016) have generated a draft assembly of the buckwheat genome using next-generation sequencing technology. They have also identified novel candidate genes involved in dimorphic self-incompatibility of the crop. Provided that the substantial buckwheat genome data-base is constructed, genomics-assisted approach will accelerate the genetic improvement of buckwheat.

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

Korak 5: Potrditev
- Potrebna je končna potrditev.

check box on the form. We advise you to check in also the Reader check box.

Submission process consists of 5 steps. Before submission, authors should go through the checklist and prepare for submission:

Step 1: Starting the submission
- Choose one of the journal sections.
- Confirm all the requirements of the Submission Preparation Checklist.
- Additional plain text comments for the editor can be provided in the relevant text field.

Step 2: Upload submission
- Upload full manuscript in the form of the Microsoft Word document file format (.doc or .docx).

Step 3: Enter metadata
- First name, last name, contact e-mail and affiliation for all authors, in relevant order, must be provided. Corresponding author has to be selected.
- Title and abstract must be provided in plain text.
- Key words must be provided (max. 8, separated by semicolons) and enter the language of the text.
- Data about contributors and supporting agencies may be entered.
- References in plain text must be provided in the relevant text filed (between each reference should be a blank line).

Step 4: Upload supplementary files
- All graphic have to be uploaded in a single ZIP file. Graphics should be named Figure1.jpg, Figure2.eps, etc.
- For each uploaded file the author is asked for additional metadata which may be provided.

Step 5: Confirmation
- Final confirmation is required.

PODROBNEJŠA NAVODILA / DETAILED INSTRUCTIONS

http://ojs.aas.bf.uni-lj.si/index.php/AAS/about/submissions#authorGuidelines