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İÇİNDEKİLER - CONTENTS

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Effects of Clinoptilolite on the Digestibility of Nutrients and Relative Organ Weights in Rat Diets

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ABSTRACT

This study was conducted to compare the effects of dietary clinoptilolite on nutrient digestibility and relative organ weights in rats. In this study, 24 adult male Sprague-Dawley rats were divided into 4 groups with 3 replicates, according to a randomized split plots design. In addition to a control group (0% clinoptilolit), 3 levels of clinoptilolite (2%, 4%, and 6%) were used in the diets, and the rats were fed (individually in cages) these diets for 56 days. Statistically significant differences were found among the groups for the digestibility rates of nutrients ($P<0.05$), except for crude fibre (CF) and acid detergent fibre (ADF). The addition of clinoptilolite in rats' feeds reduced the digestibility of crude fibre, crude ash (CA), neutral detergent fibre (NDF) and acid detergent fibre (ADF), but increased the digestibility of other nutrients. Slaughter live, organs (liver, kidney, heart, and stomach) and relative organ weights were not affected significantly by the clinoptilolite contents ($P>0.05$). In conclusion, clinoptilolites can be used in animal feed as natural toxin binders when stored under suitable conditions.

Keywords: Clinoptilolite; Feed; Nutrient digestibitiy; Relative organ weight; Rat

1. Introduction

Zeolites are used for various applications, for example; as natural toxin binders in industry, agriculture, veterinary medicine and environmental protection. Clinoptilolite is usually one of the alumino silicate minerals on the volcanic rocks (Baran & Kutay 1999; Martin-Kleiner et al 2001). Natural zeolite, especially clinoptilolite and analysim, are the most rich types in some regions of Turkey (Baran & Kutay 1999).

Different types and doses of zeolites have been studied previously in different animal diets, such as in broiler diets, with sodium bentonite (Eraslan et al 2005); in quail diets with HSCAS (Şehu et al 2005); in dairy cows adding zeolite (Dschaak et al 2010) in broiler and piglet diets adding clinoptilolite (Tang et al 2015) in rat diets adding HSCAS (Afriyie-Gyawu et al 2005), clinoptilolite (Martin-Kleiner et al 2001; Demirel et al 2011) and Tunus montmorillonit clay (TMC) (Abbès et al 2007). The clinoptilolite

protected the animals from the toxic effects of the mycotoxins (Abdel-Wahhab et al 1999).

The digestibility rates of nutrients have been studied by different researchers in various animal species, such as in a project that investigated the digestibility rates of sorghum grains in broiler diets when they were exchanged for dry matter (DM), CA, crude protein (CP) and nitrogen-free extract matter (NFE) (Adama et al 2007). The digestibility rates of wheat and sorghum grains in beef cattle diets were determined for DM, organic matter (OM), CP, EE, CF, NFE, ADF, NDF and CA (Baran et al 2008). In another study, DM and OM digestibility were similar (Baran et al 2017).

In rat diets, the digestibility rates of DM, CP, EE, and CA values were found to be 86.20, 81.66, 94.95, and 56.89%, respectively (Ahlstrom & Skrede 1998). In comparing rat diets based on rapeseed+barley and wheat bran+oats with respect to the apparent digestibility of DM and CF and the true digestibility of CP, feed intake did not affect the digestibility of the major feed components (Larsen et al 1991). However, there is limited literature related to the effects of clinoptilolite on the digestibility rates of nutrients. It has been demonstrated that improved nitrogen, OM and ADF digestibility was achieved when 5% levels of clinoptilolite were given to a high-solubility protein diet (Sweeney et al 1980). It has been stated that the apparent digestibility rates of clinoptilolite in growing piglet diets for DM, OM, CP, CF, EE, NDF and ADF ranged from 83.5-87.0%, 88.1-89.2%, 88.1-88.2%, 58.6-65.9%, 83.8-85.6%, 67.8-70.9% and 59.0-59.8%, respectively (Fokas et al 2004). Dietary clinoptilolite did not significantly affect the investigated parameters, except for an increase in EE.

This research was performed to define the effects of various contents of dietary clinoptilolite on the digestibility rates of some nutrients (DM, CP, EE, CA, CF, OM, NFE, ADF and NDF) and relative organ weights in rats.

2. Material and Methods

2.1. Zeolitic material

Zeolite purchased from Manisa, Turkey. The chemical content of clinoptilolite was as follows: SiO_2, CaO, Fe_2O_3, Al_2O_3, K_2O, MgO, Na_2O, TiO_2, MnO, LOI (loss on ignition at 1000 °C), SiO_2/Al_3O_2 as 65-72, 2.5-3.7, 0.8-1.9, 10-12, 2.3-3.5, 0.9-1.2, 0.3-0.65, 0-0.1, 0-0.08, 9-12, and 5.4-6.0%, respectively (Anonymous 2012).

2.2. Feed and feeding

Experimental diets were prepared at the Veterinary Faculty. Pelleted feed was produced for rats. All of the diets were arranged to be iso-caloric and iso-nitrogenic (National Research Council 1995). The chemical compositions of the diets are given in Table 1.

Table 1- Chemical compositions of diets used in trial

Crude nutrients	*Chemical composition, %*			
	I (control)	*II (2% zeolite)*	*III (4% zeolite)*	*IV (6% zeolite)*
Dry matter	93.55	94.15	94.40	95.10
Crude protein	22.41	22.58	22.65	22.45
Ether extact	13.36	13.76	14.49	15.54
Crude ash	8.05	9.10	10.02	10.40
Organic matter	85.50	85.05	84.38	84.70
Nit. free extract	40.23	38.56	36.04	34.66
Crude fiber	9.50	10.15	11.20	12.05
Acid detergent fiber	16.67	19.32	20.25	18.85
Neutral detergent fiber	38.08	38.76	36.65	32.71
Energy, Kcal ME kg^{-1} *	3203	3229	3211	3225

*, calculated

2.3. Animals and treatments

24 male Sprague-Dawley rats (8 weeks old and mean initial weight of 307±19 g) were used in the trial. Feed and water *ad libitum* were given. The rats were divided into 4 equal groups according to clinoptilolite rates of the diets.

2.4. Determination of crude nutrients

DM, CP, CA and EE analyses of the diets and faeces samples were conducted according to the AOAC (2000) and the crude fibre content was measured according to Crampton & Maynard (1938). ADF and NDF analyses were performed according to Van Soest (1987). The ME values of the diets were calculated by using the energy content of the feedstuff.

2.5. Digestibility analysis

Faeces samples were collected cleanly and dried daily to determine the in vivo digestibility rates of nutrients. Until sufficient amounts of faeces samples were obtained for analysis, during a 28-day sample collection period, this process was repeated. After that, all of the collected materials were ground and prepared for chemical analyses. In this trial, an indicator method was used to determine the digestibility rates of nutrients because of the difficulty of collecting all the rat faeces. The insoluble ash content in HCl was used as the indicator. The digestibility rates of the nutrients were calculated by the following equation stated by Sarı & Çerçi (1993).

$$DR\% = 100 - [(ILIF\% / ILIM\%) \times (NIM\% / NIF\%)] \times 100 \quad (1)$$

Where; *DR*, digestibility rate; *ILIF*, indicator level in feed; *ILIM*, indicator level in manure; *NIM*, nutrients in manure; *NIF*, nutrients in feed.

2.6. Relative organ weights

Determined by using slaughter and organ weights (g 100 g^{-1} BW).

2.7. Statistical analyses

The researh was arranged according to a randomized plot design with 4 groups and 3 replicates. Variance Analysis Method was used for statistical analysis of data. Duncan's Test (Duncan 1995) was used to determine differences amongst groups. For statistical calculations, SPSS 10.0 software was used (SPSS 1999).

3. Results and Discussion

The average digestibility rates of nutrients (DM, CP, EE, CA, CF, OM, NFE, ADF and NDF) are presented in Table 2. Statistically significant differences were found among the investigated groups for the digestibility rates of nutrients ($P<0.05$), except for the DM, CF and ADF levels ($P>0.05$). The adding of dietary clinoptilolite significantly increased the digestibility rates of CP, EE, OM and NFE but decreased those of CA and NDF. The highest average digestibility values of nutrients were

Table 2- Average digestibility rates of nutrients, %

Nutrients	*Groups*				*Significance*
	I (control)	*II (2% zeolite)*	*III (4% zeolite)*	*IV (6% zeolite)*	
Dry matter	62.29±1.13	63.17±1.14	65.68±0.66	65.56±2.38	0.058
Crude protein	62.66±1.20 b	63.61±1.11 b	67.00±0.92 a	67.30±1.16 a	0.013
Ether extact	92.51±0.64 c	92.83±0.38 bc	93.92±0.28 ab	94.24±0.43 a	0.037
Crude ash	47.54±1.33 a	37.81±1.37 b	33.33±0.71 c	26.96±0.69 d	0.000
Organic matter	67.96±1.09 b	70.10±1.05 b	73.84±0.62 a	74.31±1.06 a	0.000
Crude fiber	16.76±1.55	14.57±1.64	12.73±0.93	14.06±1.18	0.239
ADF	59.84±1.96	58.57±1.10	58.75±1.47	55.32±1.23	0.191
NDF	59.94±1.59 a	57.12±1.12 ab	55.54±1.00 b	49.50±1.32 c	0.000
NFE	74.05±1.23 c	82.61±1.06 b	88.68±0.81 a	91.08±1.18 a	0.000

[a, b, c, d] different letters within the same rows indicate differences among groups ($P<0.05$)

obtained for CA, CF, ADF and NDF in the control group, for DM in group III, and for CP, EE, OM and NFE in group IV. However, the lowest values were obtained for DM, CP, EE, OM, NFE in the control group, for CF in group III, and for CA, ADF and NDF in group IV.

The average slaughter live, relative organ (liver, kidney, heart and stomach) weights and their analysis of variance results are given in Table 3. Differences among the groups were not found significant for the slaughter live and relative organ weights (P>0.05).

The highest values were obtained for the slaughter live, liver, kidney and heart weights and their relative weights in the control group and for the stomach and its relative weight in group II. However, the lowest values were obtained for the slaughter, liver, kidney and stomach weights and their relative weights in group III and for the heart and its relative weight in group IV.

Our results for the nutrient digestibility rates in rat diets for DM, CP and CA values were found to be lower than the results of Ahlstrom & Skrede (1998).

Table 3- Effects of zeolite on slaughter (g), organ (g) and relative organ weights in rats (g 100 g^{-1} BW)

Parameters	*Groups*				*Significance*
	I (control)	*II (2% zeolite)*	*III (4% zeolite)*	*IV (6% zeolite)*	
Slaughter weight	358.24±41.65	352.35±26.75	345.67±6.03	353.15±30.52	0.891
Liver weight	11.95±2.56	11.60±1.35	10.89±1.07	11.29±1.25	0.691
Liver rate	3.31±0.37	3.29±0.21	3.15±0.23	3.20±0.18	0.704
Kidney weight	2.95±0.37	2.88±0.28	2.73±0.29	2.84±0.37	0.654
Kidney rate	0.82±0.05	0.82±0.07	0.79±0.07	0.80±0.06	0.762
Hearth weight	1.31±0.18	1.23±0.20	1.25±0.14	1.18±0.26	0.670
Hearth rate	0.37±0.05	0.35±0.04	0.36±0.04	0.33±0.06	0.573
Stomach weight	3.92±0.94	4.63±0.69	3.66±1.25	3.75±0.58	0.276
Stomach rate	1.08±0.14	1.31±0.13	1.05±0.33	1.08±0.26	0.237

Fokas et al (2004), found higher digestibility rates with the addition of clinoptilolite in growing piglet diets than those of ours for DM, OM, CP and NDF at 83.5-87.0%, 88.1-89.2%, 88.1-88.2% and 67.8-70.9% respectively. However, the EE values (83.8-85.6%) were lower, while those for ADF were similar to our results.

In addition, Baran et al (2008), found higher digestibility rate ranges for wheat and sorghum grain-based beef cattle diets than those of ours for DM, CF and CA at 69.48-70.50%, 59.70-60.30% and 59.70-60.10%; however, they found lower values for OM, CP, EE, NFE, ADF, and NDF at 68.90-69.30%, 69.80-70.20%, 70.90-71.40%, 73.90-74.40%, 43.20-43.70% and 46.00-46.40%, respectively.

The total DM digestibility values in this study similar level were observed by Husted et al (1968). Our digestibility rates for DM were greater than those reported by Sweeney et al (1980) and Johnson et al (1988). The higher DM digestibility values for diets containing zeolite might be due to the higher digestibility of other nutrients such as CP and a lower rate of passage. In the present work, the CP digestibility values are greater for the zeolite diets (Sweeney et al 1980). Our ADF digestibility results are similar with the findings of Johnson et al (1988). In general, the digestibility rates in this study are found lower than those of related literature findings. The reasons for these differences are due to the use of different animal species and zeolite types and doses.

Our relative organ weight results are similar to the findings of some studies that investigated the use of clinoptilolite in rat diets (Eleroğlu & Yalçın 2012; Eser et al 2012; Tang et al 2015).

The dietary clinoptilolite up to 6% did not have any adverse effects on the clinical health status of rats. Increasing the level of zeolite in animal feed increased the nutrient digestibility in diets such as DM, OM, CP, EE and NFE, except for those of CA and NDF, which decreased. However, the digestibility of CF and ADF, and the relative organ weights were not affected by clinoptilolite levels. Additionally, increasing levels of nutrient digestibility, lead to economical animal production.

4. Conclusions

Clinoptilolites can be used at the level of 2-6% in animal feed as cheap toxin binders when stored under suitable conditions, especially in poor countries. Clinoptilolite can be preferred to protect farm animals and their products from carcinogenic side effects. Various levels of clinoptilolite may be used for different animal kind to determine the safety margins and therapeutic effects, in further experiments.

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Identification of Favourable Testing Locations for Barley Breeding in South Pannonian Plain

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ABSTRACT

The aim of this study was to identify desirable, and also non-informative or highly correlated locations using GGE biplot. In this study, ten barley genotypes were tested across five locations for two growing seasons in official state trials performed by the Ministry of Agriculture, Forestry, and Water Management of the Republic of Serbia. In both growing seasons, environment had the highest influence on barley yield, explaining 77.70% in 2010/11 and 86.41% in 2011/12 growing season of the total variation. A significant grain yield variation explained by environmental effects indicated that the environments tested in our study were highly diverse. Together, PC1 and PC2 amounted 86.03% and 66.91% of the genotype and genotype × environment interaction sum of squares, in 2010/11 and 2011/12, respectively. The results indicate that Rimski šančevi was most favorable location and should be used for further multi-location trials while location Sremska Mitrovica was the least informative and it can be excluded from further trials. Excluding one of two similar environments could save resources with minimal risk to lose important information about genotypes performance. According to the results of our study, it can be concluded that GGE biplot is useful method for environment evaluation.

Keywords: Environment; GGE biplot; Interaction; Ideal environment

1. Introduction

The main objective of barley (*Hordeum vulgare* L.) breeding is a creation of new high-yielding cultivars characterized by good quality and other beneficial agronomical traits, such as optimal thousand grain weight, plant height, resistance to lodging (Dogan et al 2016; Mirosavljević et al 2016). In order to estimate the performance of promising genotypes in advanced generations, pre-registration trials are conducted for several years at different locations (Stojaković et al 2012). After the selection of superior genotypes as potential new cultivars, they are tested in official multi-location trials for two seasons conducted by the Ministry of Agriculture, Forestry, and Water Management of the Republic of Serbia. Official trials are carried out in target regions that represent the major agro-climatic conditions of the area for which the cultivars are to be realized. These trials are more detailed than breeders' pre-

registration trials, and provide more accurate information about cultivar performance.

Apart from quality and grain yield results, data collected from these trials, should also provide information about the adaptability and stability of tested genotypes (Stanisavljević et al 2013). The evaluation of tested genotypes is influenced by a genotype × environment interaction (G × E interaction), which represents differential response of genotypes to different environmental conditions. In order to identify the optimal environmental conditions for assessing and selecting promising genotypes, it is necessary to properly understand the effect of the G × E interaction (Rakshit et al 2012; Sayar & Han 2016). Nonparametric, regression and multivariate approaches have been used to understand the G × E interaction pattern. However, relatively small number of studies was conducted in order to determine desirability and representativeness of testing locations. Meng et al (2016) showed that genotype plus genotype × environment interaction (GGE) biplot enables the identification and evaluation of favorable locations. GGE biplot is widely applied and accepted by plant breeders for interpretation of the G × E interaction. The GGE biplot analysis combines two concepts-the GGE concept (Yan et al 2010) and biplot concept (Gabriel 1971). This analysis is a data visualization tool constructed by plotting two principal components, derived by the singular value decomposition of the environment-centred G × E table. The GGE analysis was previously used for a graphic analysis of multi-environmental trial data. Kaya et al (2006) tested the efficiency of the GGE model to investigate the association among nine rain-fed environments in bread wheat breeding and suggested that multi-locations trials should be carried out in a number of groups of locations sampled from the target region. It was also used by Tonk et al (2011) in order to identify discriminative locations for cultivar selection in Turkey. Kendal & Aktas (2016) examined associations among 7 testing locations across two growing seasons for barley breeding in Turkey using the GGE biplot method.

Considering that genotypes evaluation from multi-location trials is resources and time consuming process, the goal of this study is to find locations that provide desirable information about genotype characteristics. The locations that prove to be highly correlated or offer unreliable information about genotype performance could be eliminated from further cultivar evaluation trials.

2. Material and Methods

Data for this study were obtained from official registration trials conducted by the Ministry of Agriculture, Forestry, and Water Management of the Republic of Serbia. These trials were carried out across two growing seasons (2010/11 and 2011/12) at five locations: Kikinda (KI), Pančevo (PA), Rimski šančevi (RS) Sremska Mitrovica (SM) and Sombor (SO). Climatic characteristics of test locations are given in Table 1. According to Table 1, KI had the lowest rainfall level in both growing seasons and long term average. PA and RS are characterized by highest average long term rainfall. In 2010/11 and 2011/12 growing season, highest level of rainfall was recorded in SM and PA. Next to KI, SO could be singled out as location with lower level of rainfall.

Table 1- Characteristics of test locations

Locations	*Geographic position*			*Seasonal rainfall (mm)*			*Soil type*
	Latitude	*Longitude*	*Altitude (m)*	*Long term (1981-2011)*	*2010/11*	*2011/12*	
Rimski šančevi (RS)	45° 20′ N	19° 51′ E	82	472	386	361	Non-carbonate chernozem
Sremska Mitrovica (SM)	44° 58′ N	19° 36′ E	100	419	398	408	Chernozem
Pančevo (PA)	44° 52′ N	20° 39′ E	82	486	398	408	Carbonate chernozem
Sombor (SO)	45° 46′ N	19° 06′ E	87	439	360	319	Carbonate chernozem
Kikinda (KI)	45° 49′ N	20° 27′ E	82	397	355	305	Carbonate meadow soil

Ten two-rowed winter barley genotypes were used in these trials; a standard variety marked as G1 and nine advanced lines marked with symbol G2-G10.

The trials, with four replications, were arranged in a randomized complete block design in plots of 5 m^2. Plant density was adjusted to the recommended density proposed by seed companies. The standard agro-technical practices were applied in accordance to local agro-ecological conditions.

The model for a GGE biplot (Yan & Holland 2010) based on the singular value decomposition (SVD) of the first two principal components is shown in Equation 1.

$$Y_{ij} - \mu - \beta_j = \lambda_1 \xi_{i1} \eta_{j1} + \lambda_2 \xi_{i2} \eta_{j2} + \varepsilon_{ij} \qquad (1)$$

Where; Y_{ij}, observed yield of genotype i in environment j; μ, grand mean; β_j, main effect of environment j; $\mu+\beta_j$, mean yield across all genotypes in environment j; λ_1 and λ_2, singular values (SV) for the first and second principal component (PC1 and PC2), respectively; ξ_{i1} and ξ_{i2}, eigenvectors of genotype i for PC1 and PC2, respectively; η_{j1} and η_{j2}, eigenvectors of environment j for PCl and PC2, respectively; ε_{ij}, residual associated with genotype i in environment j.

Data processing was performed using Gea-R (R program) and the means were compared using Tukey's test.

3. Results and Discussion

The analysis of yield variance (Table 2) in the multi-environment trials indicated that the influences of the environment (E) and G × E interaction were significant (P<0.01). The influence of genotype (G) was highly significant (P<0.01) in the growing season 2010/11, while in the growing season 2011/12 was significant (P<0.05). In both growing seasons, the highest effect of E was observed on barley yield, and this factor affected 77.70% in 2010/11 and 86.41% in 2011/12 of the total treatments variation. Based on the ANOVA data, it is evident that percentages of the G × E interaction in the total treatment variation were 15.84% and 10.48%, in 2010/11 and 2011/12 growing season, respectively. The contribution of genotype to the total treatment variation was 6.46% in 2010/11 and 3.10% in 2011/12. According to Pržulj & Momčilović (2012), the variation in barley grain yield was mostly under control of the growing season and the genotype × year interaction. Similarly, Pržulj et al (2015) reported that in yield trials the effect of the environment affected 80-90% of the treatment variation, and the variation due the G × E interaction was higher than the genotypic variation. Further, Kendal & Dogan (2015) and Kendal & Tekdal (2016) stated had the major contributions to treatment sum of squares were environments, G × E and G, respectively. Mortazavian et al (2014) also reported that the environment constitutes the highest percent of the total yield variation, while the influence of the G and G × E interaction is usually smaller.

A remarkable grain yield variation explained by environmental effects, indicated that the environments tested in our study were highly diverse. The average grain yield ranged from 6.99 t ha^{-1} in KI to 10.42 t ha^{-1} in RS in 2010/11 and from 7.35 t ha^{-1} in KI to 10.75 t ha^{-1} in RS in 2011/12 (Table

Table 2- Analysis of variance of barley genotype yields in the growing seasons 2010/11 and 2011/12

Growing season	*Source of variations*	*Degrees of freedom*	*Sum of squares*	*Means of square*	*F values*	*% Sum of squares*
2010/11	Genotype	9	28.4	3.16	6.87**	6.46
	Environment	4	341.5	85.37	63.49**	77.70
	G × E	36	69.6	1.93	4.21**	15.84
2011/12	Genotype	9	11.9	1.32	2.30*	3.10
	Environment	4	331.3	82.83	59.45**	86.41
	G × E	36	40.2	1.12	1.95**	10.48

* and **, significant at the 0.05 and 0.01 level of probability, respectively

3 and 4). The average genotype yield varied from 8.36 to 9.70 t ha^{-1} in the 2010/11 growing season, and from 8.46 to 9.28 t ha^{-1} in the 2011/12 growing season. In the 2010/11 growing season genotype G10, followed by G5, were the highest yielding genotypes, with an average grain yield of 9.59 t ha^{-1}. In the next growing season, the genotype with the overall highest grain yield was G1, followed by G9. On average, the higher grain yield was recorded in 2010/11 in relation to 2011/12.

The first two principal components (PC1 and PC2) were obtained by partitioning the G and G × E interaction trough the GGE biplot analysis (Figure 1 and 3). PC1 accounted to 60.40% in 2010/11 and 43.09% in 2011/12 while PC2 amounted to 25.63% in 2010/11 and 23.82% in 2011/12 of the G and G × E interaction. Together, they accounted to 86.03% and 66.91% of the G and G × E interaction sum of squares, in 2010/11 and 2011/12, respectively.

According to Ding et al (2008), GGE biplot is an effective tool for: 1) analysis of mega-environment and specific genotypes can be recommended to specific mega-environments, 2) evaluation of genotype, and 3) evaluation of environment (the power to discriminate among genotypes in target environments). The selection of relevant testing locations provides the adequate information is necessary for superior genotypes

Table 3- Grain yield (t ha^{-1}) of the tested barley advanced lines in the 2010/2011 growing seasons

Genotype	*KI*	*PA*	*RS*	*SM*	*SO*	*Average*
G1	6.94^{n-q*}	8.03^{i-q}	10.29^{a-g}	9.87^{a-j}	9.83^{a-k}	8.99^{BC}
G2	6.79^{o-q}	8.96^{e-n}	8.73^{f-o}	10.20^{a-h}	10.55^{a-f}	9.04^{ABC}
G3	6.68^{o-q}	8.02^{i-q}	7.78^{k-q}	9.94^{a-i}	9.39^{b-l}	8.36^{C}
G4	6.64^{pq}	7.50^{m-q}	10.56^{a-f}	9.75^{a-k}	9.35^{c-l}	8.76^{BC}
G5	8.21^{h-q}	8.11^{i-q}	11.68^{a}	9.94^{a-i}	10.00^{a-i}	9.59^{A}
G6	6.39^{q}	7.86^{j-q}	10.85^{a-e}	10.68^{a-f}	9.22^{d-m}	9.00^{ABC}
G7	6.62^{pq}	8.04^{i-q}	11.19^{a-d}	10.06^{a-i}	8.95^{e-n}	8.97^{ABC}
G8	7.26^{m-q}	8.30^{g-q}	10.28^{a-g}	9.77^{a-k}	10.96^{a-e}	9.31^{AB}
G9	7.35^{l-q}	8.63^{f-p}	11.44^{ab}	10.37^{a-g}	9.09^{e-m}	9.38^{AB}
G10	7.01^{n-q}	8.66^{f-p}	11.41^{abc}	10.45^{a-f}	10.99^{a-e}	9.70^{A}
Average	6.99^{D}	8.21^{C}	10.42^{A}	10.10^{AB}	9.83^{B}	9.11

*, different letters indicate significant difference at the 0.05 level of probability

Table 4- Grain yield (t ha^{-1}) of the tested barley advanced lines in 2011/12 growing season

Genotype	*KI*	*PA*	*RS*	*SM*	*SO*	*Average*
G1	7.94^{f-o*}	8.24^{e-o}	10.55^{a-d}	9.72^{a-i}	9.95^{a-h}	9.28^{A}
G2	7.25^{k-o}	8.12^{f-o}	10.59^{a-d}	9.53^{a-k}	8.88^{c-o}	8.88^{AB}
G3	7.25^{k-o}	7.24^{l-o}	10.91^{a-c}	9.34^{b-l}	9.93^{a-h}	8.93^{AB}
G4	7.03^{m-o}	8.00^{f-o}	10.63^{a-d}	7.40^{j-o}	10.62^{a-d}	8.74^{AB}
G5	7.70^{h-o}	7.82^{g-o}	11.02^{a-c}	9.25^{b-m}	10.42^{a-e}	9.24^{AB}
G6	6.87^{o}	7.53^{i-o}	10.67^{a-d}	10.05^{a-g}	10.19^{a-f}	9.06^{AB}
G7	6.94^{no}	6.98^{m-o}	10.09^{a-g}	9.10^{b-o}	9.18^{b-n}	8.46^{B}
G8	8.07^{f-o}	7.16^{l-o}	10.09^{a-g}	9.19^{b-n}	9.84^{a-h}	8.87^{AB}
G9	7.30^{k-o}	8.13^{f-o}	11.33^{ab}	9.66^{a-j}	9.85^{a-h}	9.25^{AB}
G10	7.12^{l-o}	7.75^{h-o}	11.67^{a}	8.57^{d-o}	9.78^{a-i}	8.98^{AB}
Average	7.35^{A}	7.70^{D}	10.75^{A}	9.18^{C}	9.86^{B}	8.97

*, different letters indicate significant difference at the 0.05 level of probability

identification. A desirable testing location should be highly discriminative and also representative. According to Blanche & Myers (2006), a highly discriminative location is one that maximizes the observed genotypic differences between genotypes, while representative or key locations are sites that differentiate genotypes in a similar way.

On a GGE biplot, lines that connect coordinates of environment with the biplot origin are termed vectors. The angle between the vectors shows mutual relations among testing environments. If the angle between environments is obtuse, environments are negatively correlated. If angle is acute, environments are positively correlated, while environments are not associated when the angle is 90º. Since the angles of environment vectors SO with PA, and SM with RS were acute, it was concluded that they were closely correlated in the 2010/11 growing season (Figure 1). The angle between SM and SO, RS and PA, SM and PA, RS and SO was slightly obtuse or near to 90º, it means that these environments were negatively or not associated. In the 2011/12 growing season, highly correlated environments were KI with SM, and PA and RS (Figure 2). Moreover, SO was positively correlated with the environments RS and PA. Close associations between testing environments, suggest that same information about cultivar characteristics could be available from fewer testing environments, reducing the test cost (Yan et al 2015). The angle between the vectors for the environment SO, and the environments SM and KI was higher than 90º, indicating that SO was negatively correlated with SM and KI. Moreover, the presence of an obtuse angle among environments is an indicator of a strong crossover of the G × E interaction (Yan & Tinker 2006). It practically means that the selection at the location SO is irrelevant or even contra productive to the selection at SM and KI in the season 2011/12.

Some environmental factors, such as soil type and management practices are predictable, i.e. they are not different from year to year. On the other hand, the year-dependent factors, such as precipitation, temperature and disease attack, cause a high year-to-year variability. These random environmental

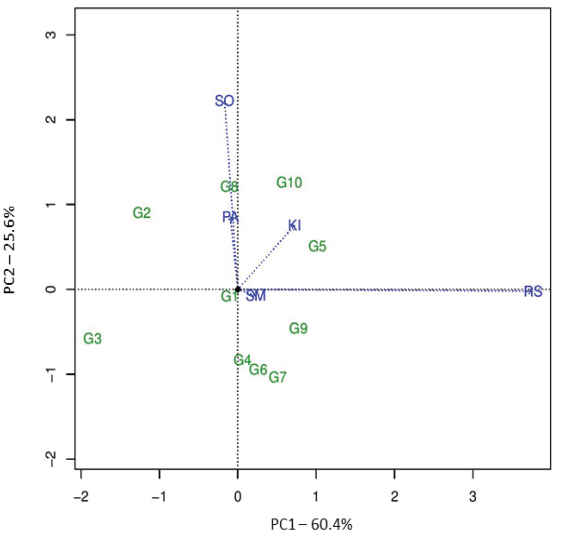

Figure 1- GGE biplot of the association among the tested environments in discriminating the genotypes in 2010/11

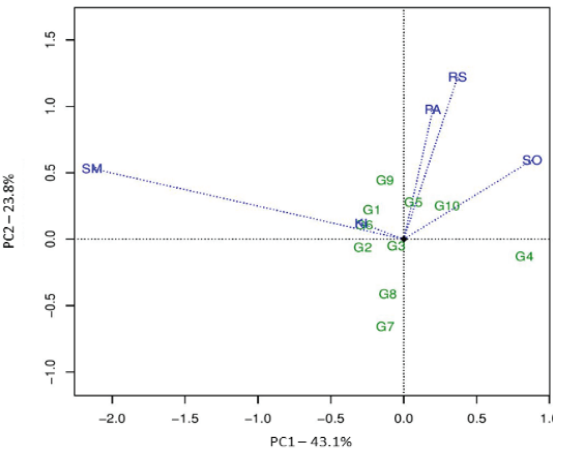

Figure 2- GGE biplot of the association among the tested environments in discriminating the genotypes in 2011/12

factors are highly variable and have a strong influence on the G × E interaction. As a result of the influence of these unpredictable environmental factors, for both years the pattern of environment groupings was not repeatable in terms of locations that were grouped together. For example, in the

season 2010/11 the location RS was not correlated with location PA, while in the season 2011/12 these two locations had a close association.

GGE biplot enables the evaluation of discriminative ability of a location. Environment points with greater vector length are more discriminative (Yan et al 2011). Environment RS had the longest vector length in the 2010/11 growing season and were the most discriminative location. In 2010/11 SM was represented by shortest vector, and had least discriminative ability. Since non-discriminative environments provide small amount of information about genotype performance, they are not desirable for genotype evaluation. Among all testing locations examined in 2011/12, the RS and SM environments were most discriminative, while KI was the least discriminative environment.

According to Figure 3 (biplot showing combined data from both growing seasons), it can be concluded that PA was the least discriminative test location in average. Further, SO and KI, and RS and PA were positively associated environments.

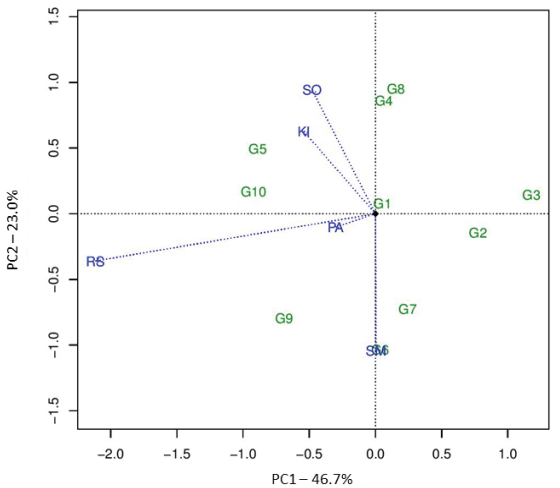

Figure 3- GGE biplot of the association among the tested environments in discriminating the genotypes according to combined data of 2010/11 and 2011/12

Another important measure that GGE biplot enables is the evaluation of environments compared to the "ideal" environment. On Figure 4 and 5, the open circle represents the average environment, defined as the average PC1 and PC2 scores of all environments. The line that passes through the average environment coordinate and the biplot origin is termed an "average environment axis" and the arrow placed on that axis represents the "ideal" environment. The "ideal" environment should be the most representative and discriminative location for conducting trials and can be used for the evaluation of other tested environments (Mitrović et al 2012). The tested environments located closer to the "ideal" environment, are more desirable, i.e. more representative and discriminative. The angle between the environment and the average environment axis shows the representativeness of the environment. The larger the angle between the axis and the environments the less representative the environment is.

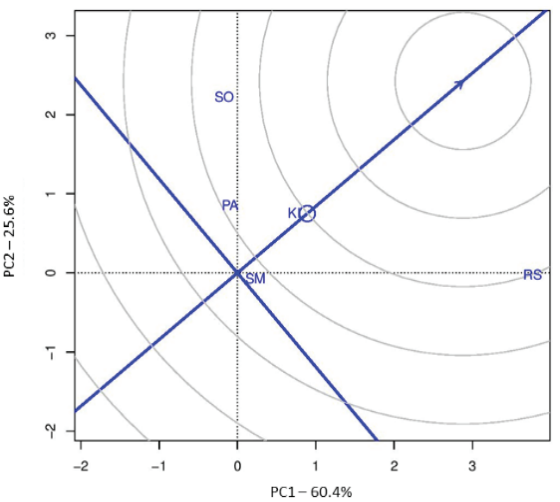

Figure 4- GGE biplot of comparison of the tested location with the ideal location in 2010/11

In the growing seasons 2010/11 and 2011/12, RS was placed closest to the "ideal" environment in relation to the other tested environments (Figure 4 and 5). Therefore, RS could be identified as the most desirable and effective location for cultivar evaluation in both growing seasons. In contrast, SM in 2010/11 and 2011/12 were the most distant from the "ideal" environment, and the least information could be acquired from this location. The most

representative environments were the location KI in the season 2010/11 and the locations KI and PA in the season 2011/12.

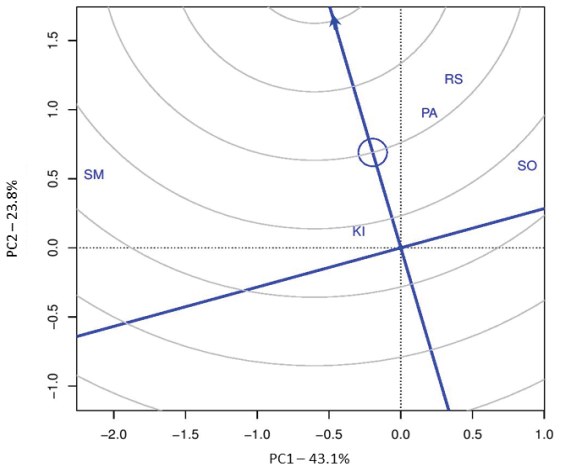

Figure 5- GGE biplot of comparison of the tested location with the ideal location in 2011/12

Based on biplot showing combined data from 2010/11 and 2011/12 growing season, RS were closest to the "ideal" environment, while SM was placed farthest from "ideal" environment (Figure 6). Moreover, PA and RS were most representative environments.

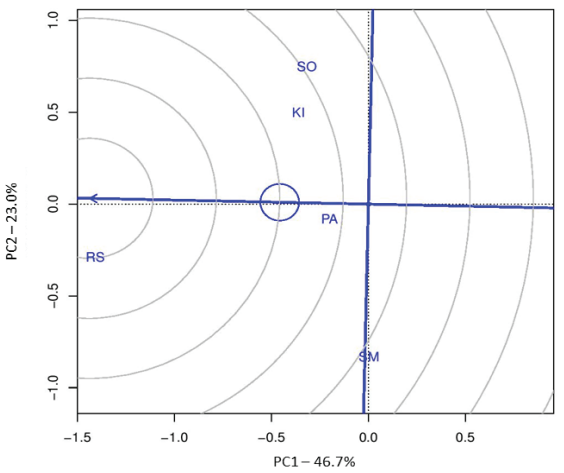

Figure 6- GGE biplot of comparison of the tested location with the ideal location according to combined data of 2010/11 and 2011/12

Although the crop yield is a result of E, G and G × E interaction effects, only G and G × E are relevant for cultivar and mega-environment identification (Yan et al 2010). GGE biplot is a data visualization tool that allows the visual interpretation of the G × E interaction, including environmental evaluation. According to Yan et al (2007), due to the discriminative ability and representativeness of GGE view, the biplot was an effective tool for environment evaluation, which was not possible with the AMMI model.

Limited seed and other resources cause that plant breeders use few locations for selection. Therefore, the identification of discriminative and representative locations is highly desirable in order to optimize genotype selection. Excluding one of two similar environments could save resources with minimal risk to lose important information about genotypes performance (Rakshit et al 2012). If the location is not similar to other locations and has a high discriminative ability and representativeness, then this location could be relevant for development of new barley cultivars because it could provide significant information about genotype traits.

4. Conclusions

According to the results of this study, it can be concluded that GGE biplot is useful method for environment evaluation. In both years, RS was placed closest to the "ideal" environment. This location was also the most discriminative in the 2010/11 growing season, and second most discriminative in 2011/12. On combined biplot, based on data from two growing season, RS was also marked as the most discriminative one. This indicates that this location should be used for further multi-location trials. The location SM was the least informative environment and placed farthest from the "ideal" environment in both growing seasons, and on biplot from combined data. These results suggest that the location SM could be replaced in further trials with another trial location from another production region in Serbia. Major difference in the characteristics of the studied environments could

be result of variation in agro-ecological conditions between two growing seasons.

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Identification of Sunflower *(Helianthus annuus* L.*)* Genotypes Tolerant to Water Stress

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ABSTRACT

The present research was carried out to determine water-stress tolerance of linoleic sunflower genotypes (P64LE119, PR63F73, P64LL62) grown under different water stress conditions [no water-stress (I_{100}); mild water-stress (I_{70}); strong water-stress (I_{35})] in the years 2015 and 2016. Variance analyses revealed significant differences between the genotypes ($P<0.01$). As the average of two years, the greatest yield was obtained from no water-stress x genotype interaction (I_{100}xP64LE119) with 4094.66 kg ha^{-1}, the lowest yield was obtained from strong water stress x genotype interaction (I_{35}xPR63F73) with 2487.81 kg ha^{-1}. Again as the average of two years, the greatest chlorophyll content was obtained from no water-stress x genotype interaction (I_{100}xP64LE119) with 49.83 spad, the lowest value was obtained from strong water stress x genotype interaction (I_{35}xPR63F73) with 34.39 spad. The greatest crop water stress index was obtained from strong water stress x genotype interaction (I_{35}xPR63F73) with 0.53, the lowest value was obtained from no water-stress x genotype interaction (I_{100}xP64LE119) with 0.21. The P64LE119 genotype with optimum water use efficiency and prominent with crop water stress index and chlorophyll content both in no water-stress and strong water stress treatments was identified as water stress-resistant and the genotype was considered to have reliable characteristics potentially to be used in further water stress-resistance studies.

Keywords: Sunflower; Water stress; Crop water stress index; Tolerance; Chlorophyll

1. Introduction

Today, agronomists and plant breeders are focused on yields rather than survival of the plants. Breeding programs are mostly implemented to develop high-yield cultivars. However, recent global warming-induced abiotic stressors have negatively influenced agricultural production activities and such impacts compelled the researchers to take new measures against the negative impacts of climate change and resultant global warming. Among the abiotic stressors, water stress, insufficient nutrition, salinity and high temperature are the leading ones (Kozlowski & Pallardy 1997). Recession in plant growth due to deficit moisture within the plant efficient root zone (through the soil profile of 0-90 cm) is defined as water stress. The initial symptoms of water stress realize at stomatal level and stomas close to prevent further moisture loss through transpiration (Flexas & Medrano 2002). Stomal closure reduces CO_2 availability in chloroplasts and negatively influences net photosynthesis rates

(Cornic 2000). Water stress is exerted on plant tissues under drought stress and this reduces photosynthesis rates significantly (Chaves 1991). Neither the soil moisture content nor the atmospheric system can accurately put forth plant inherent water status as much as crop water stress index (Reginato & Howe 1985; Gencoglan & Yazar 1999). Reginato (1983) indicated that daily crop water stress index values varied based on atmospheric demands and soil moisture contents. Water stress is experienced when the plant cover temperature was equal or greater than the air temperature (Walker & Hatfield 1979). Canopy-air temperature difference (Tc-Ta) is a significant indicator of water stress (Jackson & Reginato 1981). Choudhury & Idso (1984) carried out a water stress study on sunflower and reported significant effects of air and dew temperatures on plant cover temperature under high soil moisture conditions. Plant resistance to droughts and water stress are the primary target of plant breeders. For sunflowers, leaf canopy temperatures are the most significant parameters in measuring plant tolerance to water stress under stress conditions (Skoric 2009). Moroni et al (2012) indicated the canopy (leaf-canopy) temperature as the fastest and the most accurate means of measuring water stress and pointed out that this parameter could be used as a selection criterion in breeding studies. Crop water stress index values vary based on plant genotypes, cultivars, environmental and climate conditions (Testi et al 2008). Water stress is among the most important factors restricting plant production activities and may result in significant changes in chlorophyll content and components through hindering photosynthetic activity in plants (Mozaffari et al 1996). The parameters to be used in identification of drought or water stress should be easy, rapid, cheap and repeatable (Kaleem et al 2009; Moroni et al 2012). Oraki et al (2012) reported increased chlorophyll b levels, decreased chlorophyll a and yield levels with increasing water stress levels. Despite the studies about drought (water stress) tolerance of wheat and chickpea plants (Gunes et al 2008), the studies about plant responds to water stress in sunflower are quite limited. For sunflower, efficient selection criteria to be used in distinguishing potential status of the plants against water stress haven't been fully elucidated, yet. That is why in present study (2015-2016), 3 different irrigation treatments (I_{100}, I_{70}, I_{35}) were employed. The present study was conducted under field conditions in 2015 and 2016 to determine water stress resistance of 3 sunflower genotypes (P64LE119, PR63F73, P64LL62) grown under strong water-stress, mild water-stress and no water-stress conditions by using kernel yield, crop water stress index and chlorophyll content values.

2. Material and Methods

A pre-study was carried out under Siirt conditions in 2014 with two sunflower genotypes (PR63F73, P64LL62) and 4 irrigation treatments (I_{100}, I_{70}, I_{35}, I_0). Correlation analyses revealed that yield positively correlated with chlorophyll content (CC) (86%), water use efficiency (WUE) (74%) and soil moisture content (61%) ($P<0.01$) and negatively correlated with crop water stress index (CWSI) (79%). A negative correlation was also observed between CWSI and soil moisture content (84%) ($P<0.01$). In that pre-study, irrigation treatments were selected as I_{100}, I_{70}, I_{35} and I_0. However, I_0 treatment was not found to be assessable with regard to water-yield relations, thus removed from the study and (I_{35}) treatment was included instead to represent strong water stress conditions. Experiments were conducted under natural field conditions since it is quite hard to transfer the results of the studies carried out under controlled conditions like greenhouses or growth chambers into the practice. Sowing was performed late on 30th of May to shift the negative impacts of precipitations in May. Experiments were carried out over the experimental fields of Siirt Province during the sunflower growing seasons of 2015 and 2016. The research site has an altitude of 894 m and is located on 37˚ 58' N and 41˚ 50' E. Linoleic P64LE119, PR63F73, P64LL62 sunflower genotypes were used as the plant material of the study. Long-term and annual climate data of the research site (during sunflower growing seasons) are provided in Table 1.

Table 1- Climate data for the years of 2015 and 2016 and long-term averages (1962-2014)

Years	*Months*	*Mean maximum temperature (°C)*	*Mean temperature (°C)*	*Mean minimum temperature (°C)*	*Mean humidity (%)*	*Mean wind speed (m s^{-1})*	*Mean daily sunshine (h)*	*Total precipitation (mm)*
Average 1962-2014	May	25.20	19.40	9.00	49.30	1.00	9.10	36.90
	June	27.20	26.00	17.80	34.90	1.10	11.60	11.50
	July	35.10	30.50	23.40	30.30	1.10	12.30	0.60
	August	34.50	30.30	27.00	29.50	1.00	11.40	2.70
	September	30.00	25.10	14.70	37.40	1.00	10.10	7.00
	October	24.50	17.90	12.70	42.00	1.00	7.20	50.90
2015	May	26.62	19.29	14.52	50.87	1.00	8.70	39.60
	June	26.09	28.16	20.00	35.50	1.10	11.50	10.60
	July	34.13	31.45	24.35	32.69	1.00	12.40	0.10
	August	33.92	31.19	24.23	32.95	1.00	11.30	0.40
	September	31.23	25.43	21.50	39.90	1.10	10.00	9.20
	October	24.30	16.80	11.50	42.30	1.10	7.00	55.10
2016	May	24.69	21.29	14.59	51.77	1.00	9.30	37.70
	June	28.19	28.41	20.25	34.40	1.10	12.00	9.30
	July	36.24	33.19	25.35	29.69	1.00	12.50	0.10
	August	35.92	32.45	24.73	29.95	1.00	11.50	0.00
	September	32.23	27.43	21.65	36.79	1.10	10.00	12.20
	October	21.10	19.70	12.00	44.20	1.00	7.30	69.20

Soil samples were taken before sowing from 0-90 cm soil profile (from three depth segments as 0-30, 30-60 and 60-90 cm). Soil moisture content at field capacity (33 kPa) was determined in accordance with Klute (1986) and bulk density with Blake & Hartge (1986). Disturbed samples were subjected to organic matter, texture and permanent wilting point analyses. Water holding capacity at permanent wilting point (1500 kPa) was determined in accordance with Klute (1986). Soil physico-chemical characteristics are provided in Table 2.

Experimental soils were classified as brown forest soil with low electrical conductivity and salinity, low phosphorus content, high potassium content and medium level organic matter content and lime levels were not posing any problems for plant growth.

Irrigation water quality parameters were determined in accordance with the method specified by Tuzuner (1990). Irrigation water quality class was C_2S_1 with an average EC value of 0.34 dS m^{-1} and a pH value of 7.21. Experiments were conducted in randomized blocks-split plots experimental design with 3 replications with genotypes (P64

Table 2- Some physical and chemical soil characteristics of the research site

Properties	*Soil layer (cm)*		
	0-30	*30-60*	*60-90*
Clay (%)	62.00	58.00	55.00
Silt (%)	20.00	25.00	32.00
Sand (%)	18.00	17.00	13.00
Texture	Clay	Clay	Clay
Field capacity (Pw_{fc})	33.52	36.04	35.38
Permanent wilting point (Pwp)	24.44	26.08	25.57
Bulk density (g cm^{-3})	1.42	1.39	1.41
pH (1.25 sw^{-1})	7.50	7.66	7.91
Electrical conductivity (dS m^{-1})	1.55	1.77	1.75
Organic matter (%)	3.09	2.06	1.80
$CaCO_3$ (%)	6.40	1.90	1.90

LE119, PR63 F73 and P64 LL62) on main plots and irrigation treatments (I_{100}, I_{70} and I_{35}) on sub-plots.

Irrigation program was scheduled as to have irrigations once a week. Treatments were selected as no water-stress treatment (I_{100}) in which 100% of depleted moisture was supplied, mild water-stress treatment (I_{70}) in which 70% of depleted moisture was supplied and strong water-stress treatment (I_{35}) in which 35% of depleted water was supplied. Therefore, one full irrigation and two deficit irrigation treatments were created.

Drip irrigation was used to perform irrigations. A lateral line (20 mm and 4 atm operational pressure, 0.33 m apart 4 L h^{-1} drippers) was placed along each plant row. Soil infiltration rate was measured as 7 mm h^{-1}. Deep percolation and surface runoff were not considered. Each plot has a size of 6x2.8 m (16.8 m^2) with 4 plant rows with 70 cm row spacing and 30 cm on-row plant spacing. A buffer zone of 2 m was placed between the experimental plots as to prevent interactions.

All of the phosphorus fertilizer (pure 90 kg ha^{-1} P_2O_5) and one third of nitrogen (280 kg ha^{-1} N) were supplied at sowing. Rest of the nitrogen was given when the plants were 40-50 cm tall.

Gravimetric moisture content of each layer (0-30, 30-60 and 60-90) was converted into depth with Equation 1.

$$d = \frac{(Pw_{FC} - Pw_{AW}) \times As \times D}{100} \quad (1)$$

Where; d, soil moisture content in depth (mm); Pw_{FC} is field capacity (%); Pw_{AW} is moisture content of each layer (%); As is bulk density (g cm^{-3}); D is layer depth (mm). Volume of water to be applied was calculated by using the following Equation 2.

$$d_{T(0-90)} = d_{(0-30)} + d_{(30-60)} + d_{(60-90)} \quad (2)$$

Where; $d_{T(0\text{-}90)}$ is soil moisture at 0-90 cm soil profile (mm); $d_{(0\text{-}30)}$ is soil moisture at 0-30 cm soil profile (mm); $d_{(30\text{-}60)}$ is soil moisture at 30-60 cm soil profile (mm); $d_{(60\text{-}90)}$ is soil moisture at 60-90 cm soil profile (mm).

Volume of water to be applied to each plot was calculated by Equation 3.

$$V = d_T \times A \times U_0 \times P \quad (3)$$

Where; V is volume of water to be applied (L); A is plot size (m^2); U_0 is deficit ratio (%) and P is cover ratio (%).

Plant canopy width was divided by row spacing to get cover ratios (CR). The ratio was taken as 0.30 and 0.80 for cover ratios of 30% and 80%. The principles specified in Gungor et al (2006) were employed to find out the amount of water to be used in each plot.

Water budget method was used to calculate monthly and seasonal evapotranspiration values (Sahin et al 2007). Water use efficiency (WUE) values were calculated by using Equation 4 (Scott 2000).

$$WUE = \frac{Y}{ET_a} \quad (4)$$

Where; WUE is water use efficiency (kg da mm^{-1}); Y is yield; ET_a is evapotranspiration (mm).

Plant water consumptions were calculated by using Equation 5 (Sahin et al 2007).

$$ET_a = P + I - R_f - D_p \pm \Delta S \quad (5)$$

Where; ET_a is evapotranspiration (mm); P is precipitation (mm); I is amount of irrigation water (mm); R_f is surface flow (mm); D_p is deep percolation (mm); ΔS is the change in soil moisture (mm).

Change in CWSI and CC values of P64LE119, PR63F73, P64LL62 sunflower genotypes grown under I_{100}, I_{70} and I_{35} irrigation treatments were determined in one week intervals. CWSI and CC measurements were performed along the diagonals of each plot in four corners in three replications from the leaves close to head.

CWSI values were calculated by using Equation 6 as recommended by Idso (1982).

$$CWSI = \frac{\left[\left(T_c - T_a\right) - LL\right]}{UL - LL} \quad (6)$$

Where; *CWSI* is crop water stress index; T_c is canopy temperature (°C); T_a is air temperature (°C); *LL* is lower limit of water stress; *UL* is upper limit of water stress.

The lower limit (LL) at which plants did not experience any water stresses was calculated by the equation provided by Idso (1982) and using regression analyses between canopy-air temperature and vapor pressure deficit (VPD, kPa) (Equation 7);

$$T_c - T_a = (a - b) \times VPD \quad (7)$$

Where; *a* is intermediate section value (°C); *b* is slope of the line (kPa °C^{-1}); *VPD* is vapor pressure deficit (kPa).

Vapor pressure deficit was calculated with basic psychrometric equations (Alderfasi & Nielsen 2001). These equations are provided below;

$$e_w = 0.61078 \exp\left[\frac{17.27 T_w}{237.3 + T_w}\right] \quad (8)$$

$$e_a = e_w - \left[AP \times \left(T_a - T_w\right)\right] \quad (9)$$

Where; e_w is saturated vapor pressure at wet-bulb temperature (kPa); e_a is actual vapor pressure at air temperature (kPa); T_w is wet-bulb temperature (°C); *A* is psychrometric constant (kPa °C^{-1}); *P* is barometric pressure (kPa).

Psychrometric constant (A) was calculated from the following equation;

$$A = \left[0.00066\left(1 + 00115 T_w\right)\right] \quad (10)$$

Saturated vapor pressure was calculated by using the following equation;

$$e_a \times T_a = 0.61078 \exp\left[\frac{17.27 T_a}{237.3 + T_a}\right] \quad (11)$$

Vapor pressure deficit (VPD) was calculated as the difference of saturated vapor pressure at dry-bulb temperature from the actual vapor pressure at the same temperature;

$$VPD = \left[\left(e_a \times T_a\right) - e_a\right] \quad (12)$$

Where; e_a x T_a is saturated vapor pressure at dry-bulb temperature (kPa).

The upper limit (UL) at which plants experienced full-water stress was calculated by using the equations recommended by Idso et al (1981);

$$T_c - T_a = \left(a - b\right) \times VPG \quad (13)$$

$$VPG = \left[\left(e_a \times T_a\right) - e_a \times \left(T_a + a\right)\right] \quad (14)$$

Where; *a* and *b* are lower limits (LL) at which there are no water stress; *VPG* is slope of negative atmospheric vapor pressure required for the training of zero canopy-air vapor pressure.

CC of the genotypes was measured with a portable chlorophyll meter. Measurements were initiated when the plant cover ratio of the plots reached to 80% and performed throughout the growing season before and after the irrigations from the same plant and same leaves. Measurements were performed in days with clear sky and between 12$^{:00}$-14$^{:00}$ hours when the change in sun-ray angles the least. Chlorophyll-meter measurements were taken from the leaves just beneath the sunflower head, the device was oriented over the leaf as not to create a shade over it and 3 subsequent measurements (a total of 12 readings) were taken along the diagonal of the plot. CC increases as the value approaches to 1 and decreases as the value approaches to 0.

Harvest was performed when the seed moisture content decreased to 10% to determine the yields. Side rows and 0.5 m space at top and bottom of inner two rows were omitted as to consider side effects.

Analysis of variance (ANOVA) was performed in accordance with randomized blocks-split plots experimental design. Significant treatments were then subjected to LSD (Least Significant Difference) multiple comparison tests. Correlation analyses were carried out to identify the relationships between the traits. The directions of the relationships (positive or negative) were determined. Analyses were carried out with JUMP 5.0.1a statistical software (Der & Everitt 2002).

3. Results and Discussion

Seven irrigations were performed in all irrigation treatments. Irrigation water applied in 2015 and 2016 was measured as 550.80 and 624.46 mm in no water-stress treatments and as 216.20 and 245.09 mm in strong water-stress treatments. Seasonal plant water consumptions varied between 626.30-696.66 mm in no water-stress treatments and between 291.70-317.29 mm in strong water-stress treatments (Table 3). Higher ET_a values of strong water-stress treatments were because plants continued to benefit from the residual moisture in soil from the winter precipitations even after termination of irrigations. Water consumptions of the same plant genotypes may vary based on climate and regions and such values may even vary within the same region. Relevant differences might be due to the differences in plant genotypes, climate parameters, soil properties, method of irrigation and irrigation schedules.

The variations in yield and physiological characteristics of sunflower genotypes with irrigation water quantities are provided in Table 3, correlation coefficients between yield and other parameters are provided in Table 4. Significant differences were observed in yield, CWSI, CC and WUE values of the genotypes ($P<0.01$) and such differences were then subjected to LSD test (grouping) (Table 3). In the first year of experiments, the greatest yield in strong water-stress treatments (2657.67 kg ha^{-1}) was obtained from I_{35}xP64LE119 interaction with a low CWSI (0.31) and CC (37.13 spad) value and the lowest yield (2597.63 kg ha^{-1}) was obtained from I_{35}xPR63F73 interaction with a high CWSI (0.49) and a low CC (34.73 spad) value. The greatest yield in no water-stress treatments (4214.66 kg ha^{-1}) of the first year was obtained from I_{100}xP64LE119 interaction with a low CWSI (0.19) and a high CC (50.33 spad) values and the lowest yield (3914.65 kg ha^{-1}) was obtained from I_{100}xPR63F73 interaction with a high CWSI (0.26) and a low CC (46.25 spad) value. Genotypes also had significant impacts on yields ($P<0.01$). The greatest yield (3519.0 kg ha^{-1}) was obtained from P64LE119 genotype and the lowest yield (3398.0 kg ha^{-1}) was obtained from PR63F73 genotype. In the second year of experiments, the greatest yield (2685.66 kg ha^{-1}) in strong water-stress treatments was obtained from I_{35}xP64LE119 interaction with a low CWSI (0.37) and a high CC (36.11 spad) value and the lowest yield (2378.00 kg ha^{-1}) was obtained from I_{35}xPR63F73 interaction with a high CWSI (0.56) and a low CC (34.04) value. The greatest yield (3974.66 kg ha^{-1}) in no water-stress treatments of the second year was obtained from I_{100}xP64LE119 interaction with a low CWSI (0.22) and a high CC (49.32 spad) value and the lowest yield (3800.0 kg ha^{-1}) was obtained from I_{100}xPR63F73 interaction with a high CWSI (0.28) and a low CC (45.76) value. Variance analyses revealed that genotypes had significant effects on yields also in the second year of the experiments ($P<0.01$). Similar to the first year, the greatest yield (3393.33 kg ha^{-1}) was observed in P64LE119 genotype and the least (3225.88 kg ha^{-1}) in PR63F73 genotype. The other genotype (P64LL62) was placed in between these two genotypes in both years. As to conclude, significant interactions were observed between irrigation treatments and genotypes. Complying with the present findings, Kassab et al (2012) also reported significant interactions between irrigation treatments and genotypes. Water deficits in flowering period may cause considerable yield losses (Ali & Shui 2009). In addition, Afkari (2010), Kassab et al (2012) showed that water deficits significantly reduced plant heights, number of seeds per head, leaf area index and leaf relative water content of sunflower. Current findings comply with the results of Ali & Shui (2009), Afkari (2010) and Kassab et al (2012). However, Alahdadi et al (2011) reported substantial yield losses at short-term water deficits. Moisture deficiencies may negatively influence plant regeneration since sunflower is quite sensitive to drought stress during pollination period (Hajhassani-Asl et al 2009). Zaeifizade & Goliov (2009) showed that deficit moisture levels from budding to the end of flowering had devastating impacts on yields. In addition, Chimenti et al (2002) indicated flowering and seed maturity stages as the sensitive stages of sunflower to water-stress. Current results are in line with the findings of Hajhassani-Asl et al (2009), Zaeifizade & Goliov

(2009) and Chimenti et al (2002). Darvishzadeh et al (2010) carried out a selection study for water stress resistance of sunflower genotypes and reported that relevant genotypes exhibited similar performances both under water stress conditions and optimum conditions. Therefore in present study, the genotype P64LE119 with similar yield performance under both strong water-stress and no water-stress conditions were found to be prominent. Then, it was determined that this genotype could be used in studies to be carried out for the resistance or tolerance of sunflower genotypes to water stress and other abiotic stress factors.

In the first year of experiments, the greatest CC (37.13 spad) in strong water-stress treatments was obtained from I_{35}xP64LE119 interaction and the lowest value (34.73 spad) was obtained from I_{35}xPR63F73 interaction. The greatest CC (50.33 spad) in no water-stress treatments of the first year was obtained from I_{100}xP64LE119 interaction and the lowest value (46.25 spad) was obtained from I_{100}xPR63F73 interaction. Variance analyses revealed that genotypes had also significant effects on CC values. The greatest CC (44.15) was observed in P64LE119 genotype and the lowest value (40.17 spad) was observed in PR63F73 genotype. In the second year of experiments, the greatest CC (36.11) in strong water-stress treatments was seen in I_{35}xP64LE119 interaction and the lowest value (34.04) was observed in I_{35}xPR63F73 interaction. In no water-stress treatments of the second year, the greatest CC (49.32) was seen in I_{100}xP64LE119 interaction and the lowest value (45.76) was observed in I_{100}xPR63F73 interaction. Variance analyses revealed also for the second year that genotypes had significant effects on CC values with the greatest value (42.93) in P64LE119 genotype and the lowest value (39.70 spad) in PR63F73 genotype. The decrease in CC values was low in drought-resistant genotypes and high in sensitive genotypes (Table 3). Plants have different resistances to stress conditions and even different genotypes of the same plant may have different resistance levels (Win et al 2011) Robert et al (2016) reported decreased chlorophyll a, b and total chlorophyll contents in sunflowers under water stress. Several other researchers also reported decreased leaf chlorophyll contents under water stress conditions (Demirtas & Kirnak 2009; Zlatev et al 2010). It was also reported in previous studies that CC values might vary based on plant genotypes, cultivars, environmental and climate conditions (Testi et al 2008). Present findings comply with those earlier results.

In the first year of experiments, the greatest CWSI (0.49) in strong water-stress treatments was seen in I_{35}xPR63F73 interaction and the lowest (0.31) was observed in I_{35}xP64LE119 interaction. In no water-stress treatments of the first year, the greatest CWSI (0.26) was seen in I_{100}xPR63F73 interaction and the lowest (0.19) was observed in I_{100}xP64LE119 interaction. Variance analyses revealed that genotypes also had significant effects on CWSI values with the greatest value (0.40) in PR63F73 genotype and the lowest value (0.26) in P64LE119 genotype. In the second of experiments, the greatest CWSI (0.56) in strong water-stress treatments was observed in I_{35}xPR63F73 interaction and the lowest value (0.37) was seen in I_{35}xP64LE119 interaction. In no water-stress treatments of the second year, the greatest CWSI (0.28) was observed in I_{100}xPR63F73 interaction and the lowest value (0.22) was seen in I_{100}xP64LE119 interaction. Variance analyses again revealed that genotypes had significant effects on CWSI values with the greatest value (0.43) in PR63F73 genotype and the lowest value (0.29) in P64LE119 genotype. CWSI values of the second year were relatively higher than the CWSI values of the first year (Table 3). Drier conditions of the second year as compared to the first year increased evapotranspiration, thus CWSI values were found to be higher in the second year. Decreased CC and higher CWSI values were reported for water stress treatments (Moran et al 1994). Thusly, Khayatnezhad et al (2011) reported decreased chlorophyll contents and then reduced yields with water stress treatments in maize. Current findings comply with those earlier findings. P64LE119 with high yield, CC and low CWSI values were identified as resistant and the others were identified as sensitive.

Table 3- Changes in yield and physiological properties of sunflower genotypes

Treatments	*Yield (kg ha^{-1})***	*CWSI***	*Chlorophyll content (spad)***	*Irrigation water (mm)*	*ETa (mm)*	*WUE (kg da^{-1}-mm)***
2015 (First year)						
Irrigation treatments						
I_{100} (FI)	4071.00 a	0.21 c	48.36 a	550.80	626.30	0.65 c
I_{70} (DI)	3683.22 b	0.38 b	42.64 b	402.81	478.34	0.77 b
I_{35} (DI)	2625.33 c	0.42 a	36.11 c	216.20	291.70	0.90 a
Average	3459.85	0.34	42.37	389.94	465.44	0.77
LSD (0.05)	1.79	0.008	1.25			0.050
Varieties						
P64LE119	3519.00 a	0.26 c	44.15 a	364.37	439.87	0.80 a
P64LL62	3462.55 b	0.36 b	42.79 b	374.18	449.68	0.77 b
PR63F73	3398.00 c	0.40 a	40.17 c	383.68	459.18	0.74 c
Average	3459.85	0.34	42.37	374.07	449.57	0.77
LSD (0.05)	0.80	0.007	0.76			0,018
Varieties x irrigation treatments						
I_{100}xP64LE119	4214.66 a	0.19 h	50.33 a	526.69	602.09	0.70 d
I_{100}xP64LL62	4083.64 b	0.21 g	48.51 b	552.75	628.25	0.65 e
I_{100}xPR63F73	3914.65 c	0.26 f	46.25 c	576.94	652.44	0.60 f
I_{70}x 64LE119	3684.66 d	0.28 e	44.99 c	390.91	466.41	0.79 c
I_{70}x P64LL62	3683.33 d	0.43 c	43.39 d	409.15	484.65	0.76 c
I_{70}xPR63F73	3681.63 e	0.45 b	39.55 e	402.63	478.13	0.77 c
I_{35}x P64LE119	2657.67 f	0.31 d	37.13 f	207.23	282.73	0.94 a
I_{35X}P64LL62	2620.64 g	0.46 b	36.48 f	209.35	284.85	0.92 a
I_{35}xPR63F73	2597.63 h	0.49 a	34.73 g	225.72	301.22	0.87 b
Average	3459.85	0.34	42.37	389.04	464.53	0.78
LSD (0.05)	1.37	0.013	1.30			0.030
2016 (Second year)						
Irrigation treatments						
I_{100} (FI)	3901.33	0.24 c	47.69 a	624.46	696.66	0.56 c
I_{70} (DI)	3505.11	0.40 b	41.57 b	442.66	514.86	0.68 b
I_{35}	2538.33	0.47 a	34.86 c	245.09	317.29	0.80 a
Average	3314.92	0.37	41.38	437.40	509.60	0.68
LSD (0.05)	ns	0.017	1.72			0.005
Varieties						
P64LE119	3393.33 a	0.29 c	42.93	399.09	471.29	0.72 a
P64LL62	3325.55 b	0.39 b	41.50	416.85	489.05	0.68 b
PR63F73	3225.88 c	0.43 a	39.70	431.84	504.04	0.64 c
Average	3314.92	0.37	41.38	415.92	488.12	0.68
LSD (0.05)	48.5	0.012	ns			0.170
Varieties x irrigation treatments						
I_{100}xP64LE119	3974.66 a	0.22 h	49.32 a	601.47	673.67	0.59 e
I_{100}xP64LL62	3929.33 a	0.24 g	47.99 b	629.46	701.66	0.56 ef
I_{100}xPR63F73	3800.00 b	0.28 f	45.76 c	644.78	716.98	0.53 f
I_{70}x P64LE119	3519.67 c	0.30 e	43.36 d	430.61	502.81	0.70 cd
I_{70}x P64LL62	3499.66 c	0.44 c	42.05 e	442.46	514.66	0.68 d
I_{70}xPR63F73	3496.00 c	0.46 b	39.30 f	449.59	521.79	0.67 d
I_{35}x P64LE119	2685.66 d	0.37 d	36.11 g	232.98	305.18	0.88 a
I_{35X}P64LL62	2551.33 e	0.48 b	34.45 h	238.94	311.14	0.82 b
I_{35}xPR63F73	2378.00 f	0.56 a	34.04 h	253.55	325.75	0.73 c
Average	3314.92	0.37	41.38	435.98	508.18	0.68
LSD (0.05)	84.00	0.020	1.02			0.041

**, significant at P≤0.05 and P≤0.01; ns, not significant; means in the same column with the same letter are not significantly different

Significant correlations were observed between yield and CWSI and between yield and CC values (P<0.01). The correlation coefficients (r) for the relationships of yield with CC, CWSI and WUE are presented in Table 4a and b respectively for the years 2015 and 2016. Each year was assessed in itself to see the year-based variations in correlation and regression between the investigated traits. Significant correlations were observed between the investigated traits in 2015 (P<0.01). There was an increasing correlation between CC and yield (r= 0.925**). The regression analysis between these two parameters revealed a linear relationship as of Yield= -1253.00 + 111.00 x (CC). In this relation, 1 spad increase in CC corresponds to 1.142 kg increase in yield. Coefficient of determination was observed as R^2= 86%. In other words, the change in yield was 86% influenced by CC. There was decreasing correlation between CWSI and yield (r= -0.664**). The regression analysis between these two parameters revealed a negative linear relationship as of Yield= 4809.31 - 3887.77 x CWSI. In this relationship, 1 unit increase in CWSI corresponds to 0.921 kg decrease in yield. Coefficient of determination was identified as R^2= 44.6%.

Table 4- The correlation coefficients between yield and other parameters

a (2015)	*Yield*	*WUE*	*CWSI*	*CC*
Yield		-0.782**	-0.664**	0.925**
WUE	-0.782**		0.594**	-0.794**
CWSI	-0.664**	0.594**		-0.837**
CC	0.925**	-0.794**	-0.837**	
b (2016)	*Yield*	*WUE*	*CWSI*	*CC*
Yield		-0.825**	-0.797**	0.953**
WUE	-0.825**		0.577**	-0.821**
CWSI	-0.797**	0.577**		-0.879**
CC	0.953**	-0.821**	-0.879**	

**, P<0.01; WUE, water use efficiency; CWSI, crop water stress index; CC, chlorophyll content

Significant correlations were also observed between all parameters in 2016 (P<0.01). There was a highly positive correlation between CC and yield (r= 0.953**). The regression analysis between these two parameters revealed a linear relationship as of Yield= -926.12 + 102.65 x CC. In this relationship, 1 spad increase in CC corresponds to 0.823 kg increase in yield. Coefficient of determination was identified as R^2= 90%. In other words, the change in yield was 90% influenced by CC. There was a decreasing correlation between CWSI and yield (r= -0.797**). The regression analysis between these two parameters revealed a linear relationship as of Yield= 4840.49 - 4071.49 x CWSI. In this relationship, 1 unit increase in CWSI corresponds to 0.769 kg decrease in yield. Coefficient of determination was identified as R^2= 63.9%. In other words, the change in yield was 63.9% influenced by CWSI.

4. Conclusions

As the average of two years, the greatest yield was obtained from I_{100}xP64LE119 interaction (4094.66 kg ha^{-1}) and the lowest yield was obtained from I_{35}xPR63F73 interaction (2487.81 kg ha^{-1}). The greatest CC was observed in I_{100}xP64LE119 interaction (49.83 spad) and the lowest value was seen in I_{35}xPR63F73 interaction (34.39 spad). The greatest CWSI was observed in I_{35}xPR63F73 interaction (0.53) and the lowest CWSI was observed in I_{100}xP64LE119 interaction (0.21). There was an inverse relationship between irrigation water and CWSI and a direct relationship between irrigation water and CC. CWSI values decreased and CC values increased with increasing irrigation water quantities. However, such increase or decreases were not constant and varied based on genotypes even in no water-stress treatments. The greatest WUE (0.76 kg da mm^{-1}) was observed in P64LE119 genotype and it was placed in group A. The lowest WUE (0.71 kg da mm^{-1}) was observed in PR63F73 genotype and it was placed in group C. Therefore, the genotype P64LE119 was found to be prominent both in strong water-stress and no water-stress treatments and optimally converted applied irrigation water into the yield. The water stress-induced reduction in CC was low in water stress-resistant genotypes and high in sensitive genotypes.

In brief, in strong water-stress and no water-stress treatments of the experimental years, yield, CWSI and CC values of P64LE119 genotype were above the averages. Therefore, P64LE119 genotype was identified as water stress-resistant and can be used in further studies to be carried out for resistance to abiotic stress factors.

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Effect of *Purpureocillium lilacinum* on Root Lesion Nematode, *Pratylenchus thornei*

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ABSTRACT

Wheat (*Triticum* spp.) is highly susceptible to the root lesion nematode, *Pratylenchus thornei*, which causes excessive amounts of crop losses each year. In this research, we investigated the cumulative effect of the biocontrol agent *Purpureocillium lilacinum* (syn: *Paecilomyces lilacinus*) against *P. thornei*. Three doses of *P. lilacinum* (10^6, 10^7 and 10^8 conidia cultures mL^{-1}) with one dose of 400 *P. thornei* individuals (adults and juveniles) mL^{-1} were applied in 100 cm^3 soil under greenhouse conditions. The number of nematodes in the soil and root in addition to total nematode in soil+root were determined. Moreover, different plant parameters such as the plant height, plant fresh and dry weight, root fresh and dry weight were evaluated. Applications with the higher dose of bio-agents (100 cm^3 *P. thornei* infested soil with 10^8 conidia culture of *P. lilacinum* mL^{-1}) exhibited maximal enhancement in dry and fresh weight of shoot and reduced *P. thornei* population. As a consequence, *P. lilacinum* individually was highly effective in enhancing different plant parameters and suppressing *P. thornei* reproduction. Overall, present findings suggest that the exploitation of the biocontrol agent *P. lilacinum* could be helpful for effective management of the root lesion nematode *P. thornei*.

Keywords: *Pratylenchus thornei*; *Purpureocillium lilacinum*; Wheat; Biological control

1. Introduction

Wheat (*Triticum* spp.) is the most important crop in the world. It is cultivated in many countries and the second most important food crop in the developing world following rice. However, despite its high value, the wheat is highly vulnerable against to different types of viral, bacterial, fungal, and nematode pathogens, and insect pests. Nematodes are very important among these pests affecting wheat production. The root lesion nematodes (RLN), *Pratylenchus* species, is the most dominant group among the plant parasitic nematodes. It is ubiquitous in almost all cereal fields and causes vast crop losses every year (Nicol 2002).

RLN are widely distributed and have wide host ranges (Nicol 2002). Host suitability and abiotic factors such as soil type and moisture level chiefly govern the distribution and determine yield losses caused of RLN (Orion et al 1984). RLN compromise ninety-seven valid species distributed in almost every environmental condition in the world (Handoo et al 2008). Recently, Smiley (2010) reported that at least eight species of RLN could infect cereal crops. Among them, *P. thornei* and *P. neglectus* were reported as two very devastating species.

As a cereal root eelworm, *P. thornei* (Sher and Allen) (Tylenchida: Pratylenchidae) has been reported to feed and reproduce inside wheat roots, causing considerable loss of plant vigor and reduction in grain yield (Thompson & Clewett 1986). It is a migratory polyphagous endoparasitic nematode that causes necrotic lesions on the root systems of host crops. It is reported to be a pathogen of wheat throughout the world, with the exception of South America (Nicol 2002). *P. thornei* causes yield losses of wheat of 85%, 70%, 50% and 37% in Australia, Israel, America and Turkey, respectively (Toktay 2008; Smiley 2010; Imren et al 2015).

Several chemical nematicides have been recommended to reduce the reproduction of these nematodes. Particularly, the nematicides used to control *Pratylenchus* species have been considered less effective, environmental pollutant, toxic to the beneficial soil microflora, and expensive methods (Thomason 1987; Carneiro et al 2007).

For successful biological control, whether indigenous or introduced, the presence of antagonistic organisms in the environment is prerequisite. To date, numerous antagonistic organisms have been reported capable of reducing populations of plant parasitic nematodes (Stirling 2011). As a common soil inhabitant, various bacteria (i.e. *Pasteuria* spp.) and fungi (i.e. *Purpureocillium lilacinum*, *Pochonia chlamydosporia*) have been reported parasitizing nematodes in soil (Stirling 1991).

Purpureocillium lilacinum (Thom) Luangsaard, Hywel-Jones, Houbraken and Samson (syn: *Paecilomyces lilacinus*) (Sordariomycetes: Hypocreales) is a typical soil-borne fungus (Luangsa-Ard et al 2011). It was isolated firstly from insects in tropical regions. It has been recorded in many regions of the world specifically in warm regions (Domsch et al 1980). *P. lilacinum* was determined to cause infection in many insect, nematode and acarian species. Also, it has been described to be as efficient as commonly used nematicides (Dube & Smart 1987; Mendoza et al 2007). It is the most important egg and female parasite of plant parasitic nematodes (Morgan-Jones et al 1984; Dube & Smart 1987; Atkins et al 2005; Khan et al 2006). Furthermore, there are a lot of isolates of *P. lilacinus* and many commercial products especially the "strain 251" of *P. lilacinus* is being used as a biological control agent against nematodes all over the world (Atkins et al 2005).

The main objective of this study was to evaluate the efficacy of the fungal parasite, *P. lilacinum* and determine its effective dose of application for management of *P. thornei* on wheat in vitro conditions. This study is a pioneer research to investigate the pathogenicity of indigenous *P. lilacinum* against *P. thornei* in Turkey.

2. Material and Methods

2.1. Preparation of nematode inoculum

The root lesion nematodes, *P. thornei* which sourced from the Biological Control Research Station in Adana, Turkey, were cultured in vitro on carrot discs as described by Moody et al (1973). The nematode suspensions were prepared with sterile water. After suspending in a 50 mL of tap water in a beaker, *P. thornei* individuals (adults and juveniles) were counted using a binocular microscope. One mL of water consisting of 400 individuals was then injected to each plant a week after sowing.

2.2. Preparation of fungal source

The entomopathogenic fungus, *P. lilacinum* TR1 was provided from the Plant Protection Central Research Institute, Ankara, Turkey. *P. lilacinum* TR1 was determined (Kepenekci et al 2009) and identified by classical and molecular methods (Kepenekci et al 2015). *P. lilacinum* was isolated

from root-knot nematodes in the tomato plant roots in Sarıcakaya province (Eskişehir, Turkey). Potato Dextrose Agar (PDA) medium was used to raise a fresh culture of *P. lilacinum*. The wheat grains were inoculated with conidia scrapped from two weeks old plates. The grains were mixed following an incubation period of three weeks at 25±1 °C. To obtain a uniform suspension of fungal conidia, 500 mg of culture was mixed in 30 mL of 0.05% sterile agar solution. The number of conidia per mL was calculated by hemocytometer. The serial dilutions were prepared and the number of conidia was measured under hemocytometer to achieve the 10^6, 10^7 and 10^8 conidia mL^{-1} concentrations.

2.3. Experimental set-up

The study was carried out in 6 cm diameter pots containing 100 cm^3 of sterilized sandy loam soil (ca. 70% sand, 29% clay, and 1% organic matter). Wheat variety of Seri-82 seeds were transplanted into pots after germination on a moistened filter paper in Petri dishes. Three doses of *P. lilacinum* i.e. 10^6, 10^7 and 10^8 conidia cultures mL^{-1} with one dose of 400 *P. thornei* individuals (adults and juveniles) mL^{-1} were tested either alone or in combination. Also, the positive control (control +) *P. thornei*, and the negative control (control -) only water were added. Moreover, the number of nematodes into soil and root along with the total nematode in soil+root were counted in each treatment. Furthermore, different plant parameters such as the plant height, plant fresh and dry weight and also root fresh and dry weight were evaluated.

Each treatment was tested in a randomized complete block design with seven replicates. Plants were watered as needed and no fertilizer was added. Plants were grown under controlled conditions at temperatures between 20-25 °C, 16 hours of artificial light, and 70% relative humidity from March to May in 2013. The nematodes were extracted using a Baermann funnel after 9 weeks of nematode inoculation (Hooper et al 2005) from soil and the roots of harvested plants. One milliliter of extracted nematode suspension was counted with three replications in a counting slide at 32-fold magnification under a stereomicroscope. The number of nematodes was calculated in per plant. Fresh shoots were measured and weighed then they were dried at 70 °C for 48 hours to determine the dry weight.

2.4. Statistical analysis

Data were analyzed according to standard analysis of variance procedures using SPSS 10.0 program for Windows (SPSS 1999). Differences among treatments were tested using one-way analysis of variance (ANOVA) followed by the Duncan Test for mean comparison, if the F-value was significant ($P<0.05$).

3. Results

The experiments evaluated the effect of *P. lilacinum* on the population of *P. thornei* based on the number of nematodes in soil and root and total nematode in soil+root. Applications of various *P. lilacinum* reversed the adverse effect of nematode multiplication. Among the different doses of *P. lilacinum* conidia cultures, the maximum effect on the nematodes was recorded using the treatment having applications of higher doses of the bio-agent (Table 1).

Table 1- Effect of *Purpureocillium lilacinum* on number of *Pratylenchus thornei

P. lilacinum treatments (conidia mL^{-1})	*Number of nematodes in soil (pot containing 100 cm^3 soil)*	*Number of nematodes in root (pot containing one plant root)*	*Number of total nematode in soil+root*
10^6	3502±924.2 b	2360±25.1 c	5862±915.6 c
10^7	2458±365.1 b	2480±38.4 c	4938±262.2 bc
10^8	2192±331.5 b	1484±59.2 b	3676±353.3 b
Only *P. thornei*	5456±358.0 c	3128±117.3 d	8584±442.7 d
Only water	0 a	0 a	0 a

*, means in the same column followed by a different letter are significantly different according to LSD test ($P\leq0.05$)

Moreover, the numbers of nematodes in the soil were not significantly different based on the different doses of *P. lilacinum*. However, the number of nematodes in the soil for the control treatment (-) was significantly different to those observed for the various treatments of *P. lilacinum*. The best effects on the reducing the multiplication of nematode in the root were observed using the highest concentration of *P. lilacinum*. Additionally, the minimum nematode values were observed for the greatest concentration of *P. lilacinum* in the soil and root (Table 1).

The experiments assessed the effect of *P. lilacinum* and *P. thornei* on plant height, plant fresh and dry weight. In general, different doses of *P. lilacinum* caused positive effects on plant growth. Among the various treatments evaluated, maximal increases in the dry weight were recorded in the treatment having applications of higher doses of the bio-agents. The control (+) treatment using only *P. thornei* also had similar results to these higher *P. lilacinum* dose treatments. Conversely, the applications of *P. lilacinum* at three dosages were not found to have any significant effect on the improvement of shoot weight and plant height (Table 2).

Furthermore, the plant height and the upper parts of plant (shoot) fresh were not significantly different based on the different doses of *P. lilacinum*. Conversely, control (-) treatment resulted in significant differences in these two plant parameters. Alternatively, the dry weights (g) of plant were significantly different with the higher dose of *P. lilacinum* (Table 2).

Table 2- Effect of *Purpureocillium lilacinum* on growth characters of plants infected by *Pratylenchus thornei**

P. lilacinum treatments (conidia mL^{-1})	*Plant height (cm)*	*Fresh weight of plant (g)*	*Dry weight of plant (g)*
10^6	26.8±2.27 b	0.32±0.03 b	0.15±0.02 c
10^7	27.2±6.85 b	0.44±0.08 b	0.22±0.02 c
10^8	38.8±3.37 b	0.63±0.09 b	0.40±0.10 b
Only *P. thornei*	28.0±2.81 b	0.49±0.07 b	0.42±0.06 b
Only water	56.0±1.38 a	1.46±0.23 a	0.81±0.05 a

*, means in the same column followed by a different letter are significantly different according to LSD test (P≤0.05)

The higher dose of the bioagent was also observed to cause maximal improvement in the root fresh weight. With regard to root weight, a similar trend was also visible. Higher doses of *P. lilacinum* reduced the adverse effect of nematodes on the dry root weight. Generally, improvements in fresh and dry weight of root were produced by the higher dose of *P. lilacinum* (Table 3).

As a result, the best effects highest concentration of *P. lilacinum* on the multiplication of nematode were determined 2192, 1484 and 3676 *P. thornei* individuals (adults and juveniles) soil, root and soil-root respectively. Also, the best treatment using higher doses of *P. lilacinum* yielded maximal improvement in fresh weight of shoot and root and increase in dry weight of shoot and root respectively.

Table 3- Effect of *Purpureocillium lilacinum* on various growth characters of roots infected by *Pratylenchus thornei**

P. lilacinum treatments (conidia mL^{-1})	*Root fresh weight (g)*	*Root dry weight (g)*
10^6	0.57±0.05 b	0.17±0.03 b
10^7	0.47±0.13 b	0.34±0.14 b
10^8	1.20±0.17 a	0.78±0.06 a
Only *P. thornei*	0.44±0.08 b	0.19±0.05 b
Only water	1.45±0.11 a	0.71±0.09 a

*, means in the same column followed by a different letter are significantly different according to LSD test (P≤0.05)

4. Discussion

The biological control agent, *P. lilacinum* individually was highly effective in enhancing different plant parameters such as plant growth, fresh and dry weight of shoot and suppressing *P. thornei* population in the present study. Also, amongst the various treatments tested, applications with the higher dose of bio-agent (i.e. 100 cm^3 *P. thornei* infested soil with 10^8 conidia culture of *P. lilacinum* mL^{-1}) showed maximal improvement in fresh and dry weight of shoot and root.

Our results are in conformity with that of Rao & Malek (1973), who observed nematode-trapping fungi such as *Arthrobotrys dactyloides*, *A. arthrobotryoides* and *Dactylaria thaumesia* slowed the population increase of *P. penetrans* on alfalfa and determined that among the fungi tested, *A. dactyloides* was the most effective antagonist. Similarly, Mai et al (1977) reported different the nematode-trapping fungi such as *Arthrobotrys superba*, *A. dactyloides*, *A. arthrobotryoides* and *Dactylella doedycoides* reduced penetration of alfalfa roots by *P. penetrans*. Khan et al (2006) who observed that entomopathogenic fungi *Hirsutella rhossiliensis* suppresses the root lesion species, *P. penetrans* and *P. neglectus.* Moreover, Di Zahao et al (2013) reported that entomopathogenic fungi *P. lilacinus* impact the multiplication of different nematodes such as cyst nematodes *Globodera pallida* and *Heterodera* spp; root knot nematodes, *Meloidogyne incognita*.

Altogether, findings in the present study suggest that the exploitation of bio-control agent *P. lilacinum* could be helpful for effective management of the root lesion nematode. Based on the observations of this study it could be concluded that *P. lilacinum* had a cumulative effect on reduction of nematode population and improvement of plant growth. There are variations based on the different conidia doses of *P. lilacinum* and this difference may be the result of the variation in the experimental conditions or might be due to the environmental effects on the performance of bio-agent. It is also important in that the pathogenicity of *P. lilacinum* on *P. thornei* has not been studied under greenhouse conditions before this study. Although the result of this study gave a promising outcome, additional comprehensive research is needed to ascertain the capacity of nematode antagonists to suppress populations of *P. thornei in vivo* conditions.

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A Prototype Downdraft Gasifier Design with Mechanical Stirrer for Rice Straw Gasification and Comparative Performance Evaluation for Two Different Airflow Paths

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ABSTRACT

In this research, a prototype downdraft throatless gasifier was designed with a mechanical stirrer. The gasifier was designed for gasification of rice straw pellets. The diameter of the reactor was 350 mm and a nominal value for the heat power of biomass input was 70 kW. Rice straws which were collected from Thrace Region of Turkey gasified for determination of the designed gasifier performance in Namik Kemal University Biosystem Engineering Laboratories. The effects of airflow path and stirring process on the gasification efficiency during the gasification process were investigated. Temperatures and airflow rates observed and adjusted by controlling the air flow rate in the automation system constantly. Pellets were gasified using two different airflow paths with the same equivalence ratio of 0.2 and these were compared. Air inlet from the top showed better results than air inlet from tuyeres. For the air inlet from the top, the higher heating value of producer gas was determined as 5.047 MJ Nm^{-3} and cold gas efficiency was calculated as 65.4%. H_2/CO ratio was found as 1.385 which was higher than the air inlet from tuyeres.

Keywords: Biomass gasification; Rice straw; Stirrer; Throatless; Downdraft; Gasifier design

1. Introduction

One of the most controversial topics in the world is global climate change caused by CO_2 emissions. Therefore, greener energy sources have become significant alternatives to current energy resources and the number of the works being carried out on renewable energy has increased in current years. Biomass is one of the renewable energy sources which inspires interest for researchers (Anis & Zainal 2011). Fossil fuel resources are gradually decreasing causing the cost of petroleum-based products to increase. This problem imposes a need for an equipment that can produce an alternative source of fuel (Manguiat et al 2015). Biomass gasification occurs as a thermochemical process to produce gaseous fuel from carbonaceous feedstock which are included but not limited to pinewood,

eucalyptus wood, wheat straw, rice straw, rice husk, corn cob, corn stalk, sugarcane bagasse, poplar, hazelnut shell, coconut shell, switch grass, olive husk and so on (Demirbas 2004; Mondal et al 2011; Nwokolo et al 2016). Producer gas consists of carbon monoxide, hydrogen, carbon dioxide, methane, traces of higher hydrocarbons, such as ethane and ethylene, water vapor, nitrogen (if air is the oxidizing agent), and various contaminants, such as small char particles, ash, tar and oil (Basu 2013).

A review of the 50 gasifier manufacturers in Europe, United States, Canada showed that 75% of the designs were downdraft fixed beds while fluidized beds, updraft fixed beds and the other designs were 20%, 2.5%, 2.5%, respectively (Balat et al 2009; Zhang et al 2015).

Ma et al (2012) presented a systematic design and experimental results of a 190 kWe biomass fixed bed gasification and poly-generation pilot plant using a double air stage downdraft approach. They used a mechanical stirrer, secondary air supply, and wood chips as feedstock. It was reported that due to the secondary air supply, an enhanced tar cracking was achieved by increasing the temperature as high as 900 °C in the oxidation zone. Bridging and channelling were avoided by the use of both stirrer and reciprocating grate. In their research, the stirrer was placed in the drying zone of the reactor. The difference of our work is that the stirrer was placed in the combustion zone of the reactor.

Jain & Goss (2000) designed and fabricated four open core throatless batch fed rice husk gasifier reactors having internal diameters of 15.2, 20.3, 24.4 and 34.3 cm. Each reactor connected to a gas cleaning unit was tested for its performance characteristics. On each reactor, ten trial runs were conducted by varying the air flow rate or specific gasification rate. An experimental investigation in a downdraft gasifier was carried out by Striūgas et al (2014). They used different types of fuel and waste for a comparison of process performances and estimation of the potential to gasify various feedstock types in a single fully automated device. A number of different feedstock, including wood chips, pellets from wood, rape straw, poultry litter, dried sewage sludge and their mix with wood, were used for the investigation. The process efficiencies associated with the gasification of various feedstock and the effect of process parameters, such as temperature, pressure drop of a bed, product composition and output on the process were reported.

A review article on biomass gasification models for downdraft gasifier has recently been contributed by Patra & Sheth (2015). In the review, the importance of modeling for biomass gasification was explained and different models available for downdraft gasifiers were discussed and evaluated. The main focus was the equilibrium models for both fluidized bed and downdraft gasifiers.

In a typical design of the downdraft reactor, the biomass is fed from the top of the reactor and moves downwards as a result of its conversion and the removal of ashes through a grate at the bottom of the reactor. The literature review shows that gasification of different biomass in downdraft gasifiers entails certain difficulties with a lack of literature about the gasifier with a mechanical stirrer. Therefore, the main objective of this study was to perform experimental investigations of gasification process in the designed prototype downdraft gasifier using rice straw pellets to determine process performance and estimate the feasibility of gasification of pure rice straw pellets.

2. Material and Methods

In this research, a prototype gasifier system with an overhead controlled mechanical stirrer was designed for rice straw pellets gasification. In this section, biomass characteristics, experimental setup, and experimental procedure will be explained.

2.1. Biomass characteristics

Rice straws collected from Thrace Region of Turkey were pelleted in a firm located in Tekirdağ. Mean diameter of pellets was 6.2 mm and the mean length was 53.6 mm. Mean bulk density (ρ_b) and unit density (ρ_u) values of pellets were 562.3 kg m^{-3}

and 1226.4 kg m^{-3}, respectively (EN 15103 - 2009). Table 1 shows proximate analysis results for dry basis, original basis, and ultimate analysis results.

Table 1- Proximate and ultimate analysis of rice straw

Proximate analysis (%, wt)			
	Original	*Dry*	*Standard (ASTM D)*
Moisture	7.01	-	7582-12
Volatile matter	61.61	66.25	7582-12
Fixed carbon	14.18	15.24	3172-13
Ash	17.21	18.50	1755-01
Ultimate analysis (%, wt)			*Standard (ASTM D)*
C	39.90		5373-14
H	4.89		5373-14
O	35.27		3176-09
N	1.24		5373-14
S	0.20		4239-14

Determined lower heating value of biomass pellets (LHV_F) for original basis and dry basis were 12.77 MJ kg^{-1} and 13.90 MJ kg^{-1}, respectively. Also, the higher heating values (HHV_F) were 13.84 MJ kg^{-1} original basis and 14.89 MJ kg^{-1} for dry basis (ASTM D 5865-13). First deformation temperature, softening temperature and hemisphere temperature (ASTM D 1857/D1857) were 1045 °C, 1239 °C, and 1436 °C, respectively. A sample of rice straw pellets used in this study as biomass fuel is shown in Figure 1.

2.2. Experimental setup

A downdraft biomass gasifier was designed for rice straw pellet gasification. Characteristic parameters of the design are given in Table 2. Air was used as gasification agent. Design of gasifier allowed working with two different airflow inlets; from the top and from the tuyeres. An overhead activated mechanical stirrer was added to the reactor to determine the effects of stirring.

Table 2- Characteristic parameters of the designed gasifier

Type of reactor	*Fixed bed, downdraft, throatless*
Diameter	350 mm
Refractor layer	Yes (50 mm)
Tuyeres	10 items (Diameter 7.8 mm)
Fuel feeding	Manual (Batch type)
Grid	No
Ash removal	Semi-automatic control (HMI panel)
Fuel consumption	19±1 kg h^{-1} (for SGR 200±10 kg h^{-1} m^{-2})
Power input (biomass)	70 kW (for FCR 20 kg h^{-1})

Experimental setup consisted of gasifier reactor, cyclone, gas cooling unit and condensation tank, vacuum pump and service water tank, flare unit, measurement and control components, gas chromatography device and its components. General

Figure 1- Rice straw pellets

view of the system is shown in Figure 2 and flow chart is given in Figure 3.

Figure 2- General view of gasifier system

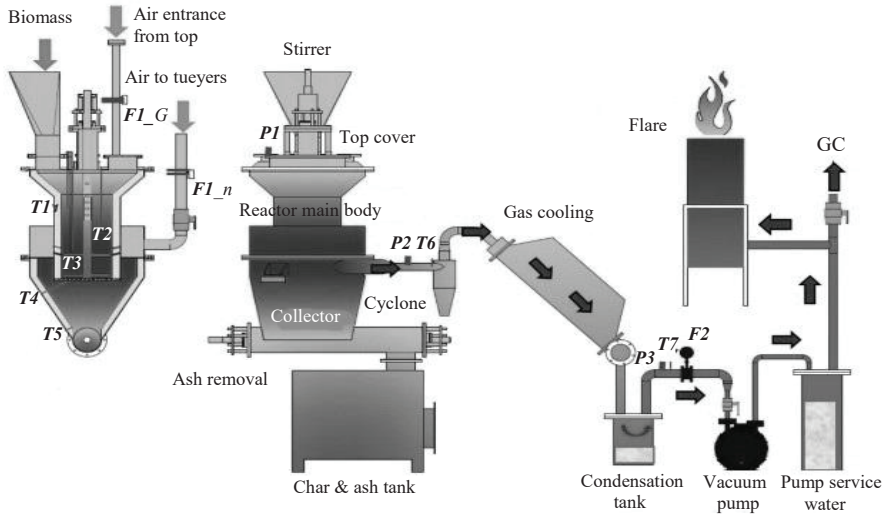

Figure 3- Flow chart of the gasification system

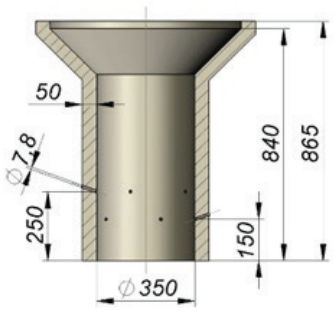

Figure 4- Main reactor body dimensions

2.2.1. Gasifier

The stratified design has some advantages over the throated design. The cylindrical construction is easy to manufacture and allows continuous flow of troublesome fuels without causing bridging and channeling (Reed & Das 1988).

The diameter of the reactor (D_R) was calculated by using Equation 1 and determined as 0.350 m. Specific gasification rate (*SGR*) and fuel consumption rate (*FCR*) were assumed as 200 kg m^{-2} h^{-1} (Jain 2006) and 20 kg h^{-1}, respectively. Other main dimensions of reactor body are shown in Figure 4.

$$D_R = \sqrt{\frac{4 \cdot FCR}{\pi \cdot SGR}} \quad (1)$$

Minimum height of the reactor (H_R) needed was calculated from Equation 2 and determined as 0.74 m for the operation lasting two hours. It was taken 0.84 m for safety.

$$H_R = \frac{FCR \cdot t}{A_R \cdot \rho_b} \quad (2)$$

Heating power of biomass (P_F) was determined by using Equation 3 and found about 70 kW while the *FCR* was 20 kg h^{-1} and the LHV_F was 12.77 MJ kg^{-1}.

$$P_F = LHV_F \cdot FCR \cdot \frac{1}{3.6} \quad (3)$$

Gasifier was designed as downdraft, fixed bed, and throatless type. The general structure included 4 units; top unit, main body, collector and ash removal unit, char and ash storage unit.

AISI 310 was selected as a construction material for the main body to overcome the corrosion problems while working at high temperatures. AISI 310 can work continuously at 1150 °C without corrosion. Reactor inner wall was constructed with refractive material to save main steel body from high-temperature effects. The thickness of refractive layer was applied as 50 mm. It was also used to overcome the adhesion problems of possible vitrified biomass to inner walls. Hycast 70 was used as a refractive material which can be used up to 1500 °C

Bridging can be prevented by stirring, shaking, or agitating the bed (Reed & Das 1988). Related dimensions are shown in Figure 5. The aim of using the stirrer was to decrease the temperatures whenever needed and to reduce the effects of agglomeration that may occur during the gasification process.

The position of the stirrer was adjustable along the reactor axis. The stirrer was overhead controlled by an electric motor that had 0.55 kW power. Rotational speed was adjustable by an inverter control placed on the human-machine interface (HMI) panel.

2.2.2. Measurement and control

Measurements made from 7 points for temperature, from 3 points for pressure, from 2 points for flow rate. Data monitoring and system control were operated by PLC and HMI control panel.

7 K-type thermocouples (Jain 2006) were used totally; 5 for reactor temperature profile, 1 for gas exit temperature and 1 for gas temperature after cooling. Figure 6 shows the positions of first 5 thermocouples along the rector height.

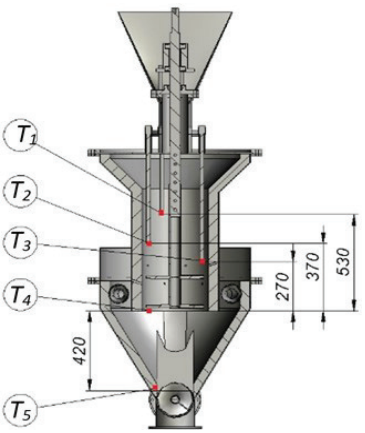

Figure 6- Thermocouple positions on reactor

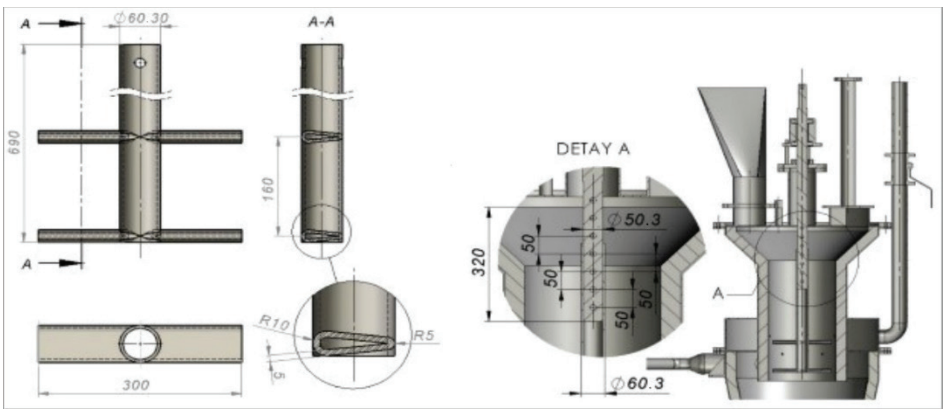

Figure 5- Stirrer construction and main dimensions

3 pressure transmitters were used in the system. P_1 was placed under the cover part of reactor body, P_2 on gas exit pipe and P_3 on the pipe after cooling. The measurable range of selected transmitters was ±1 bar.

The flow rate for the producer gas was measured by an orifice flow meter which had its own indicator with LED screen. It was suitable for the temperatures up to 200 °C. Air flow rate was also measured by an orifice flow meter that works together with a pressure differential transmitter.

Power of electric motor vacuum pump was 1.5 kW and it was controllable by an inverter. Additionally, three more motors were used to activate the stirrer, ash removal helix, and cooler fan. Each of these three components had the power of 0.55 kW. The stirrer motor was also controlled by an inverter. All on/off and invertor controls of the motors were on the control panel. Control panel included shelters, PLC modules, and HMI. HMI was 7" and connected to a computer via Ethernet. The data was stored via USB port.

2.2.3. Gas analysis

Agilent 7890B GC model gas chromatography device was used to analyze the composition of producer gas. CO, H_2, CH_4, CO_2 and N_2 concentrations were determined as volumetric percentages. In the experiments, 3 gas examples were taken in one experiment cycle time for each airflow path. One experiment cycle lasted for 1-1.5 hours. Gas sampling line was separated from the main line after flow measurement. High-purity argon gas was used as a gas carrier for sampling process.

2.3. Experimental procedure

The experiments were done for two different airflow paths with the same equivalence of ratio 0.2. The airflow paths that explained below are shown in Figure 7.

Airflow path 1 (AFP_1): Air comes into the reactor from the top, passing through the biomass bed, arrives reacting and inert char zones and leaves gasifier.

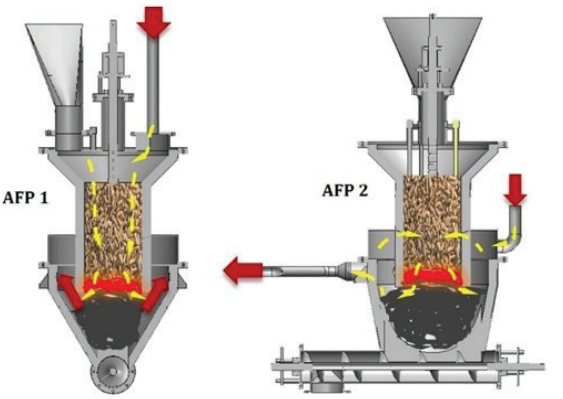

Figure 7- The airflow paths used in experiments

Airflow path 2 (AFP_2): Air comes into the reactor from the tueyers, moves through the oxidation and reduction zones and leaves gasifier.

2.3.1. Preparation

Collector was filled up to the grid level with char-ash mixture remained from previous applications. 5 kg pellets were placed on this char bed and fired by the help of some wood pieces. For the first step, both air inlets were kept open. When the flames arrived at the top level, 25 kg fuel was loaded into the reactor and the cover was closed. According to selected air path and airflow rate, air inlets were adjusted by the valves and inverter controls on HMI screen panel. After setting was completed, the experiment started.

The experiments' finish times were determined by observation of the flame level of the reactor. The observation was made by opening the valve on the biomass storage bunker. The finish times of experiments were obtained when the flame arrived at the top level of loaded biomass. During the experiment, parameters such as time, temperatures, pressures and flow rates were observed on the screen of the control panel and the data was recorded via a USB port on the panel.

The stirrer was activated when the temperature exceeded 800 °C. In other cases, the stirrer was not activated. The reactor was also tested without the stirrer by disassembly of the stirrer from the reactor.

2.3.2. Calculations

Stoichiometric ratio (SR): Stoichiometric air needed for full combustion was calculated according to ultimate analysis results by Equation 4 (Zhu & Venderbosch 2005). C_C, C_H, C_O and C_A mean the percentages of carbon, hydrogen, oxygen, and ash respectively given with ultimate analysis of biomass. SR value was determined as 3.856 kg air for 1 kg fuel.

$$SR = \left(\frac{C_C}{12} + \frac{C_H}{4} - \frac{C_O}{32}\right) \cdot \left(1 + \frac{79}{21}\right) \cdot \left(1 - \frac{C_A}{100}\right) \cdot \frac{28.84}{100} \quad (4)$$

FCR (kg h^{-1}) was calculated by Equation 5. The term m_B is defined as the mass of biomass fuel (kg) for one experiment and *t* is the measured experiment time (h).

$$FCR = \frac{m_B}{t} \quad (5)$$

Equivalence ratio (*ER*): Equation 6 was used to calculate *ER* (Reed & Das 1988). *AFR* is airflow rate measured (m^3 h^{-1}) and SR_V is stoichiometric air volume for 1 kg of biomass fuel (m^3 kg^{-1}) under experiment conditions. SR_V was taken as 3.257 m^3 air at 25 °C and 1 atm pressure.

$$ER = \frac{AFR}{FCR \cdot SR_V} \quad (6)$$

SGR (kg h^{-1} m^{-2}) is the fuel consumption rate per unit area of the reactor (m^2) (Tiangco et al 1996). Equation 7 was used to calculate SGR.

$$SGR = \frac{FCR}{A_R} \quad (7)$$

Specific gas production rate (*SGPR*): *SGPR* (N m^3 m^{-2} h^{-1}) is the producer gas flow rate (*GFR*) per unit area of reactor that calculated with Equation 8.

$$SGPR = \frac{GFR}{A_R} \quad (8)$$

LHV_G (MJ Nm^{-3}) was the lower heating value of producer gas that was calculated by Equation (9) and HHV_G (MJ Nm^{-3}) is the higher heating value of gas that was calculated by Equation (10). In these equations, it is clear that the gas compounds affected the gas calorific value are H_2, CO, and CH_4 (Waldheim & Nilsson 2001).

$$LHV_G = 10.8 \cdot \%H_2 + 12.63 \cdot \%CO + 35.8 \cdot \%CH_4 \quad (9)$$

$$HHV_G = 12.76 \cdot \%H_2 + 12.63 \cdot \%CO + 39.75 \cdot \%CH_4 \quad (10)$$

P_G (kW) is the thermal power of producer gas that calculated with Equation 11. LHV_G (MJ Nm^{-3}) and *GFR* (Nm^3 h^{-1}).

$$P_G = LHV_G \cdot GFR \cdot \frac{1}{3.6} \quad (11)$$

Cold gas efficiency (η_{CG}) equation is given with Equation 12.

$$\eta_{CG} = \frac{P_F}{P_G} \cdot 100\% \quad (12)$$

3. Results and Discussion

In this section, producer gas compositions, gasifier performance and the temperature profiles of the reactor were evaluated and compared for both airflow paths.

3.1. Gas compositions

Gas compositions and calculated lower and higher heating values are shown in Table 3. The LHV and HHV values presented in Table 3 are the values between the highest and the lowest values obtained according to the gas analysis results. As it is seen that the heating values were very close to each other but hydrogen/carbon monoxide rate was remarkably different. The H_2/CO value was desired as high as possible for environmental aspects. For this purpose, AFP_1 with the values of 5.047 MJ Nm^{-3} and 1.385 was preferred for HHV and H_2/CO respectively. In calculations, the value in the middle of the measured heating values was used.

Table 3- Producer gas compositions and heating values for AFP_1 and AFP_2

Airflow path	*Gas compositions (%)*			*LHV (MJ Nm^{-3})*	*HHV (MJ Nm^{-3})*	*H_2 (%)/CO (%)*
	H_2	*CO*	*CH_4*			
AFP_1 (from the top)	18.25	13.18	2.65	4.584	5.047	1.385
AFP_2 (from tueyers)	15.19	16.45	2.20	4.506	4.890	0.923

3.2. Gasifier performance

Performance characteristics such as FCR, SGR, GFR, SGPR, P_F, P_G and η_{CG} are given in Table 4. It is clearly seen that AFP_1 was preferable because of its cold gas efficiency value of 65.4% which is an acceptable value for a biomass gasifier. This result is in good agreement with previous publications. The previous researches on the evaluation of the energy content of paddy waste by gasification focused mostly on using rice husk as biomass feedstock due to fuel preparation cost like shredding and pelletizing.

Table 4- Gasifier performance characteristics for AFP_1 and AFP_2

Airflow path	*FCR (kg h^{-1})*	*SGR (kg h^{-1} m^{-2})*	*GFR (Nm^3 h^{-1})*	*SGPR (Nm^3 h^{-1} m^{-2})*	*P_F (kW)*	*P_G (kW)*	*η_{CG} (%)*
AFP_1 (from the top)	19.8	205.8	36.1	375.2	70.2	46.0	65.5
AFP_2 (from tueyers)	18.8	195.8	33.5	347.9	66.8	41.9	62.7

Jain & Goss (2000) studied the optimum values of SGR, LHV_g and gasification efficiency for rice husk gasification and they reported the gasification efficiency value of 65% for their gasifier under optimum conditions.

Yoon et al (2012) reported that they conducted the gasification process under the temperature range 600-850 °C with the excess air ratio of 0.2-0.32 for rice husk pellets. They determined that they reached the cold gas efficiency value of 70%.

The size of the mechanical stirrer caused the formation of a temperature bridge between the combustion and the drying zones in the reactor. In addition to this, the system was destabilized with the rapid movement of the combustion zone towards the drying zone. This situation affected the efficiency of the gasification system by shortening the time.

3.3. Temperature profiles

AFP_1 was more efficient than AFP_2 hence only AFP_1 was used in the experiments without mechanical stirrer. Measured temperatures are given in Table 5. Temperature profiles given in Figure 8 were obtained from the data saved from the first five thermocouples placed on the reactor body.

Table 5- Temperature distribution for AFP_1, AFP_2 and AFP_1 (without stirrer)

Temperature	*AFP_1 (from the top) (°C)*	*AFP_2 (from tueyers) (°C)*	*AFP_1 (without stirrer) (°C)*
T1	41	63	23
T2	44	44	21
T3	73	87	23
T4	705	687	746
T5	66	46	187

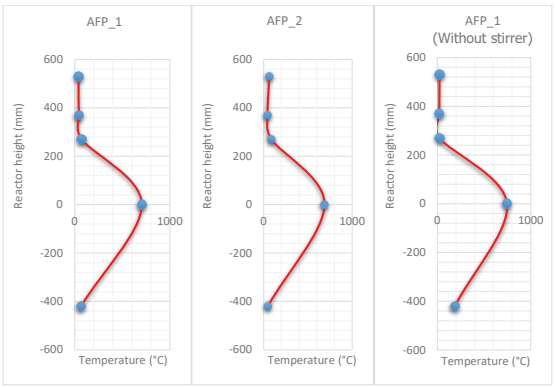

Figure 8- Temperature profiles of reactor for AFP_1, AFP_2 and AFP_1 (without stirrer)

Maximum temperature value of the reactor was 746 °C and no vitrification observed under the experiment conditions. This observation is in well agreement with a previous publication by Lin et al (1998). Their observation was that the gasification temperatures under 1000 K would recover amorphous silica materials.

The shaft of stirrer behaved like a heat bridge and took the heat from the core region to the upper cover of the reactor. In AFP_1, the fresh air that came from upside cooled down the upper regions.

3.4. Comparison of the gasifier performance due to the stirrer effect

In the experiments, it was determined that AFP_1 was more efficient than AFP_2 hence only AFP_1 was used in the experiments without mechanical stirrer.

Gas compositions and calculated lower and higher heating values are shown in Table 6. Although the results were very close to each other, using the reactor without stirrer could be more preferable when considering reactor manufacturing costs in addition to the results.

Performance characteristics such as FCR, SGR, GFR, SGPR, PF, PPG and η_{CG} are given in Table 7. It can be seen that the gasification efficiencies for both applications were very close to each other.

Table 6- Producer gas compositions and heating values for AFP_1 (with and without stirrer)

Airflow path	*Gas compositions (%)*			*LHV (MJ Nm^{-3})*	*HHV (MJ Nm^{-3})*	*H_2 (%) / CO (%)*
	H_2	*CO*	*CH_4*			
AFP_1 (with stirrer)	18.25	13.18	2.65	4.584	5.047	1.385
AFP_1 (without stirrer)	18.22	12.91	2.66	4.550	5.013	1.411

Table 7- Gasifier performance characteristics for AFP_1 (with and without stirrer)

Airflow path	*FCR (kg h^{-1})*	*SGR (kg h^{-1} m^{-2})*	*GFR (Nm^3 h^{-1})*	*SGPR (Nm^3 h^{-1} m^{-2})*	*P_F (kW)*	*P_G (kW)*	*η_{CG} (%)*
AFP_1 (with stirrer)	19.8	205.8	36.1	375.2	70.2	46.0	65.5
AFP_1 (without stirrer)	19.5	202.7	35.9	373.1	69.1	45.8	65.6

4. Conclusions

The designed gasifier for rice straw pellets successfully worked. The designed system allowed to generate clean producer gas which can be used for heating processes. The vitrification could be taken under control with designed system. In case of using additional cleaning and filtering components, electrical power generation would also be possible.

Equivalence ratio was applied as 0.2 for experiments. Two different airflow paths were tested

with this equivalence ratio and compared for the process. AFP_1 had higher cold gas efficiency than AFP_2. Furthermore, the H_2/CO value of AFP_1 was higher than that of AFP_2.

Activation of the stirrer caused a decrease in the temperature and helped to take them under control which can be considered as an advantage. On the other hand, the existence of an overhead driven stirrer axe generated a disadvantage by acting like a heat bridge between reaction zones and driving them away from the steady state. Therefore, using the overhead driven stirrer was unadvisable for rice straw gasification due to the risks for vitrification and steady-state points of view.

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Abbreviations and Symbols	
D_R	Diameter of reactor
SGR	Specific gasification rate
FCR	Fuel consumption rate
H_R	Height of the reactor
P_F	Heating power of biomass
LHV	Lower heating values
HHV	Higher heating values
SR	Stoichiometric ratio
C_C	Percentages of carbon
C_H	Percentages of hydrogen
C_O	Percentages of oxygen
C_A	Percentages of ash
m_B	Mass of biomass fuel
t	Measured experiment time
ER	Equivalence ratio
AFR	Airflow rate
SR_V	Stoichiometric air volume
P_G	Thermal power of producer gas
$SGPR$	Specific Gas Production Rate
GFR	Producer gas flowrate
A_R	Area of reactor
η_{CG}	Cold gas efficiency
AFP	Airflow path
ρ_b	Bulk density of biomass

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Combined Effects of MAP and Postharvest Salicylic Acid Treatment on Quality Attributes of Dill (*Anethum graveolens* L.) Bunches during Storage

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ABSTRACT

The effects of combinations of modified atmosphere packaging (MAP) with salicylic acid (SA) treatment on storage and shelf life quality of dill (*Anethum graveolens* L. cv. Asder) leaves were investigated. After harvest, dill leaves were dipped into an aqueous solution containing different concentrations of salicylic acid (1, 2 and 4 mM) for 2 minutes. The control group was immersed in distilled water only for 2 minutes. Treated samples were dried with blotting paper and placed in modified atmosphere package and stored at 0 ºC and 90±5% relative humidity (RH) conditions for 25 days. After cold storage, dill leaves were kept at 10 ºC and 55-60% RH for 2 days to simulate commercial practice (shelf life), and analyzed for same quality parameters performed during cold storage. Weight loss, color, respiration rate, gas composition in package, soluble solids content (SSC) and ascorbic acid content were determined initially and at 5 day-intervals. The dill bunches were also evaluated for visual quality during storage period. According to the results, SA treatment allowed dill leaves to stay green longer than those of control group. 1 mM concentration of SA was the best treatment for prolonging the storage life of dill leaves with keeping the quality.

Keywords: Dill; Cold storage; MAP; Salicylic acid

1. Introduction

Dill (*Anethum graveolens* L.), a member of celery family *Apiaceae,* is the only species of the genus *Anethum*. Dill is a valuable aromatic herb and has been used for enhancing flavor of some foods such as pickle, soups and salads (Sakaldaş et al 2010). In Europe and central Asia, people use fresh and dried leaves of dill in their food. Dill leaves are best while they are fresh because they lose their flavor rapidly when leaves are dried (Kılıç & Duyar 2016).

Leafy vegetables and herbs have a relatively short postharvest life because of the high respiration rate and rapid senescence process. Yellowing is a well-known senescence symptom of leafy vegetables and they lose their quality rapidly after harvest (Koukounaras et al 2006). Some of the reasons behind these losses are traditional approaches

to handling, lack of preservation technologies and marketing knowledge. Simple strategies for postharvest quality maintenance are required to reduce losses of economic crops (Ali et al 2013).

The modified atmosphere packaging (MAP) is widely used technology which reduces respiration rate, weight loss and decay, and prolongs the postharvest life of many horticultural products (Sakaldaş et al 2010). MAP reduces the respiration rate and other metabolic processes by modification of CO_2 and O_2 concentrations in the storage atmosphere surrounding the commodity (Kader et al 1989).

Nowadays, extend of the postharvest life by chemicals is limited because of their detrimental effect on the environment and human health. Therefore, we need to develop more safe and effective strategies (Mandal et al 2009). More attention is being paid to plant based metabolites as anti-ripening and anti-microbial agents for sustenance of maximum postharvest quality of products (Ali et al 2013). Salicylic acid (SA), a member of a group of phenolic compounds, is now considered as a hormonal substance (Awad 2013). SA has been reported to play an important role in regulating of many physiological processes (Zavala et al 2004) and in controlling quality losses of horticultural crops after harvest (Asghari & Aghdam 2010). In addition, the use of SA in pre- and post-harvest period has been reported to be effective in maintenance of quality and extension of storage life for some horticultural crops such as banana (Srivastava & Dwivedi 2000), tomato (Ding et al 2001), peach (Wang et al 2006), pomegranate (Sayyari et al 2009), and pineapple (Lu et al 2011).

There is no study available on the effect of SA treatment on dill leaves during cold storage. This research aimed to determine the role of SA on extending the storage life and maintaining quality of dill leaves during storage in MAP.

2. Materials and Methods

2.1. Plant material

The fully expanded green dill leaves (*Anethum graveolens* L.) of parsley cv. Asder were used as plant material. Dills were harvested in early morning and about 75-80 dill stems were grouped into bunches (~30 cm long, ~250 g). Foreign materials and withered yellow leaves were removed from research material. Bunches were homogenized and examined visually.

2.2. Salicylic acid treatment and storage conditions

Dill leaves were harvested from Isparta-Turkey, and transported to postharvest physiology laboratory of Fruit Research Institute, immediately. Dill bunches were pre-cooled (the external temperature of dills reduced to 4-5 °C within 24 hours) by forced air at 1 °C and 85-90% relative humidity (RH). After precooling, bunches were dipped in an aqueous solutions containing 1, 2 and 4 mM SA and Tween-20 for 2 minutes. Control group was immersed in distilled water and Tween-20 for 2 minutes. After treatments, bunches were drained and dried with blotting paper. Then bunches placed in modified atmosphere package (LDPE) were stored at 0 °C and 90±5% RH for 25 days. After cold storage, bunches were kept at 10 ºC and 55-60% RH for 2 days to simulate commercial practice (shelf life). Samples from the 0^{th}, 5^{th}, 15^{th}, 20^{th} and 25^{th} day of cold storage and shelf life were analyzed.

2.3. Quality analysis

Weight losses of dills were expressed as the percentage of loss of weight with respect to the initial weight. Weight loss was determined by the equation;

$$\text{Weight loss} = [(\text{First weight} - \text{Last weight}) / \text{First weight}] \times 100 \quad (1)$$

Respiration rate was measured in 3 bunches for each replicate. Weighed dill bunches were placed in airtight jars (1.3 L) at 20 °C for 1 hour. Then gas sample was taken from jars and injected into gas chromatographs (Agilent GC-6890N). Measurements were made in split/splitless (S/SL) of inlet in split mode with gas sampling valve with 1 ml gas sample by using fused silica capilar column (GS-GASPRO, 30 m × 0.32 mm I.D., U.S.A), with thermal conductivity detector (TCD) for respiration rate measurements by Agilent GC-6890N (U.S.A

and Canada) model gas chromatography (GC) and Chemstation A.09.03 [1417] software. Carrier gas flow was 1.7 mL min^{-1} in stable flow mode. Results were expressed as mL CO_2 kg^{-1} h^{-1}.

Soluble solids contents (SSC) of dills were measured with a digital refractometer (Atago Pocket PAL-1) and expressed as percentage (%).

Color measurement was performed at two parts of leaf surface with a colorimeter (Minolta CR-400, Japan) over 5 bunches in each replicate. Calibration was made by the standard white plate of manufacturer company. The values were expressed by the CIE L^* (brightness-darkness), a^* (+ a^*: red, - a^*: green) and b^* (+ b^*: yellow, - b^*: blue) system and the values were evaluated as L^*, a^* and b^*.

Gas concentration (O_2 and CO_2) of packages was determined by Gaspace 2 (Gas Headspace Analyzer, Systech Instruments).

Visual quality (external appearance) was determined using a scale of yellowing and freshness. Dill leaves were divided into four groups and graded as a scale of 1-4 (1 dark green-too fresh, 2 light green-fresh, 3 yellowish green-few fresh, 4 greenish yellow not fresh) (Sakaldaş et al 2010).

Vitamin C content of dills were determined by spectrophotometric method, using 2,6-dichlorophenolindophenol (Loeffler & Ponting 1942).

2.4. Statistical analysis

The experiment was set up according to the factorial randomized design with 3 replications. Main effects and interactions were analyzed and means were compared by LSD Tests at a significance level of 0.05. All analyses were performed with SPSS software package v.18.0 for Windows by General Linear Model (GLM) univariate test.

3. Results and Discussion

3.1. Weight loss

Weight loss of dills can lead to discoloration which reduces both market value and consumer acceptability. Evaporation of moisture from the surface of fresh commodities is responsible for reduction in weight (Ali et al 2013). The weight losses of dills are illustrated in Figure 1. Weight loss (%) increased with prolonging storage period and temperature. Generally, weight losses were minimized by cold storage. Weight losses of dills were at low values at the end of the cold storage but these losses reached the higher values when dills were transferred to 10 ºC. As expected in this research, the higher weight loss in shelf life condition is related to higher water vapor losses. The effects of treatments and storage periods on weight loss were significant. The weight loss of the dills treated with all concentrations of SA was relatively delayed compared to control group at the end of shelf life. MAP has been known to limit weight losses by reducing moisture loss from the package. SA application became effective in inhibiting weight loss in MAP through retarding senescence process during shelf life. The maintenance of cellular integrity by SA (Ali et al 2013) might be the reason in lowering weight loss of dill leaves in the present

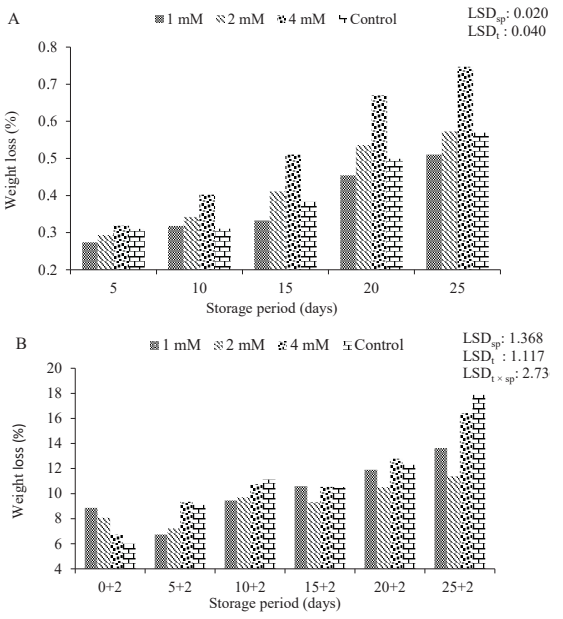

Figure 1- Effect of SA on weight loss of dill leaves stored at cold storage (A) and shelf life (B)

study. Likewise, Gill et al (2016) indicated that weight loss of crops was reduced by SA treatment.

3.2. Soluble solids content

The SSC of dills were given in Figure 2. SSC of dills was remittent during storage periods and there was no stable increase or decrease. However, SSC of dills stored at cold condition were less than those of shelf life condition. All treatment displayed a decreasing in SSC compared to initial values in cold storage condition. On the contrary of the cold storage, the SSC of dills increased with shelf life after 25+2 days. The breakdown of complex carbohydrates into more soluble sugars and reducing water content might be the reason in increased sugar concentration at shelf life condition. Likewise, according to Kluge et al (1996), sugar loss due to respiration could account for sugar increases with weight loss, and Rohani et al (1997) indicated that slower respiration rates resulted in slow degradation of complex carbohydrates into simple sugars.

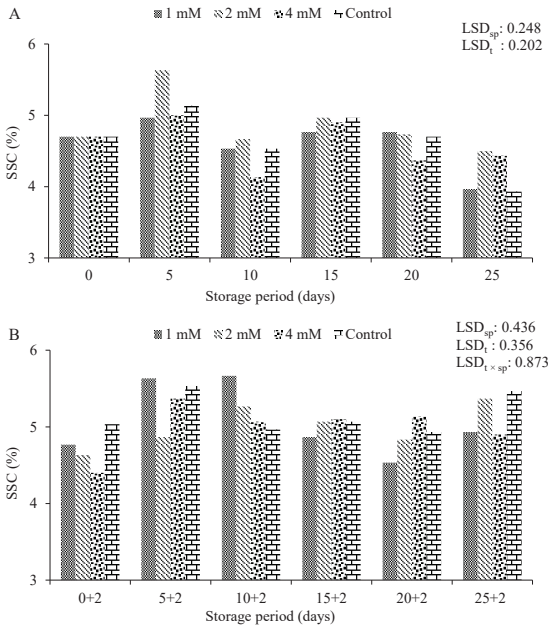

Figure 2- Effect of SA on SSC of dill leaves stored at cold storage (A) and shelf life (B)

3.3. Color

The color changes of SA-applied and control group dills during the storage were given on the Table 3. L* values, which represent brightness-darkness, of the dills were generally decreased during cold storage, except for 1 mM SA treatment. Dill leaves treated with 1 and 2 mM SA were slightly brighter than those of other treatments, especially after 15 days of cold storage. On the contrary of the cold storage, L* values increased compared to initial values after 25+2 day of storage. The increase of L* can be due to discoloration of leaves at shelf life condition (Figure 3).

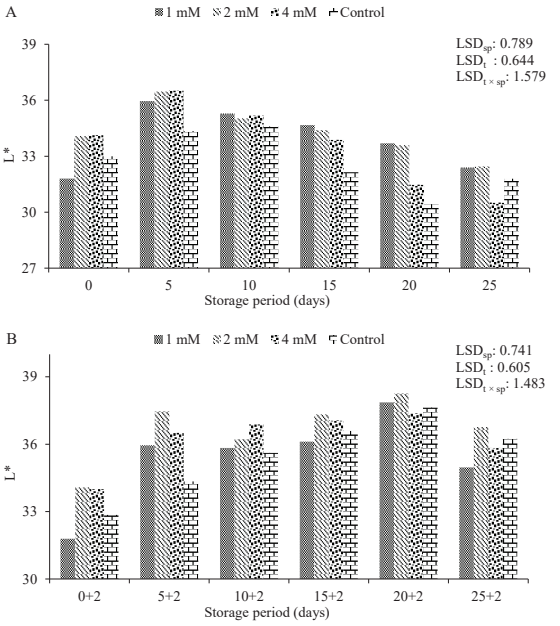

Figure 3- Effect of SA on L* values of dill leaves stored at cold storage (A) and shelf life (B)

Dill leaves were harvested at a stage when the leaves had fully green color. a* and b* values increased gradually in cold storage and shelf life. The increasing in a* and b* values were higher in the control group (Figure 4, 5). This means that the dill leaves in the control group had more yellow surface color than the others at the end of storage. The best results for maintaining color were obtained from dill

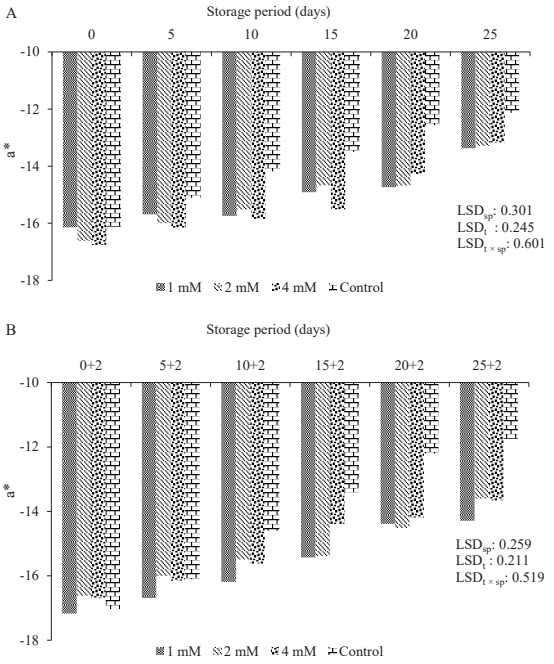

Figure 4- Effect of SA on a* values of dill leaves stored at cold storage (A) and shelf life (B)

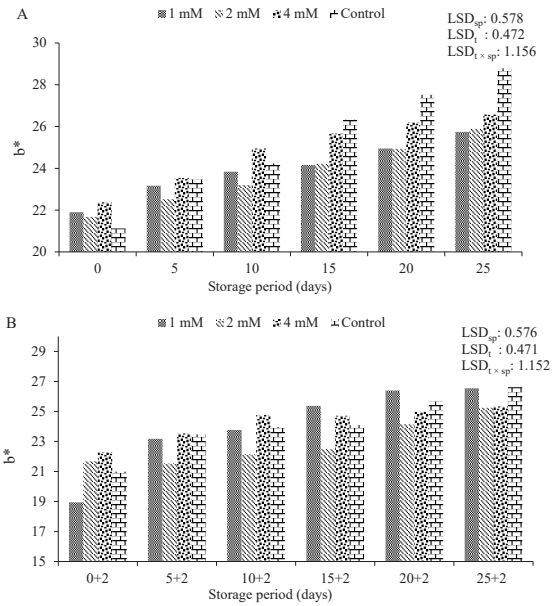

Figure 5- Effect of SA on b* values of dill leaves stored at cold storage (A) and shelf life (B)

leaves treated with 1 mM SA. These findings seem to be in accordance with the general effect of SA and MAP in delaying the senescence process during storage. Similarly, it was reported that SA treatment delayed discoloration (Peng & Jiang 2006) or prevent color change by decreasing senescence (Asghari & Aghdam 2010).

3.4. Gas composition in package

The gas composition in package is illustrated in Figure 6. The O_2 concentration of MAP fluctuated between 12.27% and 19.30% during the entire storage period. The O_2 levels on the 5th day were between 17.75% in the control group and 12.27% in the 4 mM SA treatment, while these values were found as 18.33% and 14.80%, respectively after 25 days of storage. As can be seen from Figure 6, the concentration of O_2 gas in the MAP went down to 14.93% (average value) within five days and remained fairly constant during the rest of storage period. The CO_2 level in MAP ranged from 1.20% to 3.13% throughout the storage. The lowest average

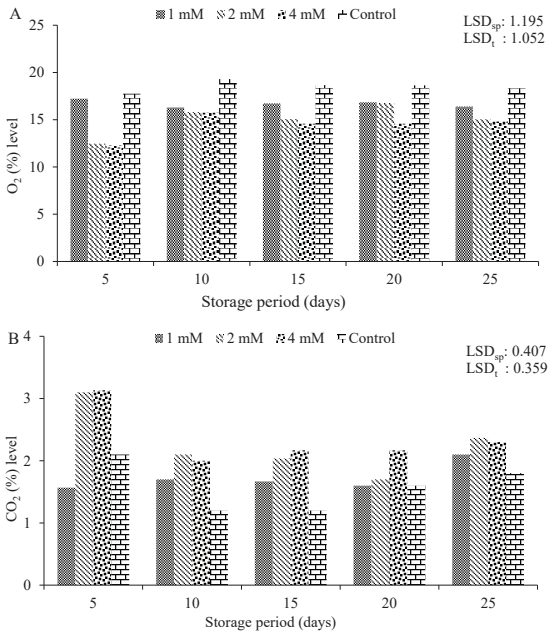

Figure 6- Effect of SA on O_2 (A) and CO_2 (B) composition in MAP

CO_2 level (1.58%) was found in the control group, while the highest one was obtained from the 4 mM SA treatment (2.35%). We observed a similar gas composition within the MAP during storage of dill leaves treated with different concentrations of SA. Although SA, in a concentration dependent manner, affected respiration rate of dill leaves, this trend could not be observed in the gas values in the package. This can be due to factors, which affect gas composition for MAP, such as weight of crops, homogeneous permeability of packaging materials, maturation stage of crops etc. These results were similar to the findings of Tano et al (2007).

3.5. Respiration rate

Respiration rate is a major factor affecting the postharvest quality loss of horticultural crops. Therefore, it is very important to maintain the respiration rate at minimum level to prolong the storage life of crops. The respiration rate of dill leaves began to increase and attained at maximum levels at the 15th days of the storage. However, on 20th and 25th day of cold storage, the respiration rate decreased. Similar results were obtained from shelf life storage. Control samples had the highest respiration rate (19.076 mL CO_2 kg^{-1} h^{-1}) followed by 4 mM (16.920 mL CO_2 kg^{-1} h^{-1}), 2 mM (14.451 mL CO_2 kg^{-1} h^{-1}) and 1 mM SA (10.130 mL CO_2 kg^{-1} h^{-1}) treatments, at the end of the cold storage. In storage for shelf life, these values were found as 15.940 mL CO_2 kg^{-1} h^{-1} (2 mM SA), 15.878 mL CO_2 kg^{-1} h^{-1} (control), 14.524 mL CO_2 kg^{-1} h^{-1} (4 mM SA) and 13.952 (1 mM SA). Lower concentrations of SA (1 and 2 mM) treatments successfully suppressed the respiration rate of dill leaves during cold storage (Figure 7). Similar trends were also observed by Norman et al (2004) in tobacco; Han et al (2003) in peach; Mo et al (2008) in sugar apple. The effect of SA may be due to retarded senescence of dill leaves during storage. Wills et al (1998) reported that SA treatment effectively reduced metabolic activity which delays crops senescence process. On the other hand, at the lower concentrations (0.1 mM or less), the effect of SA on respiration rate was transitory, but at higher concentrations, respiration rate was severely inhibited in tobacco (Norman et al 2004).

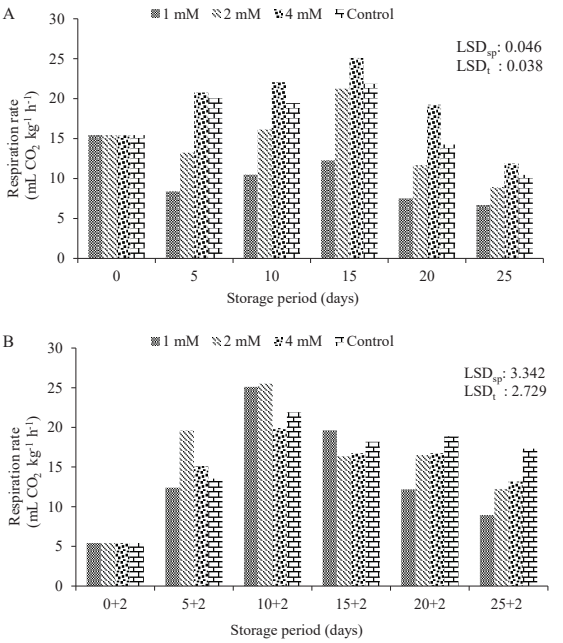

Figure 7- Effect of SA on respiration rate of dill leaves stored at cold storage (A) and shelf life (B)

3.6. Ascorbic acid

The ascorbic acid of dill significantly tended to decrease throughout the storage. Decline of ascorbic acid was strict after 15 day of storage. The best preservation of ascorbic acid was obtained from 2 mM SA treated dill bunches after 25 day of cold storage. However, no significant difference was found between SA concentrations and control group. Nevertheless, it was found that 1 mM and 2 mM SA concentrations gave the highest values of ascorbic acid compared to control after 25 days of storage (Table 1). Correspondingly, ascorbic acid was significantly decreased in control pomegranates but remained unchanged in fruit treated with the highest (2 mM) concentration (Sayyari et al 2009). In present study, the lowest ascorbic acid values (5.44-4.67 mg 100 mL^{-1}) were determined in dill leaves treated with 4 mM. Similarly, Maysoun (2016) reported that the highest SA treatments (5 mM SA) resulted in reduced ascorbic acid content compared with the control in tropical fruits. SA prevented ascorbic acid destruction in pineapple (Lu et al 2011), and it might

Table 1- Effect of SA on ascorbic acid content (mg 100 mL^{-1}) of dill leaves stored at cold storage and shelf life

Storage period (days)							
			Cold storage				
Treatments	*0*	*5*	*10*	*15*	*20*	*25*	*Means*
1 mM	11.762	10.202	8.074	1.804	3.424	1.322	6.098
2 mM	11.762	13.823	4.404	3.424	2.246	1.650	6.218
4 mM	11.762	11.298	5.749	1.730	1.585	1.138	5.544
Control	11.762	10.825	5.126	3.191	1.948	1.001	5.642
Means	11.762 A*	11.537 B	5.838 C	2.537 D	2.301 E	1.278 F	
			Shelf life				
Treatments	*0+2*	*5+2*	*10+2*	*15+2*	*20+2*	*25+2*	*Means*
1 mM	11.146	9.362	7.288	1.413	2.061	1.127	5.400
2 mM	11.146	9.803	7.419	1.588	1.287	1.027	5.378
4 mM	11.146	8.846	4.540	1.069	1.395	1.048	4.674
Control	11.146	9.275	4.397	1.370	1.090	0.984	4.710
Means	11.15 A	9.32 B	5.911 C	1.360 D	1.458 D	1.047 D	

*, means followed by different letters with in the same row are significantly different at P<0.01

be used to decrease deterioration of ascorbic acid in crops (Sayyari et al 2009).

3.7. Visual quality

The results pertaining to sensory tests of dill leaves are presented in Table 2. The visual quality of dill leaves decreased with prolonging storage period, and increasing storage temperature. Storage conditions and SA concentrations affected the visual quality of dill leaves. Dill leaves treated with 1 mM SA had the best quality compared to the other concentrations (2 mM and 4 mM). The quality loss of dill leaves was noticeable after 20 day cold storage. Similarly, Sakaldaş et al (2010) reported significant quality losses in dill leaves after 20 day cold storage. In present study, SA treated dill leaves

Table 2- Effect of SA on visual quality (1-4) of dill leaves stored at cold storage and shelf life

Storage period (days)							
			Cold storage				
Treatments	*0*	*5*	*10*	*15*	*20*	*25*	*Means*
1 mM	1.00	1.00	1.50	1.80	2.04	2.45	1.63
2 mM	1.00	1.33	1.62	1.98	1.96	3.11	1.83
4 mM	1.00	1.31	1.70	1.97	2.04	3.61	1.94
Control	1.00	1.33	1.97	2.00	2.33	3.73	2.06
Means	1.00 D*	1.24 D	1.70 C	1.94 BC	2.09 B	3.22 A	
			Shelf life				
Treatments	*0+2*	*5+2*	*10+2*	*15+2*	*20+2*	*25+2*	*Means*
1 mM	1.00	1.17	1.71	1.97	2.43	3.18	1.91
2 mM	1.00	1.39	1.93	2.15	2.63	3.72	2.14
4 mM	1.00	1.36	1.93	2.16	3.03	3.82	2.22
Control	1.07	1.40	2.15	2.27	3.39	3.85	2.36
Means	1.02 E	1.33 D	1.93 C	2.14 C	2.87 B	3.64 A	

*, means followed by different letters with in the same row are significantly different at P<0.01

almost maintained their quality until 20 day of cold storage. However these leaves reached to the non-marketable limit with 15 day cold storage plus 2 days shelf life except for 1 mM treatment (Table 3). The decreasing pattern in the score of visual quality in the present study was in agreement with previous studies by Catunescu et al (2012), who reported reductions of scores from 20.20 to 18.18 after 12 day storage of dill treated at 4 °C. In some previous researches conducted in fruits, SA treatments also decreased quality losses compared to control samples during storage (Sayyari et al 2009; Luo et al 2011; Ali et al 2013; Khademi & Ershadi 2013).

Table 3- ANOVA for dependent variables for treatments, storage period and their interactions for dill leaves

Parameters	*Cold storage*			*Shelf life condition*		
	SP	*T*	*T×SP*	*SP*	*T*	*T×SP*
Weight loss	**	**	ns	**	**	**
Respiration rate	**	**	ns	**	**	ns
Visual quality	**	ns	ns	**	ns	ns
SSC	**	*	ns	**	**	**
L*	**	**	**	**	**	**
a*	**	**	**	**	**	**
b*	**	**	**	**	**	**
Gas composition in MAP-O_2 level	*	**	ns	-	-	-
Gas composition in MAP-CO_2 level	**	**	ns	-	-	-
Ascorbic acid content	**	ns	ns	**	ns	ns

ns, represents non-significance at P<0.05; **, represents significance at the 0.01 level; *, represents significance at the 0.05 level; SP, storage period; T, treatments

4. Conclusions

According to the results of this research, SA treatment allowed dill leaves to stay green longer than those of control group. Dill leaves treated with lower concentrations of SA (1 and 2 mM) maintained their quality better than those of 4 mM SA and control treatment. The 1 mM SA was the best treatment for prolonging the cold storage and shelf life of dill leaves with keeping quality. Dill leaves treated with 1 mM SA could be stored for 15+2 days with marketable quality in MAP, but control leaves lost their commercial properties after 10+2 days. As a ubiquitous phenolic acid, a plant based metabolites and an endogenous hormone, SA could be a promising candidate for prolonging postharvest life and quality of dill leaves. However, further detailed research on this subject is needed to investigate.

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Convective Drying Kinetics and Quality Parameters of European Cranberrybush

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ABSTRACT

In this research, the effects of convective drying (60, 70, 80 and 90 °C) techniques on the drying kinetics, color, antioxidant capacity and total phenolic content of European cranberrybush were investigated in detail. To choose the best thin-layer drying models for the drying treatments, 10 mathematical models were compared for the experimental data. Depending on the evaluation by statistical tests, the Midilli et al model was determined to be the best suitable model to explain the drying behavior of European cranberrybush samples. All of the colorimetric parameters were influenced by drying temperatures. Antioxidant capacity and total phenolic content values of European cranberrybush samples displayed a significant reduction at low-temperature levels (60 and 70 °C) with regard to those at high-temperature levels (80 and 90 °C). In addition, the correlation analysis between antioxidant capacity and total phenolic content exhibited a high degree of correlation (R^2= 0.8656).

Keywords: European cranberrybush; Drying characteristics; Colorimetric parameters; Total phenolic content; Antioxidant capacity

1. Introduction

European cranberrybush (*Viburnum opulus* L.) species comes from the Caprifoliaceae plant family. Despite being grown mostly around the city of Kayseri, Turkey and called gilaburu, European cranberrybush is today common in eastern, western, northeastern, and central Europe (Yilmaztekin & Sislioglu 2015) and known as European cranberrybush (Kayaçelik et al 2015), Guelder rose or Cramp bark (Velioğlu et al 2006). It contains a high amount of polyphenolics, including phenolic acids and anthocyanins, as well as organic acids such as ascorbic and L-malic acids (Kraujalytė et al 2013). The European cranberrybush is utilized as a traditional and folk medicine by European, Asian and Native American people. It is thought that fruits features have a preventive effect on cough, cramps, stomachache, uterine infections, menstrual cramps, blood pressure, infertility, asthma, nervousness, cold, fever and water retention problems (Sagdic et al 2014). Locally, European cranberrybush fruit is used in preparing jelly, jam, marmalade and sweetmeat too (Rop et al 2010) but it is not eaten directly due to its acidic taste (Kayaçelik et al 2015).

Drying method is commonly used for the prolonged shelf-life, significant volume reduction, and product diversity, and these benefits could be extended even more, with enhancements in the quality of product and process applications. Hot air drying method has lots of benefits, for instance, decrease microbial contamination and provide a more uniform, the minimal adverse impact of weather conditions, shorter drying periods, and cheaper labor costs compared to traditional drying technique (Karabulut et al 2007). Various agricultural products have been dried by successfully applying hot air such as onion (Mota et al 2010), pear (Purkayastha et al 2013), apricot (Albanese et al 2013), cherry tomatoes (An et al 2013), mango (Murthy & Manohar 2014) and jackfruit (Saxena & Dash 2015). However, very few numbers of researches have been conducted about the drying process of European cranberrybush. The aim of the study is to specify drying kinetics of the thin layer, to examine the differences with regard to color, total phenolic content (TPC), and antioxidant activity (AC) of the dried and fresh European cranberrybush samples.

2. Material and Methods

2.1. Drying equipment and procedure

Samples of fresh European cranberrybushes were gathered from the fields of Corekdere Village, Kayseri, Turkey. The fruits were kept to dry at 4±0.5 °C till to the drying experiments. In all experiments totally matured and healthy European cranberrybushes (average diameter of 10.52±0.09 mm) were used. Their initial moisture content was determined to be 5.10 (g water g dry matter^{-1}) on a dry basis (db) by forced-air convection oven (ED115 Binder, Tuttlingen, Germany) which was drying at the temperature of 105 °C for the period of 24 hours (Hii et al 2012). Drying was continued until the final moisture content of the samples reached 0.1 (g water g dry matter^{-1}). The convective drying process was conducted in a laboratory convective oven (Whirlpool AMW 545, Italy). A rotating round plate of a glass material which has 400 mm diameter was used to put cranberrybush samples in a thin layer. For the drying procedure, the velocity of air was defined as 1.5 m s^{-1}, and air temperatures were defined as 60, 70, 80 and 90 °C. A digital balance (Shimadzu UX-6200H, Tokyo, Japan) that has 0.01 g precision was placed under the oven to measure the mass change (Giri & Prasad 2007). All of the experiments were carried out in triplicate.

2.2. Mathematical modeling of the drying data

The data about moisture content which was gathered by means of the drying experiment were converted to the moisture ratio (*MR*) and fitted by using ten thin-layer drying models (Table 1). The moisture ratio was confirmed by making use of the Equation 1.

$$MR = \frac{M_t - M_e}{M_o - M_e} \quad (1)$$

Where; M_o, initial moisture content (g water g dry matter^{-1}); M_t, moisture content at a particular time (g water g dry matter^{-1}); M_e, equilibrium moisture content (g water g dry matter^{-1}). *MR* value was simplified to Equation 2. Since, M_e values are relatively insignificant when they are compared to M_t or M_o.

$$MR = \frac{M_t}{M_o} \quad (2)$$

2.3. Color measurement

Colors of the dried and fresh European cranberrybush samples were confirmed in the color scales of *L*, *a* and *b* by the using external surface of the samples with Hunterlab Color Analyzer (MSEZ-4500L, Reston, Virginia, USA). Color measurements were stated in a three-dimensional *L**, *a**, and *b** color spaces, where *L** stands for the darkness/lightness of the sample, *a** stands for the greenness (negative (-) value) and the redness (positive (+) value), and *b** stands for the blueness (negative (-) value) and the yellowness (positive (+) value). L_0*, a_0* and b_0* represent color parameters of the fresh samples.

Table 1- Thin layer drying models used for mathematical modelling of the drying kinetics of European cranberrybush samples

No	*Model name*	*Model*	*References*
1	Henderson and Pabis	$MR = a\exp(-kt)$	Demiray & Tulek (2014)
2	Newton	$MR = \exp(-kt)$	Saxena & Dash (2015)
3	Page	$MR = \exp(-kt^n)$	Murthy & Manohar (2014)
4	Logarithmic	$MR = a\exp(-kt) + c$	Mota et al (2010)
5	Two term	$MR = a\exp(-k_0t) + b\exp(-k_1t)$	Bhattacharya et al (2015)
6	Two term exponential	$MR = a\exp(-kt) + (1-a)\exp(-kat)$	Evin (2011)
7	Wang & Singh	$MR = 1 + at + bt^2$	Arumuganathan et al (2009)
8	Diffusion pproach	$MR = a\exp(-kt) + (1-a)\exp(-kbt)$	Menges & Ertekin (2006)
9	Verma et al	$MR = a\exp(-kt) + (1-a)\exp(-gt)$	Faal et al (2015)
10	Midilli et al	$MR = a\exp(-kt^n) + bt$	Midilli et al (2002)

After the calibration of the colorimeter against standard black and white surfaces, six replicate measurements were conducted for each sample. In order to explain the color changes, chroma (C) and hue angle (α) total color difference (ΔE) values were figured out using the L_0*, a*, b*, a_0* and b_0* parameters which have been defined by Equations 3, 4 and 5 (Maskan 2001).

$$C = \sqrt{(a^2 + b^2)} \quad (3)$$

$$\alpha = \tan^{-1}(\frac{b}{a}) \quad (4)$$

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (5)$$

2.4. Preparation of sample extracts

The extraction procedure was conducted by conforming to the method of Turkmen et al (2005). Homogenized 1 g of European cranberrybush samples with 4.5 mL of water:methanol (20:80 v:v) was shaken at 140 rpm (Biosan OS-20, Latvia) for 120 minutes at room temperature. Following, the solutions were centrifuged for a duration of 15 minutes at 10,000 *g* (Sigma 3K30, UK) and the supernatants were gathered up. The two extractions were conducted with pellet by using the same conditions. After the combination of obtained supernatants, they were passed through a PTFE membrane filter of 0.45 µm in order to determine AC and TPC values of the samples. Extraction procedures were carried out in triplicate.

2.5. Determining total phenolic contents

The total phenolic content of the fruit was examined in line with the method of Igual et al (2012) with some changes on it, for instance, using gallic acid (GA) as the standard. European cranberrybush extracts (0.25 mL) were blended with Folin-Ciocalteu reagent of 1.25 mL (Sigma-Aldrich, Germany) and distilled water of 15 mL on a vortex mixer (WiseMix VM-10, Daihan, South Korea). After this mixture was stored in the dark for 8 minutes, 3.75 mL of 7.5% Na_2CO_3 was added to the mixture and then with distilled water, the volume was completed to 25 mL. Lastly, the absorbance was gauged in a spectrophotometer (Optizen 3220 UV, Mecasys, Korea) at 765 nm and then compared with a GA calibration curve (with a concentration range of 5-50 mg L^{-1}). These results were stated as mg GA 100 g^{-1} on a dry weight. All of these measurements were conducted in triplicate.

2.6. Determining antioxidant capacity

The antioxidant capacity (AC) was assessed by the DPPH (2,2-diphenyl-1-picrylhydrazyl) free-radical scavenging activity of the European cranberrybush extracts in compliance with the method defined by Alothman et al (2009) sample extract (0.1 mL)

which was appropriately diluted was put into 3.9 mL of 25 mM DPPH methanolic solution. After mixing (WiseMix VM-10, Daihan, Korea) approximately about 15 to 30 seconds and kept dark to wait at room temperature for 30 minutes, absorbance values were gauged at 515 nm (Optizen 3220 UV, Mecasys, Korea). Methanol solutions of known trolox concentrations which were between 0.1 to 1.0 mM were used in the calibration curve and the obtained outcomes were stated as µmol trolox equivalents (TE) (Merck, Germany) per 1 g dry weight. All measurements were done in triplicate as well.

2.7. Statistical analysis

The study was carried out by using the randomized plots factorial design of experimental type. During the measuring process of the examined components, three replicates were used. To analyze these results, JMP (Version 7.0, SAS Institute Inc., Cary, NC, USA) and MATLAB (MathWorks Inc., Natick, MA) software packages were used. The significance of mean differences was tested and the LSD test (Least Significant Difference Test) resulted in 5% of significance level. The model which has the lowest reduced chi-squared (χ^2) and RMSE (Root Mean Square Error) values, as well as the highest coefficient of determination, (R^2) was concluded to be the optimal model that describes the drying characteristics of pineapples in a thin layer (Chayjan et al 2015). The explanations of these statistical values are on Equations 6 and 7 (Doymaz & Ismail 2011).

$$\chi^2 = \frac{\sum_{i=1}^{N}(MR_{\exp,i} - MR_{pre,i})^2}{N - z} \quad (6)$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(MR_{pre,i} - MR_{\exp,i})}{N}} \quad (7)$$

Where; $MR_{\exp,i}$, experimental moisture ratio at the test number i; $MR_{pre,i}$, estimated moisture ratio at the test number i; N, observation number; z, total count of constants used in the drying model.

3. Results and Discussion

3.1. Drying kinetic of dried European cranberrybush

The shifts in moisture content of the European cranberrybush samples which is represented as a drying duration function at various temperatures are showed in Figure 1. Drying duration of European cranberrybush samples which were dried at air temperatures of 60, 70, 80 and 90 °C with a fixed drying air velocity of 1.5 m s^{-1} were lasted about 480, 310, 210 and 130 minutes, respectively. The outcomes of the experiment have shown that the average total drying duration for European cranberrybush at 90 °C was 250 minutes shorter than that of 60 °C. In other words, the drying time dropped 52.08% when the temperature of air raised from 60 to 90 °C. Considering these findings, it can be deduced that the increase in the drying temperature will boost the kinetic energy of water molecules and ultimately it triggers the water evaporation rate. That way, the drying duration reduces when the temperature increases. These obtained results are analogous with those asserted by Doymaz (2007) for sour cherry, Karabulut et al (2007) for apricot, Fang et al (2009) for jujube and Vega-Gálvez et al (2014) for cape gooseberry.

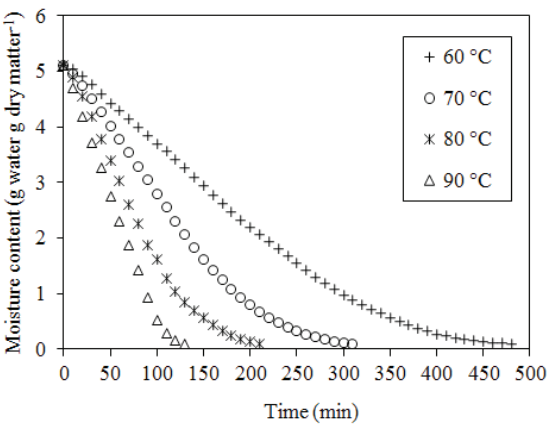

Figure 1- Drying curves of the European cranberrybush samples at different drying air temperatures

3.2. Suitability of drying curves

The obtained values from the statistical analysis, containing the model constants and R^2, *RMSE* and χ^2 values, for all thin-layer drying models are in accordance with the data about moisture ratio are shown in Table 2. Separately, in all cases, the R^2, *RMSE* and χ^2 values for all of the models being used varied from 0.9252 to 0.9996, 0.0068 to 0.0939 and 0.2908×10^{-4} to 85.1498×10^{-4}, respectively. With reference to these results, all the thin layer drying models discussed in this research sufficiently explained the drying kinetics of European cranberrybush. When the statistical values of these ten models are compared, the model of Midilli et al produced greater R^2 value and smaller *RMSE* and χ^2 values. For all drying conditions, the R^2, *RMSE* and χ^2 values of the Midilli et al model, ranged between 0.9961 and 0.9996, 0.0068 and 0.0215 and 0.2908×10^{-4} and 3.8142×10^{-4}, respectively. In compliance with the results above, the Midilli et al model was convincing in explaining the thin-layer drying curves of European cranberrybush samples. Figure 2 displays plots of experimental MR values and those estimated values which use the most appropriate models for drying duration at chosen drying conditions of European cranberrybush. It can be observed that for all of the drying conditions the estimated values obtained from the Midilli et al model offered good conformity with the experimental data. Consequently, the model of Midilli et al was considered as a preferable model to explain the characteristic features of European cranberrybush for every temperature between 60-80 °C. These findings are in good concordance with former studies. Other authors have also stated that Midilli et al is an adequate model to suit drying kinetics, including Gupta et al (2014) for aonla, Chayjan et al (2015) for hawthorn and Darici & Sen (2015) for kiwi.

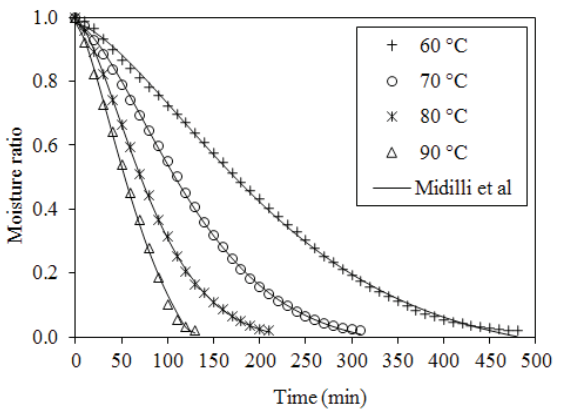

Figure 2- A comparison of the experimental and predicted moisture ratio for the Midilli et al model at different drying air temperatures

3.3. Color analysis

The color parameters of the dried European cranberrybush fruits were influenced by the different drying temperature as demonstrated at Table 3. The L*, a*, and b* chromatic parameters of fresh fruit were 24.37, 42.99, and 29.40, respectively. These values that belong to all dried samples decreased with regard to the values from the fresh European cranberrybush ($P<0.05$). Among the used four drying temperatures, the highest *a**, *b** and *L** values were acquired with the drying temperature at 60 ºC, while the greatest loss at *a**, *b** and *L** values was obtained with the drying temperature at 90 ºC. It is seen that a rise in drying temperature induced an outstanding brown products formation. In other respects, the *C* and α values were affected by the increasing drying temperature in opposite ways. Among all of the drying treatments, drying at 60 ºC generated the highest *C* value (38.03) and the lowest α value (28.04). Additionally, there was a decrease in *C* (44% at 90 ºC) and α values (18% at 60 ºC) of dried samples with regard to fresh fruit ($P<0.05$). This points that drying has resulted in discoloration of the original European cranberrybush color. Since, ΔE is a function of *L**, *a** and *b** values Equation 5, changes from 15.46 to 23.77, which were predicted to be 60 and 90 ºC, respectively. As a result, the high ΔE values acquired at high drying temperature probably due to the impact of high temperatures on heat-sensitive components such as carbohydrates and proteins, amongst others (Vega-Gálvez et al 2009). Similar impacts of high drying temperatures on ΔE values have been stated for pulp and orange

Table 2- Estimated coefficient and statistical analysis results obtained from different models for European cranberrybush samples dried at various temperatures

No	60 °C				70 °C				80 °C				90 °C			
	Model coefficients	R^2	*RMSE*	$\chi^2(10^{-4})$	*Model coefficients*	R^2	*RMSE*	$\chi^2(10^{-4})$	*Model coefficients*	R^2	*RMSE*	$\chi^2(10^{-4})$	*Model coefficients*	R^2	*RMSE*	$\chi^2(10^{-4})$
1	a= 1.135 k= 0.005415	0.9646	0.0611	36.5185	a= 1.132 k= 0.008637	0.9654	0.0605	36.0550	a= 1.125 k= 0.01299	0.9683	0.0597	35.7712	a= 1.106 k= 0.01739	0.9418	0.0828	66.0655
2	k= 0.004774	0.9452	0.0754	56.2552	k= 0.007654	0.9474	0.0754	55.7710	k= 0.0116	0.9522	0.0732	53.6611	k= 0.0157	0.9310	0.0902	78.5804
3	k= 0.0002422 n= 1.549	0.9971	0.0174	2.8085	k= 0.0004804 n= 1.557	0.9985	0.0127	1.5094	k= 0.0009948 n= 1.539	0.9994	0.0082	0.5100	k= 0.00102 n= 1.648	0.9905	0.0334	10.2447
4	a= 1.478 k= 0.002794 c= -0.42	0.9941	0.0247	5.8591	a= 1.409 k= 0.004854 c= -0.3449	0.9919	0.0295	8.5241	a= 1.349 k= 0.00793 c= -0.2804	0.9907	0.0323	10.4079	a= 2.07 k= 0.005565 c= -1.04	0.9925	0.0298	7.9318
5	a= 1.168 k_o= 0.005568 b= -0.1677 k_1= 2.252	0.9679	0.0577	32.5829	a= 22.26 k_o= 0.01547 b= -21.24 k_1= 0.01616	0.9944	0.0246	5.7625	a= 31.44 k_o= 0.02388 b= -30.42 k_1= 0.02465	0.9969	0.0188	3.3533	a= 16.92 k_o= 0.03231 b= -15.9 k_1= 0.0343	0.9786	0.0503	23.5053
6	a= 0.00005263 k= 90.69	0.9440	0.0762	57.4687	a= 0.00005115 k= 149.6	0.9456	0.0766	57.6468	a= 0.0000698 k= 166.2	0.9498	0.0750	56.3670	a= 0.0000569 k= 276	0.9252	0.0939	85.1498
7	a= -0.003384 b= 0.00000261	0.9937	0.0256	6.3707	a= -0.005496 b= 0.00000714	0.9921	0.0293	8.3594	a= -0.008439 b= 0.0000174	0.9914	0.0311	9.6667	a= -0.01043 b= 0.000019	0.9928	0.0292	7.6284
8	a= -6.815 k= 0.009203 b= 0.9038	0.9861	0.0379	13.6481	a= -11.18 k= 0.01484 b= 0.936	0.9891	0.0343	10.8789	a= -25.36 k= 0.02441 b= 0.9632	0.9973	0.0174	2.7085	a= -44.07 k= 0.03408 b= 0.9774	0.9818	0.0463	18.2005
9	a= -0.1677 k= 10.85 g= 0.005568	0.9686	0.0570	31.8743	a= -0.1858 k= 10.85 g= 0.00904	0.9720	0.0549	29.3644	a= -0.2105 k= 10.85 g= 0.01395	0.9789	0.0487	23.8672	a= -0.2174 k= 10.85 g= 0.01913	0.9541	0.0736	51.6499
10	a= 0.9813 k= 0.0002823 n= 1.499 b= -0.0001048	0.9990	0.0099	0.8480	a= 0.9817 k= 0.0004613 n= 1.551 b= -0.0000818	0.9994	0.0081	0.6113	a= 0.9923 k= 0.001005 n= 1.529 b= -0.0000611	0.9996	0.0068	0.2908	a= 0.9833 k= 0.001617 n= 1.478 b= -0.0009732	0.9961	0.0215	3.8142

Table 3- Color values of fresh and dried European cranberrybush samples

Drying method	*Color parameters*					
	*L**	*a**	*b**	*C*	*α*°	Δ*E*
Fresh	24.37±1.41[a]	42.99±1.48[a]	29.40±1.15[a]	52.09±1.65[a]	34.38±0.99[a]	-
60 °C	20.35±0.73[b]	33.57±1.10[b]	17.86±0.66[b]	38.03±1.13[b]	28.04±0.91[c]	15.46±0.93[a]
70 °C	19.57±0.38[bc]	30.10±0.73[c]	17.60±0.45[b]	34.87±0.60[c]	30.34±1.02[b]	18.14±0.53[b]
80 °C	19.05±1.00[c]	27.62±0.90[d]	16.42±0.58[c]	32.14±0.83[d]	30.76±1.19[b]	20.84±0.90[c]
90 °C	18.82±0.46[c]	24.12±0.65[e]	16.08±0.95[c]	29.00±0.75[e]	33.69±1.71[a]	23.77±0.71[d]

*L**, lightness; *a**, redness; *b**, yellowness; *C*, chroma; *α*°, hue angle; Δ*E*, total color difference; [a-e], values with different letters in same column differ significantly (P<0.05)

peel by Garau et al (2007) and for sour cherries by Wojdyło et al (2014). European cranberrybush fruits are among the fruits which are most abundant sources of anthocyanin which is the source of the red color of the fruit.

Anthocyanins are easily converted to colorless or undesirable brown degradation compounds. The most apparent factor that can affect anthocyanin stability is a thermal treatment (Moldovan et al 2012). Considering this fact, the decline in *a**, *b** and *L** values as a consequence of drying treatments of European cranberrybush samples can be intensely associated with the degradation of anthocyanins and formation of brown pigments by non-enzymatic or Maillard reaction and enzymatic reaction, particularly at higher drying temperatures (Zanoni et al 1999).

3.4. Total phenolic content

The obtained results about the changes in TPC of European cranberrybush samples caused by the various drying temperatures have been demonstrated in Figure 3. The initial TPC value in the fresh fruit was 633.56 mg GA 100 g^{-1} dry weight. After drying treatments, the TPC value declined by 14-48%. Due to drying treatments, the declines in the ingredient of total phenolic compounds were in conformance with former researches that phenolics compounds were heated labile and that continuous heat treatment may lead to irrevocable chemical modifications at phenolic compounds. It was stated that a decline in the TPC value in the course of drying also may be referred to the association of phenolics with other compounds (such as proteins) or to changes in chemical structures of the phenolic compounds that can not be obtained or confirmed by current methods on hand (Mrad et al 2012). Furthermore, from between the all dried samples, the TPC value demonstrated higher values at high-temperature levels (80 and 90 °C) with regard to low-temperature levels (60 and 70 °C) (P<0.05). In addition, some researches have also stated that long drying periods linked to low drying temperature may incite reduction of TPC (Garau et al 2007; Lopez et al 2010). One issue that was remarkable was the decrease of TPC at the 90 °C drying condition with respect to 80 °C drying condition. That was possibly due to phenolic compounds from European cranberrybush samples have lower resistance to heat at 90 °C.

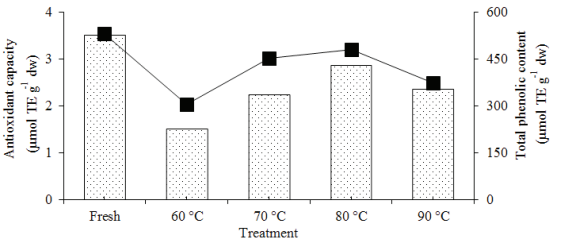

Figure 3- The effects of drying temperatures on the antioxidant capacity (▭) and total phenolic content (—■—) of European cranberrybush samples

3.5. Antioxidant capacity

Figure 3 displays the AC values for the dried and fresh samples of European cranberrybush. It was monitored that all of the drying treatments ended in a decline of AC value, with respect to

the fresh sample ($P<0.05$), in line with those ones reported for blueberry (Lopez et al 2010) and apple (Sultana et al 2012). This issue can be described by the thermal damage of antioxidant compounds during the long duration of drying treatments. Particularly, the lowest AC value was seen at 60 °C (4.54 μmol trolox g^{-1} dry weight), whereas the highest AC value was attained by drying at 80 °C (8.48 μmol trolox g^{-1} dry weight). Additionally, the production and accumulation of Maillard products which has changing the degree of AC values could also improve their antioxidant features at high-temperature levels (Que et al 2008). Furthermore, the regression analysis demonstrated that the TPC value had a positive correlation (R^2= 0.8656) with AC value of the European cranberrybush and this relation suggested that phenolic compounds may be the cause for the AC value observed in the European cranberrybush samples. Also, positive correlations between TPC and AC values have also been stated (Sultana et al 2012; Zhou et al 2016).

4. Conclusions

This study has examined the influences of convective drying method on drying characteristics, color, antioxidant capacity and total phenolic content of European cranberrybush fruits. The obtained results have indicated that superior average drying rates were attained with superior temperatures. Statistical results indicated that Midilli et al model was turned out to be the most convenient model which represents the drying curves of European cranberrybush. With respect to the color, it was likely to deduce that drying at 60 °C led to small changes in the color of European cranberrybush while drying at 90 °C led to more intense color changes. The rise of drying temperature augmented the ΔE values. Additionally, C and α value were influenced by increasing drying temperature in opposite ways. TPC and AC values were higher at high-temperature levels (80 and 90 °C) with regard to low-temperature levels (60 and 70 °C) ($P<0.05$). Additionally, a good correlation was detected between TPC and AC values (R^2= 0.8656). Finally, according to the these obtained results, in order to preserve the TPC and AC of dried samples, it can be monitored that the most suitable temperature for drying would be nearly 80 °C, which asserts that phenolic compounds from European cranberrybush samples have a higher resilience against heat decline.

Abbreviations and Symbols	
M_0	Initial moisture content, g water g dry matter^{-1}
M_t	The moisture content at a particular time, g water g dry matter^{-1}
M_e	Equilibrium moisture content, g water g dry matter^{-1}
$MR_{exp,i}$	Experimental moisture ratio at the test number I
$MR_{pre,i}$	Estimated moisture ratio at the test number i
N	Observation number
z	Total count of constant
$RMSE$	Root mean square error
R^2	Coefficient of determination
χ^2	Reduced chi-square
$a,b,c,g,n,\ k_o,k_1$	Model constants

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Induction of Herbicide Detoxifying Enzyme in Maize by Chiral 3-Dichloroacetyl Oxazolidine

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ABSTRACT

Safeners are important tools used to ensure to safe useof herbicide. The aim of this paper is to evaluate the protective effect of four safeners (R-28725, 3-dichloroacetyl oxazolidine and its two optical isomers) and investigate the mechanism of herbicide detoxication by safener. Laboratory studies were conducted to evaluate the effectiveness of safeners for protecting maize from the residues of preemergent herbicide fomesafen in Northeast Agricultural University, China. Physiological and biochemical tests were herein conducted under laboratory conditions, by using seed treatment with safeners and soil treatment with fomesafen, respectively. R-28725 provoked high glutathione level, glutathione-S-transferase activity and affinity of glutathione-S-transferase than other safeners, but R-isomer treatment resulted in complete reversal of injury caused by fomesafen.

Keywords: Herbicide safener; Herbicide detoxication; 3-Dichloroacetyl oxazolidine; Fomesafen; Glutathione-S-transferase

1. Introduction

Fomesafen [5-(2-chloro-α,α,α-trifluoro-p-tolyloxy)-N-mesyl-2-nitrobenzamide], a diphenyl-ether herbicide, controls broadleaf weeds by inhibiting protoporphyrinogen oxidase (PPOX), an important enzyme needed in chlorophyll biosynthesis. Fomesafen applied preemergence in soybean as a selective herbicide with both root and shoot activity (Peachey et al 2012). But, it has been reported that recommended dosage of fomesafen in soybeans may cause carryover injury to corn and the injury was varied significantly by plant variety and soil conditions (Rauch et al 2007; Cieslik et al 2014). When sweet corn was sown in high pH and low organic matter soils, injury of fomesafen was more serious compared with other soil types. For that reason, application of fomesafen in crops was restricted.

A key technology to increase herbicide selectivity is safeners. Herbicide safeners are synthetic compounds which can activate the tolerance of plants to herbicide without decrease herbicidal activity to weeds (Kraehmer et al 2014). The concept of safener was found by Otto Hoffman in the late 1940s. Research and development of new safeners proceeded in late 20th century, and products of safener were subsequently commercialized by agrochemical

companies. Although extensive work has been done, the mechanism of safener is not fully unveiled. Researchers have believed that structural similarity between herbicide and safener was essential for certain type of safeners (Bordas et al 2000). Research about safeners previously mainly focused on response of plants to safener. There is now a general consensus that some safeners enhanced the genes expression in plants related with exogenous compounds metabolism process such as glutathione-S-transferase (GST), cytochrome P450, and glutathione (GSH) (Matola & Jablonkai 2007; Del Buono & Ioli 2011). It has been reported that GSH-mediated detoxification was involved in the herbicide metabolism and detoxification response of plants (Riechers et al 2010). Skipsey et al (2011) found that a series of fenclorim derivatives induced GST and increased herbicide tolerance in rice. Da Silva et al (2014) also reported that fluxofenim induced GST and protect two sorghum hybrids from the injury of herbicide S-metolachlor. However, to our knowledge, there is no any report about safener for PPOX-inhibiting herbicides.

3-Dichloroacetyl-2,2-dimethyl-1,3-oxazolidine (R-28725) was proven effective in reducing herbicidal injury from ALS-inhibitor herbicides (Zhao et al 2014). Its analogue, 3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine, has a chiral carbon atom that gives rise to two optical isomers. The aim of this research was to study the bioactivity of R-28725, two optical isomers and racemate of 3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine as safeners for fomesafen. In addition, physiological and biochemical tests was conducted to assess the protective ability of these compounds and investigate the function of GSH, GST, PPOX in herbicide detoxification.

2. Material and Methods

The tested soil was *Mollisols-cryolls* clay loam type and collected from Horticulture Station, Northeast Agricultural University with a pH of 7.37 (Figure 1). The seedlings of maize cultivar, Dongnong 253 (*Zea mays* L.), was germinated and raised in a growth chamber at the Pesticide Chemistry Laboratory, Northeast Agricultural University. Fomesafen (99.5%, powder) was obtained from Aladdin Chemistry (Shanghai, China) to determine the GST activity *in vitro*. Fomesafen (250 g L^{-1}, liquid) was obtained from Dalian Songliao Chemical Industry Cmpany (Dalian, China) to use in other tests. R-28725, the racemate and two optical isomers of 3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine were synthesized in our laboratories (99.0%) (Table 1).

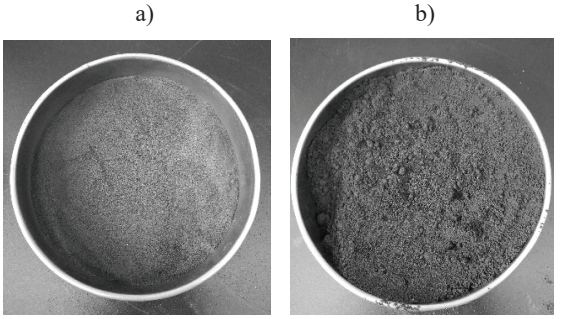

Figure 1- Tested soil (a) untreated soil (b) soil added with fomesafen

Table 1- Chemical name of safeners

Safener	*Chemical name*
R-28725	3-dichloroacetyl-2,2-dimethyl-1,3-oxazolidine
R-isomer	(R)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine
S-isomer	(S)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine
Racemate	(RS)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine

Seedlings of maize were soaking for 12 hours in solution of safeners (0, 1, 5, 10, 25, 50, 100 mg kg^{-1}), the control was soaking in water (Figure 2). Then, the seeds were germinated in dishes in a growth chamber for 24 hours (Figure 3). The recommended field application dose of fomesafen was 3.75 mg kg^{-1} and an average half-life value of fomesafen was 50 d which means that the concentration of fomesafen in soil was 0.96 mg kg^{-1} after it had been applied 100 d (Rauch et al 2007; Wu et al 2014). So, in this study, 1 mg kg^{-1} was chosen as the concentration of fomesafen in soil. Sown these seeds in paper-cups (10 cm × 15 cm), 7 seeds per cup, containing

soil added with fomesafen with a depth of 13 cm, and incubated in a growth chamber with a 12/12 photoperiod, 26.5±1 ℃ temperature, 75% relative humidity. Each treatment was replicated three times.

Figure 2- *Zea mays* seeds soaking in water and solution of safeners

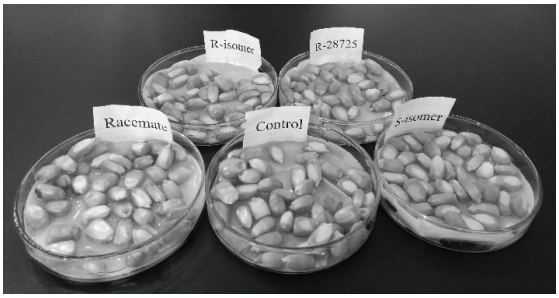

Figure 3- *Zea mays* seeds germinated in dishes

In order to calculate the recovery rate of maize, four parameters (plant height, root length, fresh weight of shoot, and fresh weight of root) of maize were measured 7 days after treatment. Recovery rate was calculated by Equation 1.

Where safener include the four safeners in this research and herbicide is fomesafen. The recovery rate of parameters of maize was calculated respectively.

The maize was washed and cutted to collect shoot and root tissues for biological assays. GSH level assay: GSH level was measured by UV-visible spectrophotomer as described previously (Ismaiel & Papenbrock 2014). To perform the determination, 5,5'-dithiobis (2-nitrobenzoic) acid (DTNB) was used in this study as chromogenic agent, absorbance data collected at 412 nm, and GSH level was calculated by comparing with standard working curve.

GST enzyme extraction and assay *in vivo*: The extraction and assay GST of was performed as described by Matola & Jablonkai (2007). The GST activity was obtained by measuring the level of conjugate composed from GSH and substrate. GST activity was expressed as level of conjugate per minute per mg of protein (μmol min^{-1} mg^{-1} protein).

GST activity assay *in vitro*: To determine the GST activity *in vitro* (against fomesafen in this study), the amount of fomesafen was determined by high performance liquid chromatography (HPLC) (Scarponi et al 2006; He et al 2010). GST enzyme was extracted from root of maize, and added with glutathione and fomesafen solution. After cultivated 2 hours, residue of fomesafen in this mixture was measured through HPLC. The GST activity *in vitro* was expressed as amount of fomesafen decreased per minute per mg of protein (nmol min^{-1} mg^{-1} protein).

$$\text{Recovery rate}(\%) = \frac{\text{Parameter of maize treated by safener and herbicide} - \text{Parameter of maize treated by herbicide}}{\text{Parameter of maize untreated} - \text{Parameter of maize treated by herbicide}} \quad (1)$$

Kinetic parameters of GST assay: The procedure described by Scarponi et al (2006) was followed to measure kinetic parameters of GST with modification. The GST activity was determined over a range of 1-chloro-2,4-dinitrobenzene (CDNB) concentration (0.13-4.14 mM) at a single GSH concentration of 5 mM.

PPOX enzyme extraction and assay: To investigate the effect of safener to target enzyme, PPOX activity was determined as described previously (Labbe et al 1985). PPOX activity was expressed as amount of protoporphyrin IX composed from protoporphyrinogen IX catalyzed by PPOX per hour per mg of enzyme (nmol h^{-1} mg^{-1} protein).

Statistical analysis: All data was performed by SPSS statistic software to determine statistical significance at 95% confidence level (P= 0.05) by Duncan's multiple-range test. All data reported were expressed as mean±standard deviation of three replicates.

3. Results and Discussion

3.1. Parameters of maize

The parameters of maize were significantly decreased by the treatment of fomesafen. When fomesafen applied at 1 mg kg^{-1} in soil, caused 34.42% to 39.36% decrease to root and shoot of maize, respectively.

The maize response to different concentrations of these four safeners was studied to get appropriate concentration of safeners offering maximum protection from fomesafen. The recovery rate of maize can be found in Figure 4. Significant differences were observed for recovery rate of maize to different concentration of safener in this study. Appropriate concentration of safener significantly decreased

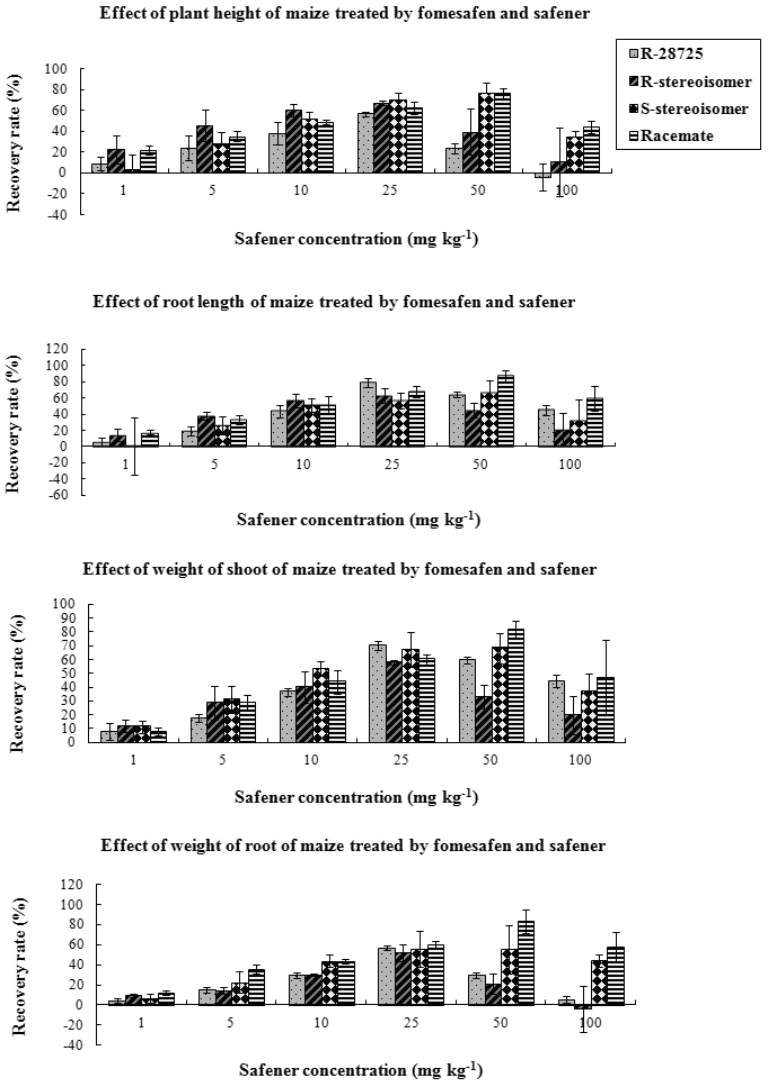

Figure 4- Recovery rate of parameters of maize effected by fomesafen and safener

herbicidal injury from fomesafen. The optimum concentration of safener for R-28725, and R-isomer were 25 mg kg^{-1}, and for S-isomer, and Racemate were 50 mg kg^{-1}. The results of recovery rate of maize soaked in the optimum concentration of safeners indicate that the order of protective ability of safeners was as follows: Racemate > R-28725 > S-isomer > R-isomer. Optimum concentration for each safener was then applied to maize for subsequent testing.

3.2. GSH level

Hatzios & Burgos (2004) reported that safeners could elevate the conjugation of herbicide with GSH through increasing the level of GSH in plants. Therefore, the level of GSH in plant was taken as an important index to check the protective ability of safener (Table 2). GSH levels in root and shoot of maize increased 43.4% and 30.1%, respectively, for fomesafen treatment compared with control. The level of GSH in root of maize treated by Racemate or R-28725 combined with fomesafen increased significantly by 84.0% and 101.2% compared with control, respectively, while the level of GSH in shoot of maize treated by Racemate combined with fomesafen increased 77.1% compared with control.

3.3. GST activity

Response of GST in maize treated by safener and fomesafen were investigated to discover the its role in detoxification process. The GST activity *in vivo* of maize treated by Racemate or R-28725 combined with fomesafen increased 88.6% and 85.0% compared with the control, respectively. The GST activity *in vitro* of maize treated by R-isomer or R-28725 increased 328.7% and 299.2% compared with the control, respectively (Table 3). The results of GST activity of maize indicate that R-28725 induced GST affinity for substrate significantly.

3.4. Kinetic parameters of GST

Further research was conducted for kinetic parameters of GST. The kinetic parameters V_{max} (the maximal reaction rate of detoxification reaction) and K_M (the concentration of substrate when the velocity of detoxification reaction is half of the maximum velocity) of GST were calculated by linear regression (Table 4). V_{max} value of GST for maize treated by R-28725 was raised to 2.02 times that of control and K_M value was decreased to 59.8% of control, indicating the strong inducement of GST caused by R-28725. It

Table 2- Effect of safeners and fomesafen on GSH level

Treatment	*GSH level in root ($\mu g\ g^{-1}$)*	*GSH level in shoot ($\mu g\ g^{-1}$)*
Control	3.268±0.2532 d	9.281±1.6320 c
Fomesafen	4.686±0.2233 c	12.071±0.5690 c
R-isomer+Fomesafen	4.732±0.1381 c	14.373±0.1657 b
S-isomer+Fomesafen	4.474±0.1797 c	13.231±0.2767 b
Racemate+Fomesafen	6.012±0.2349 b	16.491±0.2611 a
R-28725+Fomesafen	6.574±0.3296 a	14.262±0.2086 b

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05). The values correspond to averages of three replicates

Table 3- Effect of safeners and fomesafen on GST

Treatment	*GST activity in vivo ($\mu mol\ min^{-1}\ mg^{-1}$ protein)*	*Treatment*	*GST activity in vitro ($nmol\ min^{-1}\ mg^{-1}$ protein)*
Control	1.67±0.038 e	Control	0.254±0.0455 d
Fomesafen	2.26±0.064 d	Fomesafen	0.071±0.0158 e
R-isomer+Fomesafen	2.90±0.020 b	R-isomer	1.089±0.1075 a
S-isomer+Fomesafen	2.54±0.088 c	S-isomer	0.574±0.0294 c
Racemate+Fomesafen	3.15±0.075 a	Racemate	0.795±0.1000 b
R-28725+Fomesafen	3.09±0.120 a	R-28725	1.014±0.0340 a

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05). The values correspond to averages of three replicates

was consistent with the results of GST activity. Other safeners also induced the affinity of GST to substrate of conjugated reaction in some extent.

Table 4- Effect of safeners and fomesafen to kinetic parameters of GST

Treatment	V_{max} *(nmol min^{-1} mg^{-1} protein)*	K_m *(mmol L^{-1})*
Control	0.790±0.0300 e	1.950±0.0557 b
Fomesafen	0.567±0.0292 f	2.973±0.1429 a
R-isomer	1.437±0.0231 b	1.320±0.0100 d
S-isomer	0.913±0.0666 d	1.673±0.0569 c
Racemate	1.090±0.0346 c	1.570±0.0173 c
R-28725	1.597±0.0907 a	1.167±0.1443 d

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05). The values correspond to averages of three replicates

3.5. PPOX activity

Fomesafen harms plants via inhibition of PPOX, but safener protect plants by reducing the amounts of herbicide to reach to the targeted site in the plant (Rushing et al 2013). For that reason, PPOX activity is clearly critical for the resistant ability of plants. The effect of safeners and fomesafen on the PPOX activity was determined to investigate the protective effectiveness of safeners (Table 5). The results showed that fomesafen inhibited PPOX activity in maize significantly by 56.6% compared with the control and safeners could elevate PPOX activity of maize significantly. It is noteworthy that R-isomer could reverse the effects on maize caused by fomesafen totally.

Table 5- Effect of safeners and fomesafen on PPOX activity

Treatment	*PPOX Activity (nmol h^{-1} mg^{-1} protein)*
Control	0.505±0.0312 a
Fomesafen	0.219±0.0161 d
R-isomer+Fomesafen	0.519±0.0406 a
S-isomer+Fomesafen	0.237±0.0173 d
Racemate+Fomesafen	0.403±0.0196 b
R-28725+Fomesafen	0.332±0.0172 c

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05). The values correspond to averages of three replicates

While fomesafen contribute to improving crop yield, it can also pose a risk to those plants that are sensitive to them (Cieslik et al 2014). For that reason, effective safeners were developed to protect plants. But no safener has been developed to protect plant from diphenyl-ether herbicide. In order to develop safener for fomesafen, the protective effects of four safeners were studied in our laboratory. The results conclusively demonstrated that the maize injured by fomesafen was effectively protected by these safeners. The maize seeds that had been soaked in solution of safener were safe from fomesafen treatment. The results indicate that the application of these safeners produced high recovery rates of growth level of maize ranged from 51.80% to 87.21% with fomesafen applied at 1 mg kg^{-1} in soil.

For evaluation the enhancement of detoxification of maize, induced by safeners, the GSH level, GST, and PPOX activity of maize treated by fomesafen and safener were investigated. Our study has shown that these safeners caused enhancement of GSH level, GST, and PPOX activity of maize and affinity of GST enzyme to substrate. Enhancement of GSH level in root of maize, GST activity and affinity of GST to CDNB caused by R-28725 was greatest which caused 1.40-fold, 1.46-fold and 2.82-fold increase to GSH level, GST activity *in vivo* and V_{max} of GST, respectively. It is safe to say that these safeners induced the conjugation of herbicide with GSH catalyzed by GST to some extent. Consistent with previous studies, safeners significantly change the affinity of GST to substrate of conjugation reaction (Scarponi et al 2006). Fomesafen resulted in inhibition to plant by inhibiting PPOX activity. So, PPOX activity is an important index to maize treated by safener and fomesafen. R-isomer treatment resulted in complete reversal of injury caused by fomesafen. This might suggest that the protective ability of safener not only depends on the GSH and GST in maize (Jablonkai 2013).

4. Conclusions

From this study, it can be concluded that seed treatment with these four safeners present protective ability to injury caused by fomesafen. In addition, the excellent efficacy suggests that these safeners should be considered

for reducing of herbicide toxicity in maize. Therefore, these compounds can be a useful tool to protect maize from the injury of herbicide and improve selectivity between crop and weed. This study is the first one on the effect of chiral 3-dichloroacetyl oxazolidine and their interaction with fomesafen. However, in-depth studies are still needed to determine the exact mechanism of the enhancement of protective ability.

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Rice Bran or Apple Pomace? Comparative Data Analysis of Astaxanthin Bioproduction

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ABSTRACT

Modeling and optimization of high value-added astaxanthin pigment bioproduction statistically by *Sporidiobolus salmonicolor* ATCC 24259 from two substantial wastes, rice bran (RB) and apple pomace (AP) was aimed in this study. The experimental data was obtained at constant inoculum rate (2%) and particle size (0.85 mm) for both wastes by conducting 17 runs, which were generated by Box-Behnken design. 33.41 µg astaxanthin gRB^{-} and 77.31 µg astaxanthin gAP^{-} were produced as the maximum amount at the end of fermentation period, 10 days. Apple pomace was concluded as the optimized waste for the production of astaxanthin based upon the highest yield. Predicted response results of response surface methodology (RSM) and radial basis function-neural network (RBF-NN) were compared in order to evaluate the accuracy of two methodologies on non-linear behavior of the astaxanthin bioproduction. RBF-NN became prominent with its well-suited to apple pomace fermentation system by resulting in quite low 0.8495, root mean square error (RMSE), 0.3349, mean absolute error (MAE), and 0.9985, correlation coefficient (CC) as best measures of a model performance.

Keywords: Apple pomace; Rice bran; Astaxanthin; Radial basis function-neural network

1. Introduction

The wastes of the cereals and fruits are obtained after post-harvesting and processing of them industrially, which are mayor columns of solid agro-industrial wastes. There are several methods for the waste management including composting, pyrolysis, combustion, gasification, land filling, animal feed *etc*. (López et al 2005; Arvanitoyannis 2010; Baino 2014; Panesar et al 2015). Bio-processes are alternative and good way of utilization of the wastes, not just owing to their rich organic content, also for good anchorage feature, easy accessibility, abundantly available, low cost what kind of properties solid state fermentation (SSF) system requires (Pandey et al 2000; Couto & Sanromán 2006; Couto 2008). SSF of the wastes is a highly attractive process to study due to providing environmental solutions of solid wastes, technique performance, wide usage and execution areas, feasibility, low cost and labor, product manifoldness and so on. However, the advantages in SSF are not efficient just for themselves. Also revealing certain fermentation conditions and productivity of the system is important by applying an experimental design, modeling and optimization statistically. In this manner, response surface methodology and artificial neural network (ANN) methodologies show

up in order to evaluate the bio-processes. RSM is a methodology which uses the collection of numerical and statistical analyses by fitting the experimental data to a polynomial equation and describes the single and combination relations of the variables and responses mathematically and graphically as a result (Aslan & Cebeci 2007; Baş & Boyacı 2007). ANN is a mathematically modeling tool aimed to figure out complex non-linear relationships by simulating human brain learning process. Radial basis function is a particular data-based modeling neural network type that is frequently preferred to model bio-processes. RBF-NN has three layers: an input layer, a hidden layer with non-linear RBF activation function, and a linear output layer. It introduces high-speed training rate, thus a quickly developed model is submitted (Warnes et al 1998; Dutta et al 2004). Consequently, the bio-processes could be improved, and productivity of the system may be increased by applying of these two strategies, RSM and RBF-NN as single or together to compare.

Rice bran and apple pomace as two of the most came out of the wastes have been used to produce bio-molecules such as ethyl alcohol, citric acid, microbial colors, enzymes *etc*. in SSF systems with different type of microorganisms (Laufenberg et al 2004; Joshi & Attri 2006; Gupta et al 2011). Astaxanthin as a red color xanthophyll carotenoid is commercially produced as a targeted bio-molecule and used in preferentially food, feed, aquaculture, nutraceutical, pharmaceutical and poultry areas (Ambati et al 2014).

In this presented work, astaxanthin, which is very valuable carotenoid health wise, commercially and industrially, was chosen to be produce from rice bran and apple pomace by pure culture of *Sporidiobolus salmonicolor*. Box-Behnken experimental design was performed to obtain the experimental data, whereas RSM and RBF-NN methodologies were employed to get the predicted data. Comparison of the methodologies was carried out by several terms mathematically and statistically to reveal the predictive capability of them.

2. Material and Methods

2.1. Wastes and microorganism culture

RB and AP were supplied from Gaziantep and Adana provinces respectively. They were stored at cold storage (+4 °C) in polyethylene packages. RB was directly sieved to size 0.85 mm; AP was dried by tray drier, milled and then sieved in order to obtain a uniform material for fermentation system. Freeze-dried *Sporidiobolus salmonicolor* ATCC 24259 yeast was purchased from the American Type Culture Collection (Manassas, USA). The yeast was transformed in YM broth and maintained both in YM (Yeast-Malt) broth and agar. The composition of the media; 3 gL^- yeast extract (Merck, Germany), 3 gL^- malt extract (Merck, Germany), 5 g L^- peptone (Merck, Germany), 10 g L^- dextrose (Sigma-Aldrich, Germany) for the broth, and 20 g L^- agar agar (Merck, Germany) for the agar were mixed and filled up with bi-distilled water. Fresh culture of ATCC 24259 was prepared by growing at its optimum growth conditions; 18.0 °C and 6.0 pH with regard to the ATCC protocol for 24 hours. Fresh culture at 2% (40.5 $CFUg^-$) inoculation rate was used for the inoculum.

2.2. Extraction and spectral analysis

A spectral scanning was done for astaxanthin (AX) standard (Chromadex, USA) which was purchased dissolved form in methanol and the wavelength of the maximum pick point was determined. Different concentrations (dilution series) of the AX standard with pure methanol (Sigma-Aldrich, Germany) were used to prepare standard curve. Spectrophotometric measurements of the series were carried out against pure methanol at the maximum wavelength. Standard curve of AX pigment was obtained using absorbance values versus the concentrations of the standard. Raw material and fermented content were subjected to pigment analysis. Sample was mixed with pure methanol at 1:4 ratio. The mixture was waited for 2 hours, 5 mL of the liquid phase of the mixture was taken and centrifuged at 6000 rpm for 10 min. The supernatant was analyzed by double beam UV/VIS Spectrophotometer (Lambda

25 UV/VIS Spectrophotometer, USA) against the pure methanol blank (Babitha et al 2007). AX concentration was calculated with regard to the equation of the standard curve. The results were explained as the mean of triplicate measurements and presented as microgram astaxanthin per gram dry waste (μg AX gdw^{-}).

2.3. Modeling and optimization

Response surface methodology in Design-Expert Version 7.1.5 (Minneapolis, USA) was used to generate an experimental design. Box-Behnken design (BBD) was selected using three independent variables; temperature (x_1), moisture content (x_2) and pH (x_3) at three levels. Temperature and pH levels from ATCC protocol, and moisture content levels from water activity requirement (0.60-0.88) for the growth of the yeast were selected. The depended variable, response, was astaxanthin amount for the design. There were 17 runs with 5 center points conducted through the design (Table 1). Equation (1) introduces polynomial quadratic model as second order equation where y is response or dependent variable; β_o is model constant; $\beta_1, \beta_2, \beta_3$ are linear coefficients; $\beta_{12}, \beta_{13}, \beta_{23}$ are cross product coefficients (present the interactions between the variables); β_{11}, β_{22}, β_{33} are quadratic coefficients; independent variables: x_1 is temperature, x_2 is moisture content and x_3 is pH. The predicted data was obtained from the analysis of RSM methodology.

$$y=\beta_o+\beta_1x_1+\beta_2x_2+\beta_3x_3+\beta_{12}x_1x_2+\beta_{13}x_1x_3+\beta_{23}x_2x_3+\beta_{11}x_{11}+\beta_{22}x_{22}+\beta_{33}x_{33} \quad (1)$$

The experimental data of BBD was exposed to Gausiian function with 0.75 spreadability in RBF-NN in MATLAB Version 7.10 (USA) using the equation below (2) in order to estimate the data with 3 input layer-one hidden layer with 17 nodes-1 output layer (3-17-1) topology.

$$a_{hk}= exp\ (-\|x_h-x_k\|^2/\sigma_h^2) \quad (2)$$

Where; a_{hk} is basis function or activation of h-th unit in the hidden layer; x_h is unit center or n-dimensional position of the center of h-th (n as input number); x_k, mean or center of RBF element, σ_h is standard deviation or local scaling constant. Input variables as

Table 1- The design matrix and response results in RSM and RBF-NN

Run	x_1	x_2	x_3	*RB*			*AP*		
				E	*RSM–P*	*RBF-NN–P*	*E*	*RSM–P*	*RBF-NN–P*
1	18	70	7	4.28	8.15	4.28	6.72	1.13	6.72
2	18	80	6	10.83	15.12	15.12	25.82	26.08	26.08
3	23	70	6	7.95	4.22	7.95	10.99	19.61	10.99
4	18	90	7	27.29	24.52	27.29	18.16	23.91	18.16
5	13	80	5	9.73	9.87	9.73	31.78	34.81	31.78
6	23	80	5	11.75	12.70	11.75	8.48	5.61	8.48
7	18	80	6	9.86	15.12	15.12	26.94	26.08	26.08
8	23	80	7	9.86	9.72	9.86	5.22	2.20	5.22
9	13	80	7	8.92	7.97	8.92	24.74	27.61	24.74
10	13	90	6	17.15	20.88	17.15	77.31	68.69	77.31
11	18	70	5	5.23	8.01	5.23	13.17	7.43	13.17
12	18	80	6	25.71	15.12	15.12	23.17	26.08	26.08
13	18	80	6	13.41	15.12	15.12	26.82	26.08	26.08
14	23	90	6	25.10	28.01	25.10	16.47	13.75	16.47
15	13	70	6	9.69	6.77	9.69	16.55	19.27	16.55
16	18	90	5	33.41	29.54	33.41	22.62	28.21	22.62
17	18	80	6	15.79	15.12	15.12	27.65	26.08	26.08

E, experimental data; *P*, predicted data

the fermentation parameters (x_1, x_2, and x_3) and output variable as the astaxanthin amount (y) were utilized for the non-linear mapping in RBF-NN. Integration analyses of the experimental and the predicted data were performed to judge the quality of predictions by RMSE, MAE, CC, R^2 and adjusted R^2 measures, whose expressions were referred to Willmott (1982).

2.4. Solid state fermentation

100 gram total amount of the waste and bi-distilled water in 250 mL Erlenmeyer flask was prepared as fermentation content. Amount of the water to be added was determined depending on initial and last moisture content of the solid waste. pH value of the water to be according to the experimental design was adjusted by HCl (Merck, Germany) and NaOH (Merck, Germany) solutions. Water and solid waste was mixed in flasks, which were autoclaved (HMC HV-85L Autoclave, Germany) at 121.0 °C for 15 minutes. After inoculation, incubation was maintained at the design temperature degrees during the fermentation period.

3. Results and Discussion

Bio-production of astaxanthin from RB and AP was accomplished by *Sporidiobolus salmonicolor* applying SSF technology. Experimental data obtained according to BBD matrix and predicted data generated by RSM and RBF-NN was presented in Table 1. Individual and interaction effects of the independent variables on the response were investigated well thanks to response surface plots, model equation coefficients and probability analysis in RSM by DoE. Therefore, the relationships between the solid state fermentation parameters and astaxanthin amount produced could be explained. The quadratic equations in the coded values for both wastes were given below.

RB quadratic equation with coded coefficients:

$$y=15.1201+1.1456x_1+9.4745x_2-1.2215x_3+2.4226x_1x_2-0.2715x_1x_3-1.2909x_2x_3-3.8191x_{11}+3.6694x_{22}-1.2364x_{33}$$

AP quadratic equation with coded coefficients:

$$y=26.08-13.65x_1-2.65x_2+10.89x_3+0.95x_1x_2-13.82x_1x_3+0.5x_2x_3+3.32x_{11}-11.84x_{22}+0.93x_{33}$$

Whereas the model was significant only at $P<0.1$ for RB system, it was significant at all probability intervals for AP system as seen in Table 2. Lack of fit expression is expected as 'not significant', which is good for fitting of the model. It was observed that a reverse result for both fermentation systems at all probability intervals. Significant lack of fit result for AP system meant that there was a bad fitting. It could be also seen the optimized fermentation conditions through the experimental and predicted at the same table. The highest astaxanthin amount, 77.31 µg AX gdw^- was produced from the apple pomace system at the highest moisture content, lowest temperature and middle pH levels. It was understood that there was almost a perfect fitting between the experimental and predicted optimized results. However, lack of fit result for AP system had been commented as a bad fitting that caused the first confusion. In the mean time, RB system produced maximum 33.41 µg AX gdw^- at the highest moisture content level.

Table 2- Optimum RSM results for rice bran and apple pomace fermentation systems

Optimized conditions		*RB*		*AP*	
		E	*P*	*E*	*P*
Temperature (°C)		18.0	20.52	13.0	13.0
Moisture content (%)		90.0	90.0	90.0	90.0
pH		5.0	5.0	6.0	5.87
Max yield (µg AX gdw^-)		33.41	29.54	77.31	68.69
Tools	P <	Prob > F			
Model	0.1	+		+	
	0.05	-0.0995		+0.043	
	0.01	-		+	
Lack of fit	0.1	-		+	
	0.05	-0.5726		+0.024	
	0.01	-		+	

Response surface plots (Figure 1 and 2) demonstrated the interaction of the fermentation parameters. It could be surely said that pH parameter had no effect on the bioproduction utilizing the wastes

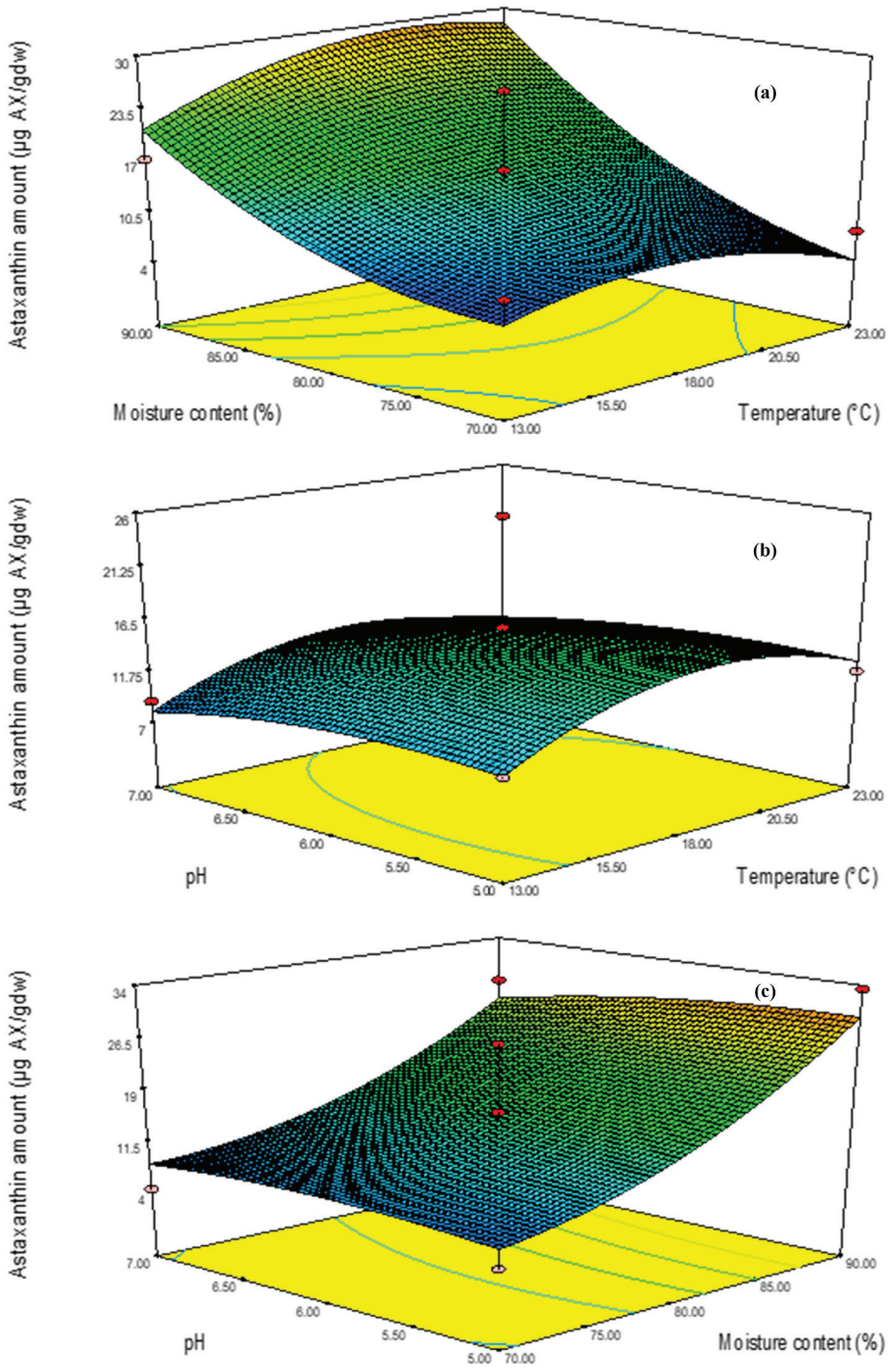

Figure 1- Response surface plots (a, b, c) of the interactions for RB fermentation system

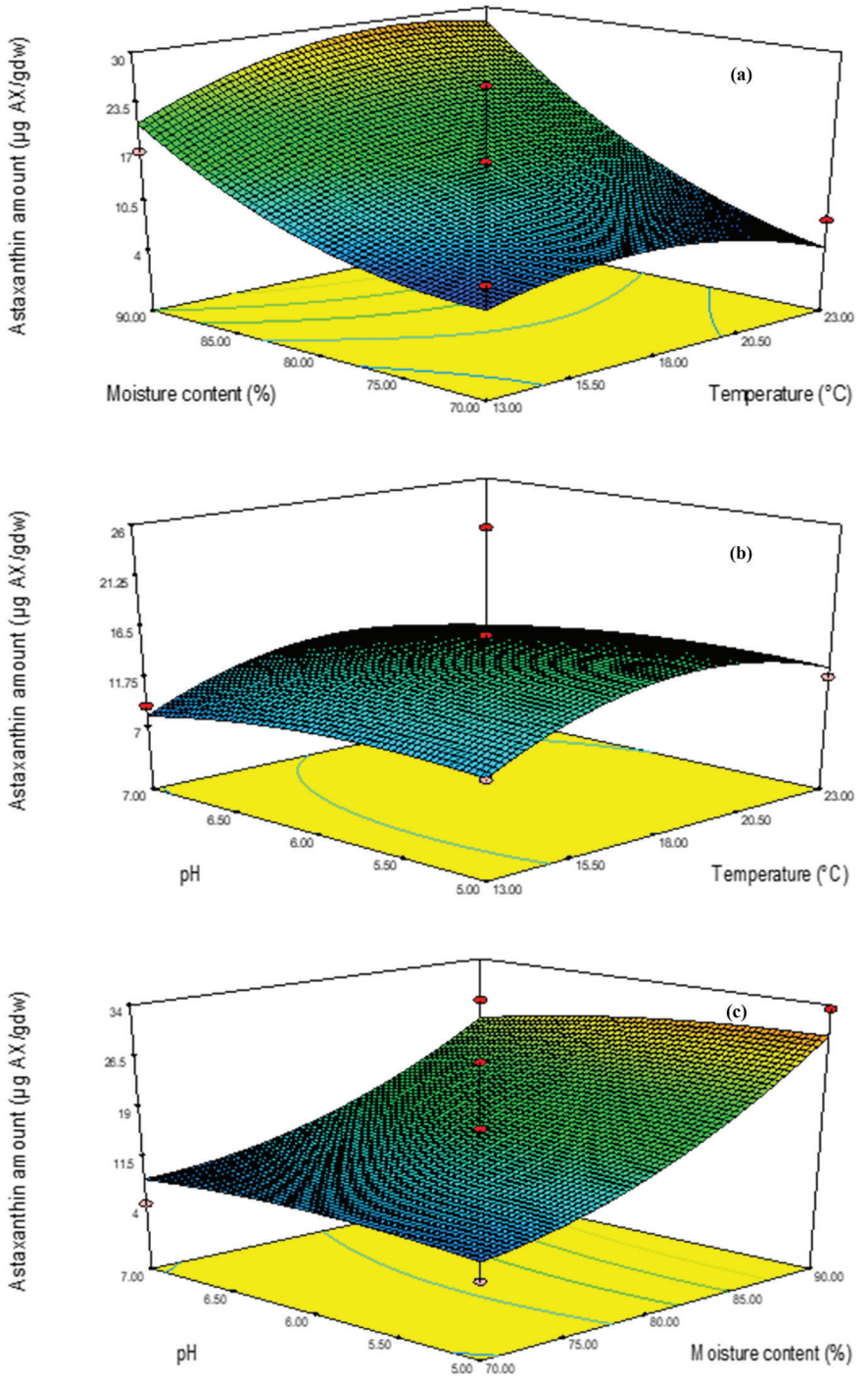

Figure 2- Response surface plots (a, b, c) of the interactions for AP fermentation system

as single or in company. Moisture content had the most significant effect on the astaxanthin production from both wastes. It was clearly seen that moisture content was a great inducer. Temperature parameter with moisture content showed that mild values of it had low significant effect. On the other hand, a slight increasing at temperature with pH meant significancy on both bioproductions. Prediction quality (or capacity) of two methodologies was needed to be revealed in terms of comparing, and also overcoming some incoherency. For these purposes, the parameters in Table 3 were estimated for each waste fermentation system. Small RMSE and MAE, high CC, R^2 and Adj. R^2 values indicates well approximation for predicting and exhibits good fitting. When the comparison results were evaluated between themselves and among each other, RBF-NN showed more successful predictions for the solid state fermentation of the rice bran and apple pomace. Although R^2 value, which means well replication of the experimental data to predict by the model, was much higher for AP system, the model fitting (lack of fit) had showed not-significant result at even all probability intervals. This caused the second confusion. It is known that RSM is a widely used and does a good job for microbiological, fermentation, biotechnological processes. However, the power and quality of RSM needed to be confirmed that this study brought out clearly. RBF-NN as a local approximator offered us a superior prediction owing to high accuracy, particularly for AP system that was mathematically supported (Table 3). It has been indicated by many studies in literature that neural networks applications has a real superiority over RSM (Warnes et al 1998; Dutta et al 2004; Fang & Horstemeyer 2007; Desai et al 2008; Liu et al 2009; Deshmukh et al 2012). Figures 3 and 4 depicted the comparison of generalization ability of RSM and RBF-NN for both wastes by regression measurements. They also substantiated the good predictor characteristic of RBF-NN.

Table 3- Comparison of RSM and RBF-NN methodologies

Parameters	*RB*		*AP*	
	RSM–P	*RBF-NN–P*	*RSM–P*	*RBF-NN–P*
RMSE	3.8608	3.0819	4.4648	0.8495
MAE	2.6976	1.1857	3.3409	0.3349
CC	0.8943	0.9381	0.9591	0.9985
R^2	0.7783	0.8588	0.9198	0.9971
Adj. R^2	0.7636	0.8493	0.9145	0.9969

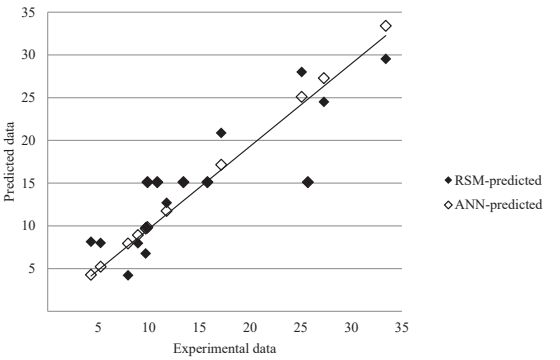

Figure 3- Comparative parity plots of RSM and RBF-NN data sets for RB

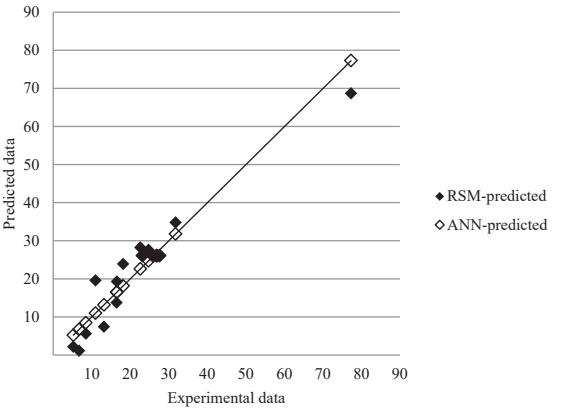

Figure 4- Comparative parity plots of RSM and RBF-NN data sets for AP

4. Conclusions

The following statements have been concluded from solid state fermentation of RB and AP by *S. salmonicolor* yeast: 1. Optimizing the fermentation conditions and resource in the scope of an experimental design were achieved for a valuable bio-product, astaxanthin. 2. Moisture content was

assigned as the most significant parameter for both waste fermentation systems. 3. Apple pomace was determined as a good resource due to resulting considerably high astaxanthin yield. 4. RSM and RBF-NN are both popular applications for the evaluation of fermentation systems statistically and mathematically, for which were performed in this study. Eventually, RBF-NN displayed a superior role on prediction of the original data set that several comparison parameters assisted. RBF-NN may be used substituted for RSM.

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Design of a Nozzle-Height Control System Using a Permanent Magnet Tubular Linear Synchronous Motor

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ABSTRACT

In agricultural spraying, keeping the spray at the correct height reduces pesticide drift and provides uniformly distributed pesticide accumulation on the target plant. In this study, an agricultural nozzle-height control test system was developed using a permanent magnet tubular linear synchronous motor (PMTLSM) that can adjust the height between the spraying nozzle and the plant. The developed system was experimentally tested in the laboratory environment and under field conditions. According to the experimental results, the nozzle height coefficient of variation (CV) value decreased from 16.77% to 5.17%, while the uniformity of distribution in the forward direction increased from 56.57% to 86.11% at 12 km h^{-1} under field conditions. Under test conditions it was found that the developed system keeps the distance between differently sized plants and the nozzle at the set point with minimum error.

Keywords: Agricultural spraying; Nozzle height control; Permanent magnet tubular linear synchronous motor

1. Introduction

The rapid increase in the world population, high-quality food demand and the expectation of maximum yield from accessible agricultural land leads to more intensive farming practices (Matthews 2008). Agricultural diseases and pests reduce product yield, grain size, storage time and quality, and also causes rapid spread of disease (Bisesi & Koren 2003). For this reason, the use of pesticides for high yield and high-quality food is an inevitable tool (De Schampheleire et al 2007; Matthews 2008). Although pesticides have important benefits for the development of agriculture, they also have many negative effects on people, animals and the environment (Belforte et al 2011). During the pesticide application, the required dose throughout the entire area must be properly applied to the target. Incomplete application causes negative effects such as weeds and harmful insects to be sustained by decreasing pesticide efficiency. On the other hand, excessive application leads to contamination of the soil and surface waters, and excessive pesticide residues. Since pesticides contain intense active ingredients, excessive pesticide residues on plants causes crop damage (Ozkan & Reichhard 1993; Marck & Luycx 1993). The quality of spraying is determined by characteristics such as mean diameters, uniformity of distribution, drop frequency and the coating ratio of the droplets

collecting on the target surfaces. It is desirable that the amount of pesticide drift to the off-target area must be as low as possible, as the accumulation rate and coating rate is high for each treatment (Ozkan 1995; Gil & Badiola 2007).

For high quality spraying, it is necessary to keep the spraying height constantly at an appropriate value. Spraying at too high a level results in spray drift, while a low-level setting causes untreated slivers and excessively sprayed strips in areas underneath the nozzle. The total effect appears to be very poor accumulation and deterioration in uniform distribution (Yoshida & Maybank 1971). For this reason, it is of utmost importance that the treatment height is kept at a reasonable value to provide treatment in one go, to reduce pesticide drift, to obtain a more uniform pesticide distribution, to avoid disruption of the spray pattern and to provide adequate coverage (Wang et al 1993; Womac et al 2001; Wen & Kidd 2005; Qasem 2011).

In published literature, the distance between the nozzles and also the nozzle angle is taken into account. For wide-angle nozzles, a lower spray height is preferred. For example, for a nozzle spacing of 50 cm, a spray height of 50 cm is recommended at a 110° nozzle angle (Langenakens et al 1999; Wilson et al 2008). However, when the spray is being applied, the spray height is constantly changing due to fluctuations in the land structure, to hills, tyres and vertical vibrations. Thus, the quality of the spraying is adversely affected (Langenakens et al 1995; Ramon et al 1997; Langenakens et al 1999). Based on a study conducted by Langenakens et al (1995), the spray deposit can vary between 0% and 1000% for vertical boom vibrations. When passive and active suspension systems are used, the level of spraying quality is increased by reducing the vertical vibrations and height errors (Ramon et al 1997). Passive suspension systems are based on balance, central rotation and damping suspension systems and do not require any power source (Frost 1984; Klein & Kruger 2011). On the other hand, active suspension systems use sensors and actuators to balance the boom arm. If a height deviation is detected by the sensor, the actuator adjusts the set point by moving the boom in a downward or upward direction (O' Sullivan 1986; Klein & Kruger 2011).

Many theoretical and experimental studies have been carried out on passive and active suspension system applications (Musillami et al 1982; Frost 1984; O'Sullivan 1986; Frost & O'Sullivan 1986; Marchant & Frost 1989; Kennes et al 1999; Deprez et al 2002; Deprez et al 2003; Anthonis et al 2005; Sun & Miao 2011; Koc & Keskin 2011; Pontelli & Mucheroni 2012). These systems control the vertical height of all or parts of the boom arms carrying the spray nozzles, and they keep the boom parallel to the ground. In these studies, hydraulic cylinders were also used as actuators.

This study differs from previous studies because it uses a PMTLSM to control the vertical motion of the nozzle, and for the independent adjustment of the height of a single spray nozzle and ability to track differently sized plants on a row.

2. Material and Methods

The test bench used to perform laboratory tests is presented in Figure 1. The variable speed conveyor belt with different sizes of artificial plants is 410 cm long and 60 cm wide. The conveyor belt speed is measured by a wheel-type incremental encoder (Autonics ENC-1-1-V-5, South Korea). The data acquisition card (National Instrument NI DAQ 6211, USA) and the graphical programming language (National Instrument LabVIEW 2013, USA) were used to read the distance information from the sensor and to calculate the analog voltage information commands to be sent to the analogue inputs of the motor servo drive. Vertical movement of the nozzle is provided by the high-performance PMTLSM (LinMot P01-23x160H-HP-R20, Switzerland) and is given in Figure 2. In the PMTLSM, high-speed linear motion is produced by direct electromagnetic force, there are no mechanical parts such as a mechanical gear and a belt-pulley system. In addition, there is no need for an oil tank, pump, filter or liquid transmission pipes. In addition, the motor's tubular configuration provides benefits such as easy installation, accuracy, high repeatability, high thrust density, low weight / force ratio and quiet operation from the direct drive linear motion system (LinMot 2016).

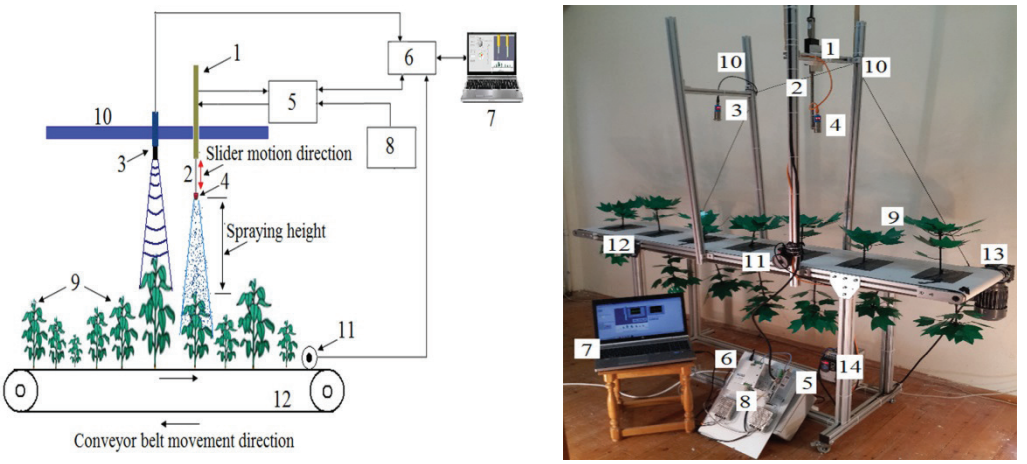


Figure 1- The laboratory test setup (schematic diagram in left, picture in right): 1, PMTLSM; 2, Slider; 3, 1st ultrasonic sensor; 4, 2nd ultrasonic sensor; 5, servo driver; 6, DAQ board; 7, laptop; 8, power supply; 9, artificial plants; 10, bridge; 11, wheel type encoder; 12, conveyor belt; 13, induction motor with reduction gear; 14, frequency converter

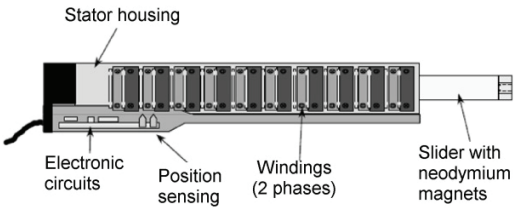


Figure 2- Assembly of PMTLSM

The servo drive (Linmot B1100 GP-HC, Switzerland) has a built-in, internal, proportional integral derivative (PID) controller that controls the linear travel position of the PMTLSM slider. The linear motor-defining parameters, PID controller gains, slider acceleration and maximum velocity information are entered manually into the driver via the servo drive software (LinMot Talk software version 6.0). The servo driver has maximum velocity and limited acceleration interpolation. In this interpolation process, the traditional PID controller output is damped to provide trapezoidal trajectory tracking according to the set acceleration and maximum velocity values (Figure 3). Thus, the slider travels from the current position to the target position at the maximum velocity with limited acceleration thereby avoiding unwanted position deviations. When the acceleration increases, the isosceles trapezoidal angles also increase, as seen in Figure 4. Thus, a motion profile is produced that will enable the target to be reached in a shorter time.

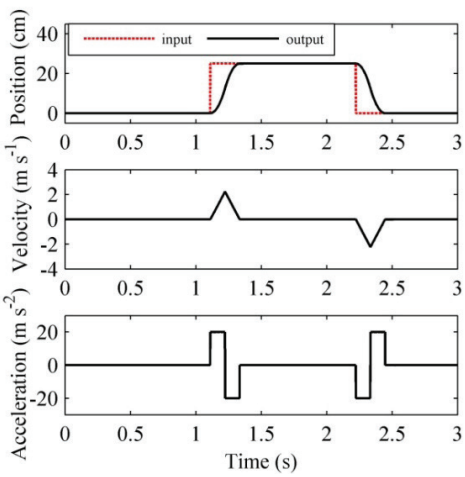


Figure 3- PMTLSM trapezoidal motion profile (for 25 cm position, 2.3 m s^{-1} velocity, 20 m s^{-2} acceleration)

The first of the two ultrasonic distance sensors (Sick UM30 213113, Germany) in the system measures the profile of the plant while the second ultrasonic distance sensor measures the distance between the plant and the slider following the height control process. Tests were performed to determine the calibration characteristics of the ultrasonic sensor with an analog output voltage from 0 to 10 V, a detection range from 20 to 130 cm. The calibration characteristics obtained from distances measured using a fixed object is shown in Figure 5. The mathematical equation obtained by using the calibration characteristic of the ultrasonic distance sensor is given in Equation 1.

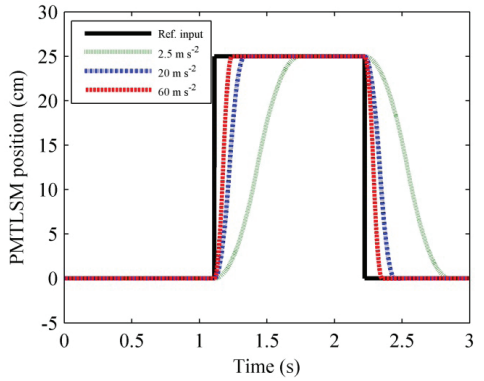


Figure 4- Trapezoidal motion profile for different acceleration values (Reference input, 0.45 Hz square wave signal)

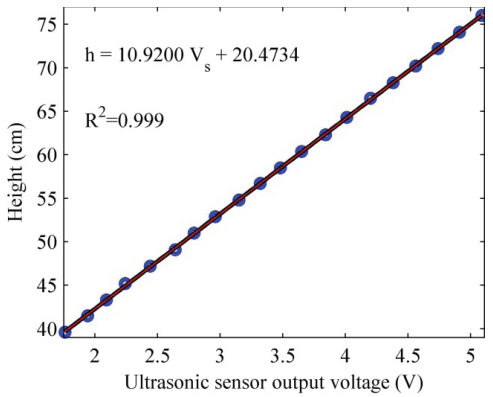


Figure 5- Calibration characteristic of the ultrasonic distance sensor

$$h = 10.9200V_s + 20.4734 \quad (1)$$

Where; V_s is the voltage (in volts) read from the sensor, and h is the height (in cm). This equation is used to calculate the distance between the sensor and the object according to the voltage measured from the sensor output. For synthesis of the drive signal a test was performed using a measured height. The characteristic figure obtained according to the test result is given in Figure 6. Using this characteristic, Equation 2 was obtained.

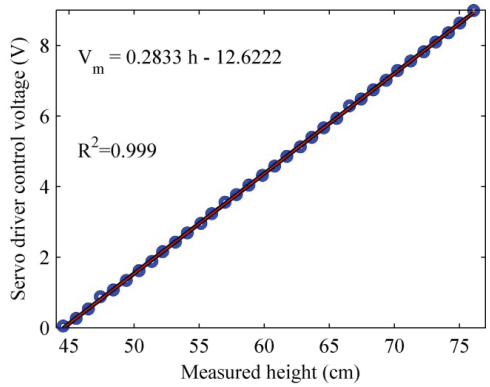


Figure 6- Servo driver analog control signal characteristic according to height

$$V_m = 0.2833h - 12.6222 \quad (2)$$

Where; h is the height (in cm) and V_m is the control signal (in volts). This equation provides a calculation of the analog control signal to be applied to the servo drive inputs to achieve the slider's 50 cm reference height using the distance information obtained from the sensor. Since the servo drive inputs are suitable for a 0 to 10 V analogue voltage, the control signal is limited between these values. In the PID position controller, the proportional gain, integral gain and derivative gain parameters significantly affect the performance of the control. These parameters are set 2.5 A mm^{-1}, 0 A mm^{-1} s^{-1} and 7.5 A s m^{-1} respectively. Since there is an oscillation risk in the slider position at steady-state conditions, integral gain is set to the zero as recommended by the PMTLSM manufacturer.

In the laboratory experiments, the conveyor belt was operated at speeds of 1, 2, 3, 4 km h^{-1}. At every speed, the PMTLSM acceleration was set manually via the servo drive software to be 2.5, 5, 10, 20, 40, 60 m s^{-2}, respectively.

The coefficient of variation (CV) and linear motor RMS current values (the root mean square values of motor current values calculated for each test) were used to test the performance of the system. CV is taken as the deviation from the average height. The RMS current is an important criterion in determining the amount of energy consumed and the amount of warm-up time for the electric motors. The linear motor RMS current values were calculated using the data received from the servo drive.

Analysis of variance (ANOVA) was used to investigate: 1. Whether there is a difference between the CV averages, and 2. Whether there is a difference between the PMTLSM RMS current averages. When a significant difference occurred, the LSD multiple comparison test was used to identify which subgroups caused the differences.

After laboratory experiments, field experiments were carried out on land where real plants were found in different sizes. For the field test, the system developed in the laboratory was adapted to a tractor (Figure 7). In the field tests the driving speed was set at 4, 8 and 12 km h^{-1} detected by a GPS device (Aselsan ASN3040, Turkey), because Langenakens et al (1995), indicated that the tractor speed changes between 3 and 12 km h^{-1} in spraying applications. The PMTLSM acceleration is set to 20 m s^{-2}, which was found to be the best value in laboratory tests. The 2nd ultrasonic sensor on the test setup was replaced with a conical type nozzle for spray tests on the field. A conventional type of hand pump knapsack sprayer (Basar Memeto, Turkey) was used as the sprayer at 4.9 bar of service pressure. Data for the wind speed, air temperature and average humidity during field experiments were recorded at 5.9 m s^{-1}, 23 ˚C and 41%. Water-sensitive paper (WSP) cards (26×76 mm, Syngenta, Switzerland) are used to visualize, measure and map out the spray distribution and for analysis of the spray coverage area. Spray droplets leave a blue stain on the yellow surface of the WSP (Salyani et al 2013). In this study, spray distribution was charecterized by measuring spray coverage on the WSP. Evans et al (1994), found a strong correlation between the spray coverage obtained from image analysis and the mass deposits obtained by chemical analysis provided that the spray coverage is fairly uniform. Six WSP cards were located at approximately 30 to 65 cm directly under the nozzle and water was used as the spray liquid. In accordance with previous studies (Salyani et al 2013) only a sample area of 2×2 cm was used at the center of the WSP cards for image analysis. WSP cards were analyzed using the Matlab image processing toolbox. The percentage of wetted area or spray coverage was calculated for the samples after the spray application. WSP card images were converted to binary values by converting the image to gray scale and then a threshold was applied to assign a value of 0 or 1 to pixels based on their intensity. Spray coverage area was calculated as the ratio of the number of pixels exposed to water, divided by the total number of pixels (Sama et al 2016).

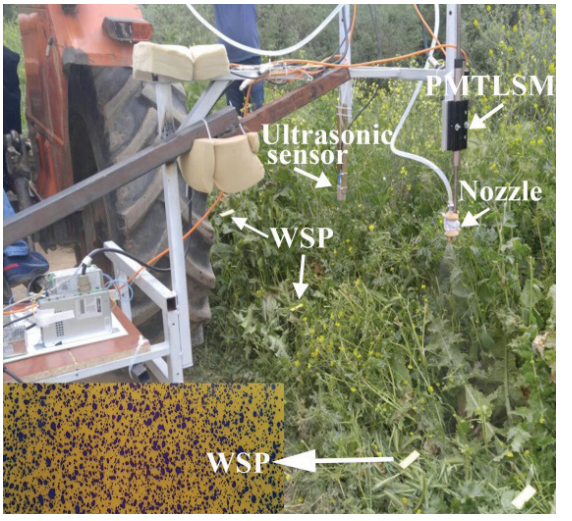


Figure 7- The field test setup

3. Results and Discussion

3.1. Laboratory tests results

The CV value was calculated using the data in Figure 8 at 10.79% for the average height of 50 cm when control was not applied to the system. Some graphs related to the data obtained from the experiments are given in Figures 9 to 11. It can be seen from the figures that the system tries to keep the nozzle at about the 50 cm set height for different accelerations and conveyor speed values. The tests were performed for three repetetions at 4 different conveyor belt speeds and 6 different PMTLSM accelerations. The results of the variance analysis for the data obtained in all experiments are shown in Table 1. This table indicates that the change in PMTLSM acceleration and conveyor speed, and the interaction between them, had a statistically significant effect ($P<0.05$) on the CV and PMTLSM RMS current. The statistical importance of interaction is that it indicates how the effect of increasing acceleration differs according to the changing conveyor speed values. According to the LSD test shown in Table 2, the CV value decreased when acceleration was increased. This decrease was not statistically significant ($P>0.05$) between 20 m s^{-2} and 60 m s^{-2}. Variations of the CV values according to accelerations and conveyor belt speeds are shown in Figure 12. It is seen from the figures that the rate of decrease of CV varies according to the speed. The effect of acceleration is more visible at higher conveyor speeds. When the acceleration is increased from 20 m s^{-2} to 60 m s^{-2}, the CV values decrease only for 4 km h^{-1} of speed, while others are changed little. It can be said that the increase in the amount of vibration generated in the motor causes a reduction in the acceleration effect after 20 m s^{-2}. According to the conveyor belt speed, if the speed was further increased in value by over 2 km h^{-1}, the increase in the CV value became statistically significant ($P<0.05$). It can be said that this is caused by the ultrasonic sensor which is negatively affected by the increasing conveyor speeds (Iida & Bursk 2002; Zaman et al 2007; Koc & Keskin 2011). The effect of PMTLSM acceleration and conveyor speeds on the PMTLSM RMS currents are presented in Table 1, Table 2 and Figure 13. The results of Table 1 indicate that the change of PMTLSM acceleration and conveyor speed, and the interaction between them, affect the PMTLSM RMS current ($P<0.05$). From the results of Table 2 it was found that the effect of conveyor speed was found to be statistically significant ($P<0.05$) for acceleration values greater than 10 m s^{-2}. The PMTLSM RMS current increases as the acceleration increases. The increase in the RMS current was found to be significant ($P<0.05$) when the acceleration was increased from 5 m s^{-2} to 60 m s^{-2}. This is due to the fact that PMTLSM tries to respond very quickly to the measured height, which changes very rapidly. RMS current increases when the conveyor speed increases, When the speed increases from 2 km h^{-1} to 3 km h^{-1}, the increase in the RMS current is not statistically significant ($P>0.05$). A current value of 2.48 in Figure 13 represents the maximum allowed RMS current for the PMTLSM. It can be seen from the figure that the PMTLSM RMS current increases at the 40 m s^{-2} and 60 m s^{-2} acceleration values as the belt speed increases, but there is no increase for smaller acceleration values. The maximum allowed instantaneous current value of the motor used in this study is 11 A, but the maximum continuous current is 2.48 A. Because the warming of the motor windings (caused by losses in the copper) is proportional to the square of the current (Wang et al 2012), the increase in the RMS current after the limit value causes the motor windings to overheat and damage the winding insulation and demagnetize the permanent magnets in the slider. For this reason, the RMS current has a limiting factor as the motor acceleration value increases. This means that the acceleration value must be kept less than 60 m s^{-2} in the system. According to statistical analysis results obtained, the PMTLSM acceleration and conveyor belt speed were found to be important variables affecting the CV and the RMS current values.

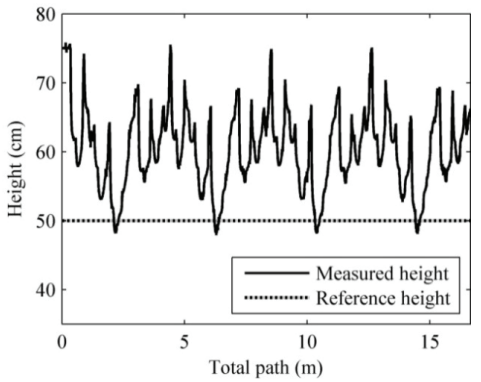


Figure 8- The measured height between by 2nd ultrasonic sensor and artificial plants along the total path (artificial plant profile)

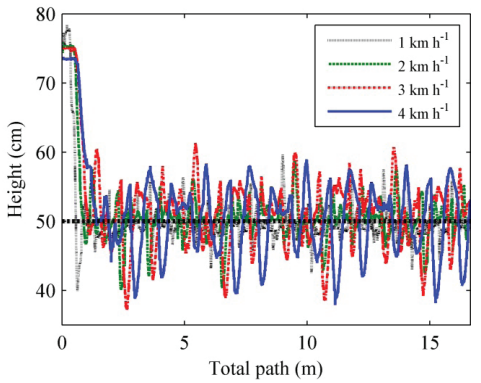


Figure 9- The height measured by 2nd ultrasonic sensor at all speeds for acceleration of 2.5 m s-2

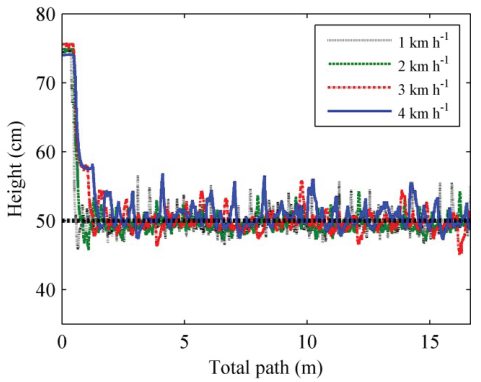


Figure 10- The height measured by 2nd ultrasonic sensor at all speeds for acceleration of 20 m s-2

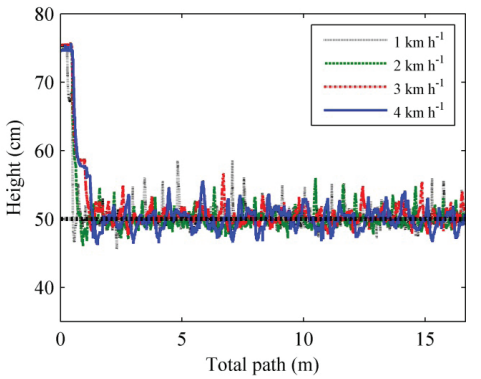


Figure 11- The height measured by 2nd ultrasonic sensor at all speeds for acceleration of 60 m s-2

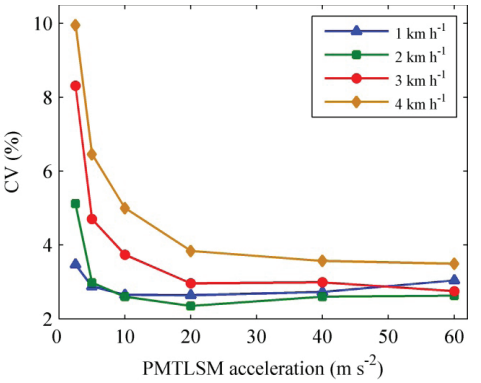


Figure 12- The relationship between PMTLSM acceleration and CV for different conveyor speed

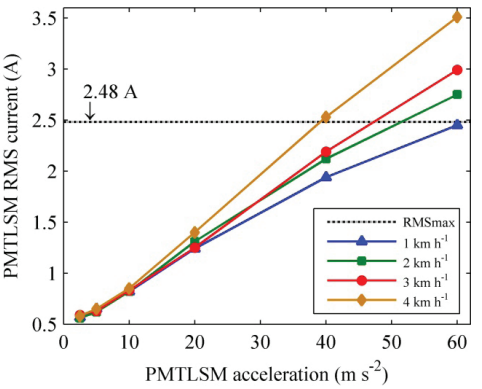


Figure 13- The relationship between PMTLSM acceleration and PMTLSM RMS current for different conveyor speed

Table 1- The results of variance analysis of the mean values of the CV and PMTLSM RMS current

Source of variation	*CV*			*PMTLSM RMS current*	
	Degree of freedom	*Sum of squares*	*F ratio*	*Sum of squares*	*F ratio*
Acceleration (A)	5	0.983	95.96*	54.64	1691.60*
Speed (S)	3	0.687	111.72*	0.91	47.08*
A×S	15	0.247	8.04*	1.49	15.36*
Error	48	0.098		0.31	
Total	71	2.015		57.35	

*, significant at P<0.05

Table 2- Mean values and significance groups of CV and PMTLSM RMS current

Acceleration	*CV (%)*					*PMTLSM RMS current (A)*				
	1 km h^{-1}	*2 km h^{-1}*	*3 km h^{-1}*	*4 km h^{-1}*	Mean	*1 km h^{-1}*	*2 km h^{-1}*	*3 km h^{-1}*	*4 km h^{-1}*	Mean
2.5 m s^{-2}	0.54^{efg}	0.71^{d}	0.92^{b}	1.00^{a}	0.79^{A}	0.57^{j}	0.56^{j}	0.59^{j}	0.58^{j}	0.58^{E}
5.0 m s^{-2}	0.46^{h}	0.47^{gh}	0.67^{d}	0.81^{c}	0.60^{B}	0.65^{j}	0.62^{j}	0.63^{j}	0.65^{j}	0.64^{E}
10 m s^{-2}	0.42^{hi}	0.41^{hi}	0.57^{e}	0.70^{d}	0.52^{C}	0.82^{i}	0.82^{i}	0.83^{i}	0.85^{i}	0.83^{D}
20 m s^{-2}	0.42^{hi}	0.37^{i}	0.47^{gh}	0.58^{e}	0.46^{D}	1.24^{h}	1.31^{gh}	1.25^{h}	1.40^{g}	1.30^{C}
40 m s^{-2}	0.44^{hi}	0.41^{hi}	0.47^{gh}	0.55^{ef}	0.47^{D}	1.94^{f}	2.12^{e}	2.19^{e}	2.53^{d}	2.20^{B}
60 m s^{-2}	0.48^{fgh}	0.42^{hi}	0.44^{hi}	0.54^{efg}	0.47^{D}	2.45^{d}	2.75^{c}	2.99^{b}	3.51^{a}	2.92^{A}
Mean	0.46^{C}	0.47^{C}	0.59^{B}	0.70^{A}		1.28^{C}	1.36^{B}	1.41^{B}	1.59^{A}	
$LSD_{0.05}$	A: 0.037 S: 0.030 AxS: 0.074					A: 0.066 S: 0.054 AxS: 0.132				

A, PMTLSM acceleration; *S*, Conveyor belt speed; uppercase letters represent groups of A and S means, lowercase letters represent groups of A×S interaction means; (P<0.05)

The average height values for each trial in laboratory experiments were found to be between a minimum of 49.47 cm and a maximum of 51.05 cm, as shown in Table 3. It can be seen from the table that the nozzle-height control system developed in this study was able to keep the nozzle within a very close mean of the set point of 50 cm in height.

Table 3- The average height values for each trial in laboratory experiments

Acceleration	*Mean height (cm)*			
	1 km h^{-1}	*2 km h^{-1}*	*3 km h^{-1}*	*4 km h^{-1}*
2.5 m s^{-2}	50.54	50.01	50.03	49.99
5.0 m s^{-2}	50.12	50.01	49.64	49.47
10 m s^{-2}	49.51	50.03	50.68	51.05
20 m s^{-2}	50.12	50.09	50.88	50.95
40 m s^{-2}	50.14	49.70	50.64	50.27
60 m s^{-2}	50.05	48.82	50.98	50.22

3.2. Field tests results

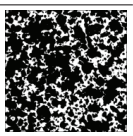
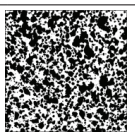
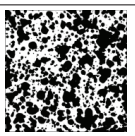
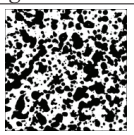
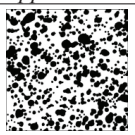
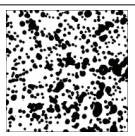
The average height and CV values obtained from experiments are shown in Table 4. CV value of 16.77% were obtained while the nozzle was stationary (without nozzle height control). However, when height control is applied, this CV value decreases to 5.17%, 4.98%, 4.09% respectively for driving speeds of 12, 8 and 4 km h^{-1}. The reason for the increase in CV value with the increase in speed can be explained as follows; The increase in the driving speed reduces the measurement accuracy of the ultrasonic sensor (Iida & Bursk 2002; Zaman et al 2007; Koc & Keskin 2011), causing the test platform vibration to increase (Langenakens et al 1999; Pontelli & Mucheroni 2012). WSP card samples obtained from field trials for three driving speed are shown in Table 5, where the number under each sample represents the percentage of the

Table 4- Mean and CV values of nozzle height for different driving speeds in the field tests

Control situation	*Driving speed (km h^{-1})*	*Mean (cm)*	*CV (%)*
With height control	4	49.77	4.09
	8	49.94	4.98
	12	50.70	5.17
Without height control	--	50.33	16.77

wetted area. The mean of wetted area percentage (WA) and Uniformity of Distribution (UD) were calculated over the six samples for each speed (Table 5). The information in Table 5 reveals that the percentage of wetted area or spray coverage is different in the forward direction when the height control is not applied. At the lowest nozzle height of 30 cm, it could be clearly seen that for all speeds,

Table 5- Mean of wetted area and uniformity of distribution for different driving speeds in the field tests

Speed	*Sample number (Nozzle height)*							
km h^{-1}	*1(30 cm)*	*2 (40 cm)*	*3 (50 cm)*	*4 (55 cm)*	*5 (60 cm)*	*6 (65 cm)*	*WA (%)*	*UD (%)*
Nozzle-height control not applied								
4							50.43	74.83
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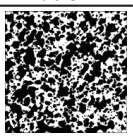
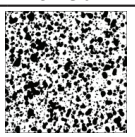
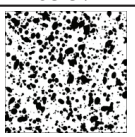
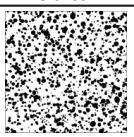
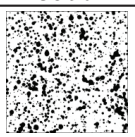
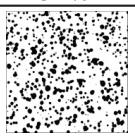
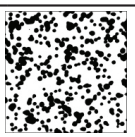
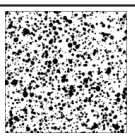
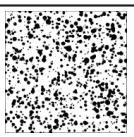
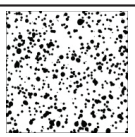
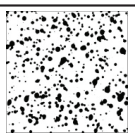
8							33.79	58.21
	58.51	40.65	32.02	28.89	22.24	20.44		
12							28.5	56.57
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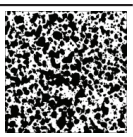
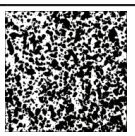
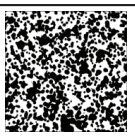
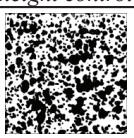
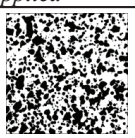
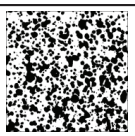
4							49.59	88.06
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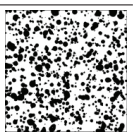
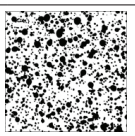
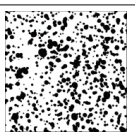
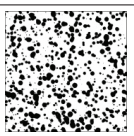
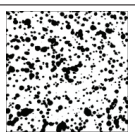
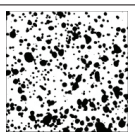
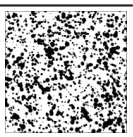
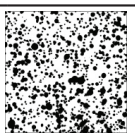
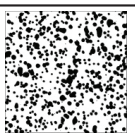
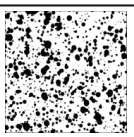
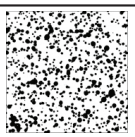
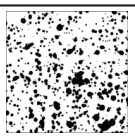
8							28.56	91.33
	32.62	30.59	27.55	27.44	26.51	26.66		
12							26.23	86.11
	30.35	29.28	27.67	25.79	23.71	20.62		

the wetted area percentage was the largest on the sample card for sample number 1, while for the highest nozzle height of 65 cm the wetted area percentage was the least on sample number 6. It is indicated in the study of (Al-Gaadi 2010) that when the distance between the nozzle and the target was small, the highest volume and application rate was found over a narrow area directly under the nozzle. On the other hand, when the height between the target and the nozzle increases, the spray volume and application rate was decreased directly under the nozzle and a larger area was sprayed. Yoshida & Maybank (1971) emphasized that for shorter distances in droplet movement towards the target, larger droplets at higher speeds can hit the target, and droplet bouncing and fragmentation can occur. This situation may negatively affect the distribution uniformity and the accumulation amount on the target. It may be said that when the distance between the nozzle and the target is large, spray-drift-which means that the sprayed droplets goes out of the target area-is a reason for the wetted area percentage to be small. Balsari et al (2017), pointed out that spray-drift increased significantly when the spraying height was increased from 30 cm to 50 cm, and from 50 cm to 70 cm, and the effect of boom height is independent of the nozzle type. Lardoux et al (2007), indicated that the dose obtained in L ha^{-1} decreases when the height increases. Losses depend on evaporation, drifting and dispersal of droplets. It is shown from Table 5 that, when speed increased, the WA value decreased for all trials. The biggest WA value was obtained at 4 $km\,h^{-1}$ and the smallest was obtained at 12 $km\,h^{-1}$. Wolf et al (1997), reported that increased speed decreased the spray deposit under the nozzle center. Ooms et al (2003), found that there is a strong correlation between horizontal motion and longitudinal spray coverage in the laboratory and under field conditions, and they indicated that spray coverage is inversely proportional to the horizontal speed. They also stated that the speed increase tends to increase the risk of spray-drift. Results from field measurements reported by Miller & Smith (1997), indicated that spray-drift increased aproximately 51% when speed was increased from 4 to 8 $km\,h^{-1}$. Langenakens et al (1995), emphasized that at a speed greater than 4 $km\,h^{-1}$ the amount of the chemical has to be increased to achieve the desired effect. As it can be seen from Table 5, when the proposed nozzle-height control was applied, the Uniformity of Distribution (UD) values were increased in the forward direction according to the fixed nozzle-height condition from 74.83% to 88.06% for 4 $km\,h^{-1}$ speed, from 58.21% to 91.33% for 8 $km\,h^{-1}$ and from 56.57% to 86.11% for 12 $km\,h^{-1}$. From this, it can be concluded that as the nozzle height control is applied, system produces better spray distribution in the forward direction.

4. Conclusions

In this study, a spray nozzle-height control system developed using a PMTLSM was tested in the laboratory environment and under field conditions. According to the results obtained, it can be said that the developed height control system is affected by the PMTLSM acceleration and speed. For this system, the optimal acceleration value was found to be 20 m s^{-2}, according to the data obtained from laboratory experiments. Both the laboratory tests and field tests showed that the CV value was reduced when the nozzle height control was applied. In the field tests when the height control is applied, the uniformity of distribution increased significantly in the forward direction. According to the results, if this system is mounted on a conventional pesticide sprayer, excessive and incomplete spraying of the pesticides will be reduced when applied in the field.

Acknowledgements

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Effects of Cultural Conditions on Exopolysaccharide Production by *Bacillus* sp. ZBP4

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ABSTRACT

Microbial exopolysaccharides (EPSs) are of great interest for the application in various industries due to their gelling, stabilizing, emulsifying, and antioxidant properties. In the present study, EPS production of 12 *Bacillus* strains were investigated and the best producer, namely *Bacillus* sp. ZBP4, was selected for further studies in order to determine the effects of fermentation conditions on the biosynthesis of EPSs. Beet molasses was used as substrate in the experiments. The highest amount of EPS was obtained at 60 g L^{-1} molasses concentration within 24 h. Optimum temperature and pH were determined as 45 °C and 5.0, respectively. Various carbon sources (glucose, starch, lactose, whey, mannitol, sucrose, beet molasses) have been tested for EPS production and beet molasses was found as the best. Using inorganic nitrogen source (ammonium sulfate) caused a decrease in the production of EPS. Tryptone gave the highest EPS yields amongst the organic nitrogen sources (yeast extract, peptone, tryptone) tested. Considerable increase in EPS production (1071 mg L^{-1}) has been observed when the experiment was conducted under the optimized conditions (using tryptone and 60 g L^{-1} molasses at pH 5.0 and 45 °C in 24 h) which was 143 mg L^{-1} before the optimization studies.

Keywords: *Bacillus*; Exopolysaccharide (EPS); Beet molasses

1. Introduction

A group of microorganisms including the strains of bacteria, yeast and molds, are able to secrete high molecular weight polymers into their surroundings called as exopolysaccharide (EPS) (Koçberber & Dönmez 2008; Fang et al 2013). Microorganisms produce EPSs as a response to harsh conditions in order to prevent cell damage (Donot et al 2012). Main structures of EPSs are comprised of monosaccharides, particularly glucose, galactose and rhamnose (Welman et al 2003). The interest in EPSs have increased considerably in the recent years, because of their physiological, chemical and rheological properties which make them suitable for a wide range of commercial applications in different fields, such as food, petroleum, cosmetics, textile, bioremediation and pharmaceuticals (Freitas et al 2011; Singh et al 2011; Öztürk et al 2014; Zhou et al 2014).

Even though numerous microorganisms can secrete EPSs, bacteria are considered as the best producers owing to the quality and quantity of EPSs

(Kumar 2012). During the recent decades several *Bacillus* strains reported to produce EPSs, such as *B. licheniformis* (Sing et al 2011), *B. subtilis* (Shih et al 2010) and *B. firmus* (Salehizadeh & Shojaosadati 2003). Levan is the best known EPS produced by *Bacillus* sp. which is mainly produced from sucrose. It is highly soluble in water, and has biological activity (e.g: anti-tumor and anti-inflammatory). It can be applied in food, feed, medicine, and cosmetics (Freitas et al 2011; Donot et al 2012).

The yield and the composition of EPSs produced by microorganisms are mainly dependent on the cultural conditions such as temperature, pH and medium composition (Tallon et al 2003; Çelik et al 2008). Sugars are mostly used for the production of EPSs as carbon source. However, there are researches concerning the utilization of cheaper carbon sources, generally agro-industrial wastes or by-products (Göksungur et al 2004; Freitas et al 2011). Molasses is a by-product of sugar industry either from the sugar cane or sugar beet. In Turkey, sugar industry relies on beet, hence beet molasses is readily available and the most preferred substrate in the fermentation industry. It has high sugar content (approximately 50%) that can be fermented by yeasts and bacteria. In addition, it contains nitrogen, vitamins and minerals which support fermentation (Yilmaz et al 2012; Abdul Razack et al 2013).

The aims of the current study were; a) to investigate the EPS production capabilities of some *Bacillus* strains that were isolated from various food and soil samples, b) after selecting the best EPS producing isolate, to produce EPS using molasses by this strain (*Bacillus* sp. ZBP4) and c) to determine effects of process conditions (temperature, pH and substrate concentration) and some nitrogen and carbon sources on the EPS production.

2. Material and Methods

2.1. Material

Molasses was obtained from Adapazarı Sugar Factory in the 2014 season. Nutrient agar, nutrient broth, trichloroacetic acid (TCA), phenol, yeast extract, peptone, and tryptone were purchased from Merck (Darmstadt, Germany) and 3,5-dinitrosalycylic acid (DNS) was purchased from Sigma (USA).

2.2. Cultural conditions and selection of EPS producing microorganisms

The isolates which were maintained in nutrient broth containing 50% glycerol at -18 °C, were activated using nutrient agar plates at 35 °C for 24 h. Single colonies from the plates were inoculated into 30 mL nutrient broth in 100 mL Erlenmeyer flasks. Then, they were cultivated aerobically (120 rpm) at 35 °C for 24 h to be used as seed culture for the production of EPSs.

For the screening of EPS producing microorganisms, a medium containing (as g L^{-1}); glucose 10, yeast extract 5, K_2HPO_4 1.5 and $MgSO4.7H_2O$ 1 was prepared. Unless otherwise mentioned, production experiments were conducted in 100 mL Erlenmeyer flasks containing 30 mL medium. The pH of the medium was adjusted to 7.0 prior to sterilization at 121 °C for 15 min. Then, the flasks were inoculated with 5% fresh cultures (having 2.0 optical density at 600 nm) and incubated at 35 °C on a shaking incubator for 24 h. EPSs produced by the isolates were extracted as described below and the amounts of total sugars were determined using phenol-sulfuric acid method (Dubois et al 1956). In screening experiments, *Bacillus* sp. ZBP4 isolate has produced the highest amount of EPS. Hence, it has been selected for further studies. This bacterium was previously isolated from a soil sample taken from the potato cultivation field and identified as *Bacillus subtilis* ZBP4 based on 16S rDNA sequence analysis. The sequence is available in GenBank (Acc No. KX811594) (Avci et al 2017).

2.3. Preparation of molasses

Raw molasses was diluted with deionized water to a ratio of 1:1 (w w^{-1}). After the adjustment of pH to 4.0 using 2 N HCl, it was boiled for the decantation of impurities. Upon cooling, it was first filtered through regular filter paper followed by centrifugation at 9000 rpm for 10 min. The resulting supernatant was used as substrate for EPS production.

2.4. Production of EPS from molasses

A basal medium was prepared which contained; molasses 40 g, yeast extract 5 g, K_2HPO_4 1.5 g and $MgSO_4.7H_2O$ 1 g in 1000 mL of deionized water. Initial pH of the medium was adjusted to 7.0, except pH experiments. Five percent (v v^{-1}) fresh seed culture having optical density of 2.0 (at 600 nm) was used to inoculate flasks. The effect of temperature on the EPS production was determined by incubating the bacterium in the basal medium at varying temperatures ranging from 30 to 45 °C on a shaking incubator at 120 rpm for 24. In order to determine the effect of pH on the production of EPSs, initial pH of basal media were adjusted to different pH values between 4.0 and 9.0 by using either 2 N HCl or 2 N NaOH. Incubations for pH experiments were carried out at 35 °C on a shaking incubator at 120 rpm for 24 h. For the determination of the effect of substrate concentration, basal media were prepared using molasses at concentrations ranging from 10 to 60 g L^{-1} and the pH was adjusted to 7.0. The bacterium was grown in these media at 35 °C on a shaking incubator at 120 rpm for 24 h. The effect of nitrogen sources was studied by replacing yeast extract from the basal medium with ammonium sulfate, tryptone, and peptone (5 g L^{-1}) and one experiment was also performed without any nitrogen source. Selected carbon sources (starch, lactose, mannitol, glucose, whey) were also tested separately by replacing the molasses from the basal medium. The concentration of each carbon source was 30 g L^{-1}. In the experiments regarding the effects of nitrogen sources, the initial pHs of the media were adjusted to 5.0 by using 2N HCl, and all incubations were conducted with shaking at 120 rpm, 45 °C for 24 h.

Finally, an experiment was conducted at the optimized conditions where a medium containing; molasses 60 g, tryptone 5 g, K_2HPO_4 1.5 g and $MgSO_4.7H_2O$ 1 g in 1000 mL of deionized water, was prepared with an initial pH of 5.0. The bacterium was incubated in this medium at 45 °C for 24 h on a shaking incubator at 120 rpm. Sample was taken at the end of the incubation and subjected to analysis of EPS.

2.5. EPS extraction

EPS extraction was done according to Koçberber & Dönmez (2008) with some modifications. Briefly, 5 mL sample was boiled for 15 min to inactivate enzymes in the fermentation broth. Upon cooling, TCA (4%; w v^{-1}) was added into the sample, and subsequently vortexed and centrifuged at 9000 rpm, 4 °C for 30 min to precipitate proteins. Supernatant was transferred into a clean tube and the same volume of 99.5% cold ethanol (at 4 °C) was added. The mixture was stored at 4 °C overnight. Then it was centrifuged at 9000 rpm, 4 °C for 30 min to precipitate the EPS. Supernatant was removed and pellets containing the EPSs were dissolved in 1 mL of deionized water and used as crude EPSs.

2.6. Determination of EPS amount

Total sugars were determined using phenol-sulfuric acid method described by Dubois et al (1956) using a glucose standard curve. Same samples were also subjected to reducing sugar analysis by 3,5-dinitrosalycylic acid (DNS) method (Miller 1959) to detect trace sugars in the samples and the results were subtracted from the total sugars determined by phenol-sulfuric acid method.

2.7. Statistical analysis

All the experiments were conducted at least in duplicate and values as mean±SD were reported. Duncan's Multiple Range Test was applied for the determination of the significance among the means ($P<0.05$).

3. Results and Discussion

3.1. Screening of Bacillus strains for EPS production

Twelve *Bacillus* strains that were isolated from soil and some food samples were screened for their EPS productions. Two of the isolates did not produce EPSs and the rest of them produced varying amounts ranging from 13.4 to 143.1 mg L^{-1} (Table 1). EPS producing bacteria formed mucoid colonies on agar media that was also an indication of the formation of EPSs (Figure 1). The best EPS producer, *Bacillus* sp. ZBP4, was used for the production of EPSs from molasses.

Table 1- Production of exopolysaccharides by *Bacillus* strains isolated from various sources from glucose; the initial pHs of the media were adjusted to 7.0 and incubations were performed on a shaking incubator (120 rpm) at 35 °C for 24 h

Bacteria	*Amount of EPS (mg L^{-1})*
Bacillus sp. BAST2	13.4
Bacillus sp. BMZE2	43.5
Bacillus sp. BMZE3	81.9
Bacillus sp. BMZE4	39.8
Bacillus sp. ZGT1	0.0
Bacillus sp. ZGT3	40.0
Bacillus sp. ZGT5	0.0
Bacillus sp. ZGT9	48.8
Bacillus sp. ZBP4	143.1
Bacillus sp. ZBP10	99.2
Bacillus sp. GİT2	48.5
Bacillus sp. BAT3	72.2

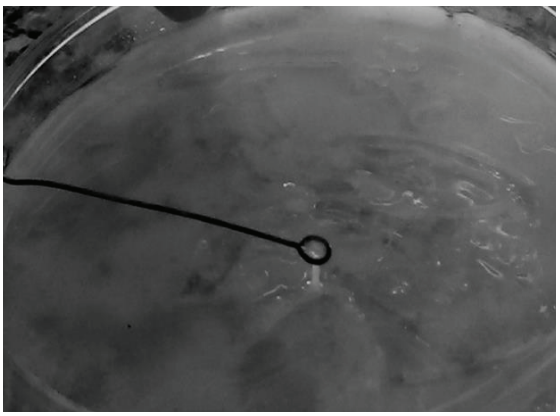

Figure 1- Mucoid appearance of exopolysaccharide (EPS) produced by *Bacillus* sp. ZBP4 on nutrient agar

3.2. Effect of growth parameters on EPS production

3.2.1. Effect of substrate concentration on EPS production

Optimization of the growth parameters effecting the EPS production have been investigated using beet molasses as carbon source which contained 46.3% (w w^{-1}) total sugars. Initially, optimal molasses concentration was determined using 10-60 g L^{-1} molasses in the basal medium. The lowest EPS production has been detected at 10 g L^{-1} concentration which was 68 mg L^{-1}. Significant increases in EPS production have been observed with increasing concentrations ($P<0.05$) and it reached its maximum (426 mg L^{-1}) at 60 g L^{-1} molasses concentration which corresponds to 27.8 g L^{-1} total sugars (Figure 2). It has been indicated that EPS production was stimulated by the excess of carbohydrate in the medium and limitation of carbon sources diminishes EPS synthesis (Van Geel-Schutten et al 1998; De Vuyst & Degeest 1999). A number of reported researches concerning the effect of substrate concentration suggest that optimum substrate concentration varies depending on the individual microorganism. For instance, Çelik et al (2008) tested the effect of substrate concentration on EPS production by *Pseudomonas aeruginosa* G1 and *Pseudomonas putida* G12 using xylose and found the maxima as 368 mg L^{-1} and 262 mg L^{-1} at 3% (w v^{-1}) and 2% concentrations, respectively. *Halomonas anticariensis* produced EPS better at 1% (w v^{-1}) glucose concentration (Mata et al 2006). *Bacillus licheniformis* produced maximum EPS (~600 mg L^{-1}) with 2% sucrose (w v^{-1}) (Larpin et al 2002). On the other hand, there are microorganisms requiring much higher substrate concentrations, such as *Rhizobium rodiobacter* which produced 2834 mg L^{-1} EPS on 10% whey (Zhou et al 2014).

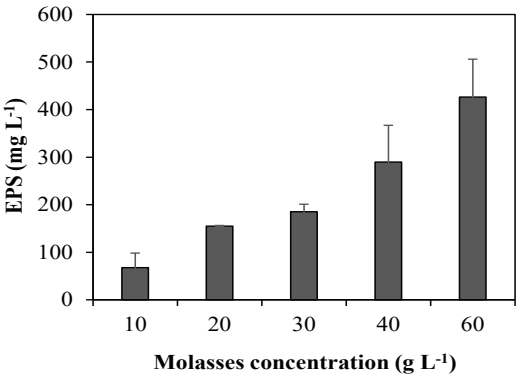

Figure 2- Production of exopolysaccharide by *Bacillus* sp. ZBP4 at varying molasses concentrations; the initial pHs of the media were adjusted to 7.0 and incubations were performed on a shaking incubator (120 rpm) at 35 °C for 24 h

Production of EPS was also determined during longer incubation periods and a drastic decrease (ca 30-40%) has been detected at 48 h and 72 h of the incubation compared with the production at 24 h (data not shown). The decrease in EPS yield after a certain incubation time is due to the activity of glycohydrolases secreted by the microorganisms into fermentation medium which catalyze the degradation of EPS (Li et al 2014).

3.2.2. Effect of temperature on EPS production

Temperature for EPS biosynthesis is crucial (Lui et al 2009). Thus, the impact of temperature on EPS production was determined by incubating the bacterium in the basal medium at temperatures ranging between 30 and 45 °C. Figure 3 depicts the EPS amounts produced at varied temperatures and growth of the bacterium which were given as optical density (OD_{600}) measured by using a spectrophotometer. Interestingly, temperatures beyond the optimum growth temperature (33-35 °C) of *Bacillus* sp. ZBP4 promoted the production of EPS, and the production became the lowest at temperatures 35 and 37 °C where the strain ZBP4 grows well. Means of the values determined at different temperatures were statistically different ($P<0.05$). Various temperature dependencies were reported in the literature. For instance, Wang et al (2011) obtained maximum amount of EPS with *Paenibacillus* sp. TKU023 at 37 °C while having lower yields at low temperatures which is not in accord with our findings. Lui et al (2009) reported the best EPS production temperature for *Paenibacillus polymyxa* as 24 °C. It is known that EPSs help the cell in protecting it from stress conditions such as temperature, pH, and light intensity, thus its production is a direct response to environmental conditions (Donot et al 2012). In our study, higher production rates of EPSs at lower or higher temperatures can be attributed to that phenomenon because *Bacillus* sp. ZBP4 produced the maximum amount of EPS at 45 °C, which is reasonably higher than the optimum growth temperature of the bacterium.

3.2.3. Effect of pH on EPS production

The pH of the medium is an important parameter affecting the cell membrane and structure thereby nutrient uptake and EPS production are also influenced (Liu et al 2009). In this regard, EPS production was performed at different pH values and the results showed that EPS biosynthesis has been significantly affected by the pH of the medium ($P<0.05$) (Figure 4). The highest EPS production was achieved at pH 5.0 and it decreased with increasing pH values. pH 4.0 and pH 9.0 resulted in the lowest

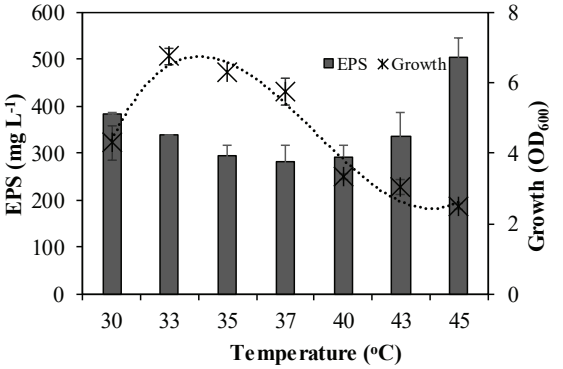

Figure 3- Growth and exopolysaccharide production by *Bacillus* sp. ZBP4 at different temperatures; the initial pH of the media were adjusted to 7.0 and incubations were performed on a shaking incubator (120 rpm) for 24 h

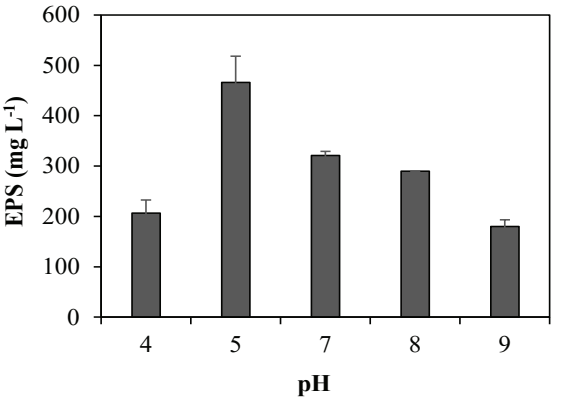

Figure 4- Effect of initial medium pH on the production of exopolysaccharide by *Bacillus* sp. ZBP4; incubations were performed on a shaking incubator (120 rpm) at 35 °C for 24 h

EPS productions. Levansucrase is an extracellular enzyme that catalyzes the production of levan from sucrose and it was reported that it is produced when the pH of the medium is acidic (Castillo & Lopez-Mungia 2004; Donot et al 2012). That can be possible explanation why our strain had produced EPS maximally at pH 5.0. Moreover, as mentioned above, harsh conditions forces microorganisms to secrete EPS for protection.

The medium composition affects the EPS secretion by microorganisms to a great extent (Çelik et al 2008). Carbon sources display different effect on catabolic repression and secondary metabolisms (Liu et al 2009). In this study, effects of different carbon sources on EPS production were tested by replacing molasses in the basal medium with glucose, lactose, starch, whey and mannitol. *Bacillus* sp. ZBP4 produced EPS on all the carbon sources tested in varying amounts. Yet, molasses was the best carbon source followed by whey and glucose which resulted in reasonably higher yields than the other carbon sources tested. Levan is synthesized by the enzyme levansucrase from sucrose (Donot et al 2012). Since the major sugar constituent of molasses is sucrose, most probably the stain ZBP4 is producing levan. However, when pure sucrose was used, the production of EPS decreased dramatically. It can be suggested that the amino acids found in molasses significantly promoted the production. The same case was also seen with whey which contains lactose and proteins. EPS production was doubled when whey was used compared with lactose. Similar results were obtained by Abdul Razack et al (2013) who produced EPS by *B. subtilis* from cane molasses and sucrose and they found 4.86 and 2.98 g L^{-1} EPSs, respectively. As being economically viable and fermentable by many microorganisms, either cane or beet molasses were successfully used in a number of studies (Göksungur et al 2004; Abdel-Aziz et al 2012; Sirajunnisa et al 2012; Abdul Razack et al 2013). Mannitol gave the weakest yields followed by starch and lactose.

Several nitrogen sources (tryptone, peptone, yeast extract, ammonium sulfate) were tested in order to find out the best one for EPS production by *Bacillus* sp. ZBP4 (Table 2). Ammonium sulfate that was used as inorganic nitrogen source led to decrease in EPS synthesis. Similarly, some researchers have also found decreased EPS yields with inorganic nitrogen compounds and it was proposed that some amino acids cannot be synthesized from inorganic nitrogen sources (Abdul Razack et al 2013). Amongst the organic nitrogen sources used, tryptone was found as an excellent ingredient with which 974±72 mg L^{-1} EPS was obtained while it was 525±2 mg L^{-1} when the yeast extract was used. Peptone seemed to be ineffective on EPS production which gave almost the same amount of EPS when there is no nitrogen supplement in the medium.

Table 2- Effects of carbon and nitrogen sources on the production of exopolysaccharides by *Bacillus* sp. ZBP4; the initial pH of the media were adjusted to 5.0 and all the incubations were carried out on a shaking incubator (120 rpm) at 45 °C for 24 h

Carbon sources	*EPS (mg L^{-1})*	*Nitrogen sources*	*EPS (mg L^{-1})*
Glucose	450^{d*}±10	Ammonium sulfate	388^{a}±67
Starch	171^{b}±42	Tryptone	974^{c}±72
Lactose	220^{bc}±9	Yeast extract	525^{b}±2
Whey	452^{d}±25	Peptone	448^{ab}±42
Mannitol	103^{a}±22	Without nitrogen**	432^{ab}±19
Sucrose	243^{bc}±21		
Molasses	505^{d}±41		

*, means with different letters in each column are significantly different for each sample (P<0.05); **, only molasses was used in the medium without nitrogen supplementation

4. Conclusions

EPS production by *Bacillus* sp. ZBP4 was investigated using molasses as substrate. The effects of substrate concentration, pH, temperature, and carbon and nitrogen sources on the production of EPSs were determined. The strain produced the highest amount of EPS in the medium having an initial pH of 5.0, at 60 g L^{-1} molasses concentration, at 45 °C in 24 h. Use of tryptone as nitrogen source has markedly enhanced the secretion of EPSs. The microorganism produced 1071 mg L^{-1} EPS under the optimized conditions. The results showed that

the isolate *Bacillus* sp. ZBP4 can be good candidate for the production of EPSs and it was able to utilize molasses both as carbon and nitrogen sources. However, there is a need for more studies in this topic.

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Drainage Water Salt Load Variations Related to the Salinity and Leaching Ratios of Irrigation Water

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ABSTRACT

Solubility of salts and leaching fractions have different effects on drainage water quality. Knowing the quality of drainage water is extremely important in terms of environmental factors and quality of water resources for the reason that this water is transferred to various sources.

In this study, were studied the changes of drainage water salinity and salt load under lyzimeter (soil columns) conditions using different irrigation water salinity and with different leaching fractions. The study was carried out with sunflower in PVC soil columns with 40 cm diameter and 115 cm length with 3 different irrigations and 5 irrigation waters with different salinity level. The three irrigation treatments were 75%, 115% and 135% of the required irrigation water. The irrigation water salinities were 0.25 dS m^{-1} as control treatment, 1.5 and 3.0 dS m^{-1} with NaCl+$CaCl_2$ salts and 1.5 and 3.0 dS m^{-1} with NaCl+$CaSO_4$ salts as saline treatments.

In this study investigated drainage water quality variations and salt load with irrigation water and some individual ions load and their leaching by drainage water as well. Drainage water salinities variated with both irrigation water salinity and leaching fractions. It was higher under the effect of soluble salts and with the 15% leaching fraction. However, salt load was higher at 35% leaching fractions level. When discuss the individual ions; while Cl^-, (CO_3^{-2}+HCO_3^-) and Ca^{+2} were accumulated in the soil profile, SO_4^{-2}, Na^+ ve Mg^{+2} were leaced from the profile, and all these ions variated by interaction for irrigation waters, and by leaching fractions for the drainage water.

Keywords: Irrigation water quality; Drainage water quality management; Solubility of salts; Leaching fraction; Salt load; Lysimeter experiment

1. Introduction

In most of the world's irrigated lands, there occur salinity and drainage problems due to irrigations and today it is known that irrigation is almost impossible without adequate drainage. Drainage is also significant in controlling salinity problems. The fact that accumulated salts can only be leached from the profile via leaching water, requires the absence of drainage problems in the area. Drainage water returns to natural resources or gets used in irrigating lower plains. Therefore, the quality of drainage water become important for water resources, for irrigation practices and also for the environmental point of view.

Salts affect soil water salinity according to their solubility. High solubility salts are highly concentrated salts that are easily soluble and harm the growth of plants. Conversely, low solubility salts are ones that never reach a high enough concentration to harm the plants (Ayers & Westcot 1988; van Hoorn & van Alpen 1988).

The estimation of drainage water below root zone is essential for evaluating risks of soil salinity in dry areas, developing irrigation management and tracking damage of agricultural chemicals in the ecosystem (Bond 1998; Walker et al 2002). The quality of drainage water reflects the quality of water table and the components of the drained water. Drainage water in irrigated dry places contains salts such as NaCl and $CaSO_4$ and may include elements such as Se, B and Ar. The core of sustainable irrigated agriculture is plant root zone salinity management and the requirement for controlled drainage implements to this end (Ayars et al 2006).

Regarding sustainable irrigated agriculture, leaching is a necessary procedure in order to avoid the accumulation of soluble salts in the root zone. The salinity of drainage water is increased with the leaching of salts from the root zone. Therefore, despite an increase in irrigation efficiency, that is to say, a decrease in leaching ratio might mean an increase in below root zone salinity concentration, it would also mean a decrease in drainage water salt load (Oster & Rhoades 1978). Northey et al (2006) have stated that there is a relation between the changes in salinity of the water table and depth. Generally, as depth increases, salinity in irrigated areas increases.

The increase of world's population and expansion of irrigated areas on one hand, and global warming and decrease of clean water resources on the other, make it unfortunately obligatory for poor quality water to be used in certain irrigation areas (Şener 1993). In the long run, 50% of the irrigation water needs will be met with rotational drainage water usage (Rhoades 1983). In Egypt, drainage water is diluted before usage. Therefore, additional water resources are created and as inhomogeneous water distribution becomes balanced, irrigation efficiency rises significantly (Wolters & Bos 1990). In a study done in Konya, where irrigation agriculture is common, 22% of farmers use irrigation water from drainage canals (Çiftçi et al 1995). Heng et al (1991) have carried out an experiment to determine the leaching losses of Cl^-, SO_4^{-2}, NO_3^{-1}, K^+, Mg^{+2}, Ca^{+2} and Na^+, and found that Cl^- was the dominant anion in the drainage water, with losses total concentration of 100 kg ha^{-1} per year. The leaching loss of $S0_4$ was concentration of 13 kg S ha^{-1} per year from the paddock fertilized with superphosphate compared with 3 kg ha^{-1} per year from the elemental S-fertilized paddock.

Irrigation with saline water and applying leaching fractions affected the mass balance of soil salinity constituents. Yurtseven at al (2014) stated that increasing leaching fractions in relation with the irrigation water salinity, caused to change soil Cl^-, SO_4^{-2}, HCO_3^-, Ca^{+2}, Mg^{+2}, and Na^+ balances; while Cl^-, SO_4^{-2}, Mg^{+2} and Na^+ washed out with leaching water, HCO_3^- and Ca^{+2} accumulated in SCL soil profile.

In this study, the leaching tendency of individual ions and salt loads have been analyzed in regards to irrigation water containing different solubility salts as well as drainage water salinity under varying leaching fractions, in homogeneous soil in lysimeters.

2. Material and Methods

The experiment was held in Ankara University Agricultural Faculty at Dışkapı Campus, as an outdoors cultivation between June 9th-September 16th 2015 with sunflower. Lysimeters were set up with corruge PVC tubes of 40 cm diameter and 115 cm height. The soil, was provided from the top 20-60 cm profile of the surrounding cultivated area and sieved and put into lysimeters in equal amounts according to their bulk density. The soil used was sandy-loam and its bulk density 1.35 g cm^{-3}, pH 8.03, EC_p (at 1:2 saturation) 1.92 dS m^{-1}, field capacity 25.1%, and wilting point 15.2%. The sunflower

used was "Meriç", an original variety from Thrace Agricultural Research Institute of Turkey.

As treatments 5 salinity levels (S) and 3 irrigation amounts (L) have been used in a total of 45 lysimeters. Experiments were done as a factorial experiment in fully randomized design with 3 replications. Irrigations were done with water containing at various rates of NaCl, $CaCl_2$ and $CaSO_4.2H_2O$ salts. Irrigation water salinity levels were S_1= top water (0.25 dS m^{-1}), S_2= 1.5 dS m^{-1} NaCl+$CaCl_2$, S_3= 3 dS m^{-1} NaCl+$CaCl_2$, S_4= 1.5 dS m^{-1} NaCl+$CaSO_4.2H_2O$ and S_5= 3 dS m^{-1} NaCl+$CaSO_4.2H_2O$. Irrigation water was applied with leaching fractions as 115% (L_{15}) and 135% (L_{35}). Field experiments also had a treatment of limited irrigation, i.e. 75% of the required amount (L_{075}) but, this treatment was not included to this drainage water quality evaluation since it didn't produce drainage water. Drainage waters were collected from the leaching treatments of L_{15} and L_{35}.

This experiment implemented 8 irrigations during growing season and these were dated according to TDR (Trace) and gravimetric soil water analyses, considering of 40% usage of available soil water. After each irrigation, drainage water was collected from plastic cups previously put at the bottom of soil columns. EC measurements were taken with ECmeter (YSI-3000) according to USSL (1954). The anion and cation analysis were taken via ion chromatography (DIONEX IC-1600) apparatus according to Anonymous (1993).

Salt load values of drainage waters were calculated by multiplying the total drainage volume (mm) and drainage water salinity (EC, dS m^{-1}) and denoted as ECmm (van Hoorn & van Alpen 1988). Salt load values regarding ion concentrations were denoted as mmol as the multiplication of water volume (liter) and appropriate ion concentration (Yurtseven et al 2014).

3. Results and Discussion

During the growing season, irrigation was done 8 times and the average salinity levels were 0.27, 1.58, 3.12, 1.95 and 3.45 dS m^{-1} for S_1, S_2, S_3, S_4 and S_5 treatments, respectively. The S_4 and S_5 treatments had higher salinity than predicted. It was because the added salts had various solubilities and jips solved in a different manner than chloride salts. Anyway the differences of the water salinity levels than predicted weren't extremely high to effect the design of the experiment.

3.1. Drainage water salinity

Examined drainage water salinity resulted in L_{15} having salinity between 15.11 dS m^{-1} (S_5) and 17.84 dS m^{-1} (S_3) and for L_{35} treatment having salinity between 12.81 dS m^{-1} (S_1) and 17.47 dS m^{-1} (S_3). The changes in drainage water salinity is statistically significant for both treatments irrigation water salinity and leaching fractions (Figure 1). Drainage water salinity has been highest (17.84 dS m^{-1}) for treatment S_3 which was containing the highest level of soluble salts. This value is approximately 17% higher than the average of other treatments. The reason behind this, is the fact that chloride salts are highly soluble and easily leachable (Yurtseven et al 2003). Though salinity level in S_5 treatment was high and same as S_3, NaCl+$CaSO_4.2H_2O$ salt composition inhibited the effect of drainage water salinity and provided the salinity level similar to that S_2 and S_4 treatments which were low salty irrigation waters.

The leaching fraction affected the salinity of drainage water (Figure 1). The average drainage water salinity in L_{15} treatment was higher than L_{35} treatment of about 10%. Lower level of leaching fractions resulted in average a more concentrated drainage water because of highly soluble salts. Since soil was sandy-loam and 15% of leaching fraction was enough to provide an efficiently leaching the profile, drainage water became more concentrated for treatments with lower leaching fractions (Oster & Rhoades 1978; Yurtseven et al 2011).

3.2. Salt load

As salt load, salts that were added to soil columns with irrigation water and the salts that were collected from the profiles as leached water via drainage were evaluated.

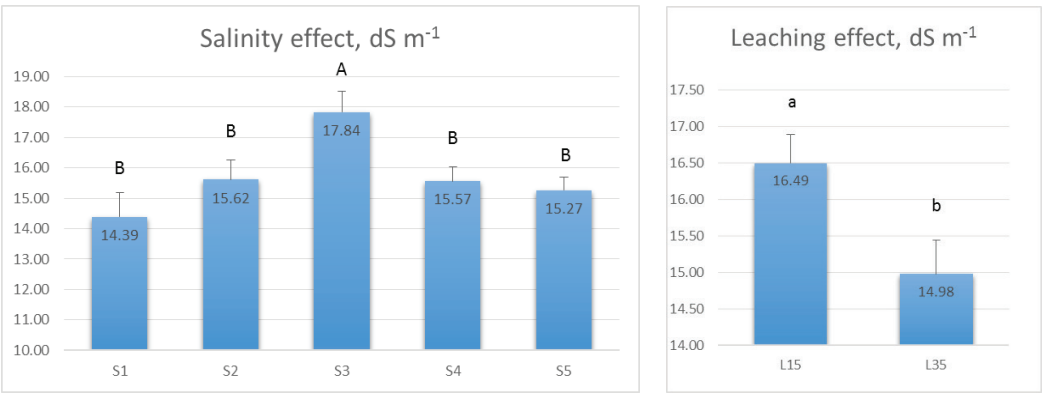


Figure 1- Salinity and leaching effects on drainage water EC values (dS m^{-1})

Salt load values varied between 2370 ECmm (S_1) and 3918 ECmm (S_3) for L_{15}, and between 5434 ECmm (S_1) and 6900 ECmm (S_5) for L_{35}. S_3 and S_5 had higher irrigation water salinity (3 dS m^{-1}) and caused the highest drainage water salt load. As previously explained, the EC values of drainage water from the L_{15} treatment had higher average drainage water salinity with lower leaching fraction. However, when looking at salt loads, L_{35} having a higher leaching fraction, had the highest salt load values, i.e. highest total salt leached from the profile occurred. Even having low salinity level of irrigation water, providing a higher volume of drainage water related with the high level of leaching, consequently resulted a higher level of salt loads (Figure 2). Increase in leaching fractions from 15% to 35% resulted in an average increase of 84% in salt load. Results are consistent with which were given in Yurtseven et al (2014).

While in drainage water only leaching fraction was significantly important, interaction was significantly important for irrigation water salt load.

When examining salt load values of irrigation water (ECmm), the variation was between 281 for S_1L_{15} and 6367 for S_5L_{35} treatments. For all treatments except for S_3L_{15} and S_5L_{15} the total salt load leached with drainage was higher than loaded with irrigation water. The difference in salt load between irrigation water and drainage water was proportionally lower for treatments with higher salt and lower leaching fractions (Figure 3). Considering the total salt load,

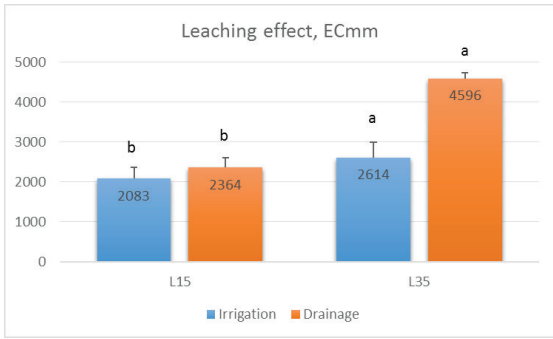


Figure 2- The effect of leaching fractions on drainage water salt load (ECmm)

leaching has been high in all treatments except S_3 and S_5. This proved that it is possible to obtain high enough leaching with irrigation water salinity till 1.5 dS m^{-1} salinity level. For high leaching at 3 dS m^{-1} salinity level, 35% leaching fraction is needed. In general says, 15% leaching fraction is enough to able to leach salts from the soil and which are loaded with irrigation water as well (Ayers & Westcott, 1988). In this study it has been seen that the low level of leaching fraction was quietly not enough for high salinity levels (S_3 and S_5), but was noticeably enough for all of other treatments.

Salt load values were considered in concentration ($mmol_c$) form for the salinity components as well. Therefore, highly active ion chloride and some of ions and components such as Na^+, Ca^{+2}, Mg^{+2}, SO_4^{-2} and CO_3^{-2} were examined (Yurtseven et al 2014).

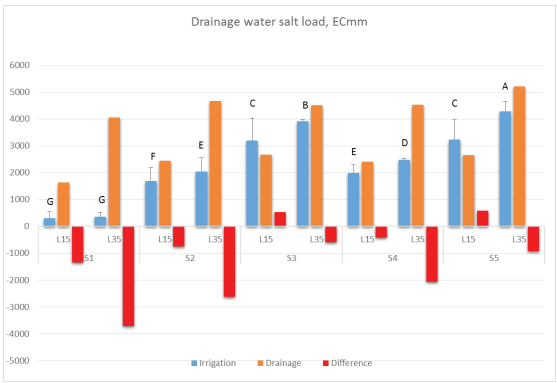


Figure 3- Changes in salt load (ECmm) values in drainage water in relation with irrigation water salinity and leaching

3.2.1. Chloride load

Figure 4 shows the interaction of chloride with the treatments. Leaching of chloride was insufficient and chloride has accumulated in all treatments. Chloride load by the irrigation water were greatly affected by the interaction between irrigation water salinity and leaching fractions (Yurtseven et al 2014). S_2 and S_3 treatments had the highest irrigation chloride levels because of the highest level of chloride salt addition (Heng et al 1991). These values were 1448 $mmol_c$ for S_2L_{15} while 4170 $mmol_c$ for S_3L_{35}. As a higher volume of water was applied to L_{35} treatments, they had a higher level of Cl^- compared to L_{15} treatments. Drainage Cl^- levels however, were affected by irrigation water salinity and leaching ratios individually (Figure 5).

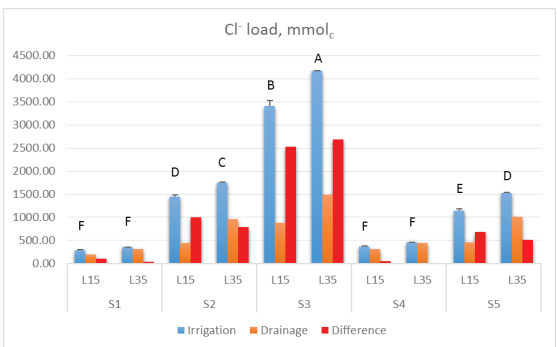
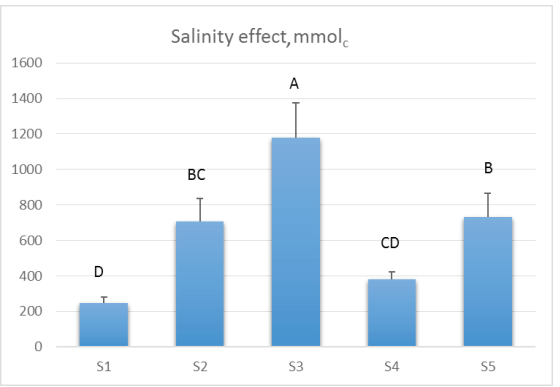


Figure 4- Chloride accumulation as $mmol_c$ related to irrigation water salinity and leaching ratio



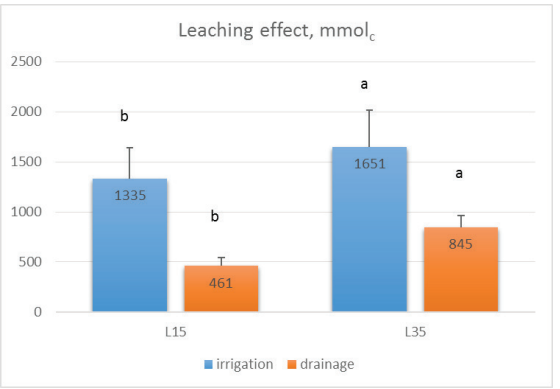


Figure 5- Salinity and leaching effects on drainage water Cl^- load ($mmol_c$)

S_3 treatment had the highest level of Cl^- in drainage water (1177 $mmol_c$) followed by S_5 and S_2 treatments. In other words, S_3, which was loaded with highest level of Cl^- by irrigation water, caused drainage water with the highest level of Cl^- load as well. For the experimental conditions, add more Cl^-, led to the highest level of leaching. L_{35}, with the highest level of leaching fraction, had the highest leaching of Cl^-. Cl^- load levels of L_{15} and L_{35} treatments are 461 and 845 $mmol_c$, respectively and the difference is at a level of 83%. Chloride coming with irrigation is approximately 2.8 times the Cl^- load leached with drainage.

3.2.2. Sulphate load

Drainage water SO_4^{-2} load variations are shown in Figure 6. All treatments had a high level of SO_4^{-2} load leached from the profiles via drainage water.

It can be said that SO_4^{-2} leaching was the case for all treatments (Yurtseven et al 2014). S_1, S_2 and S_3 treatments had higher leaching sulphate load rates. This is the result of the sulphate that originally have in the soil, and leached out from the soil with leaching water. In S_4 and S_5 because of the sulphate salt loading, the sulphate leached by the drainage water was relatively less or not leached at all (S_5L_{15}). That is, SO_4^{-2} loaded by irrigation water for S_4 and S_5 was high so at the end the total SO_4^{-2} leached (loaded with irrigation-leached with leaching fraction) became relatively small but, in fact the total sulphate leached from the soil were the highest.

There is an interaction effect on irrigation water sulphate loads. While salinity (S) has no effect on sulphate that was being leached with drainage water, leaching ratios were significantly important and L_{35} had higher sulphate load (Figure 6). As the leaching fraction increased from 15% to 35%, the increase in the sulphate loads of the irrigation water were 15.7% while on the drainage water was 85.4%. Leaching ratios for L_{15} and L_{35} treatments sulphate loads are 1177 and 2948 $mmol_c$ respectively. In average for all treatments SO_4^{-2} leached with drainage were 2.7 times the SO_4^{-2} that came with irrigation.

3.2.3. Alkalinity load

As the soil profile alkalinity, CO_3^{-2}+HCO_3^- leaching was analyzed. It was seen that drainage masses were insufficient for the leaching of the alkalinity. When analyzing alkalinity leaching, the interaction between CO_3^{-2}+HCO_3^- load in irrigation treatments is important (Figure 7). Drainage water alkalinity load showed significant changes depending on leaching

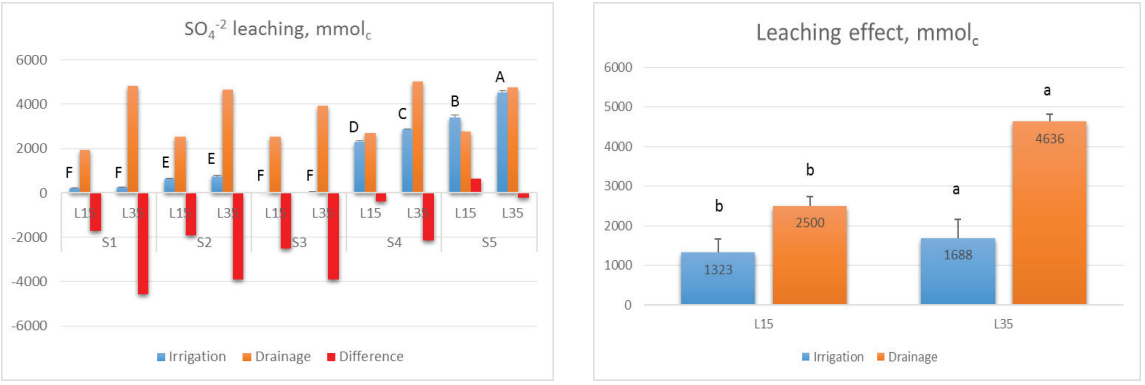


Figure 6- Sulphate leaching related to the irrigation water salinity and leaching ratio as $mmol_c$

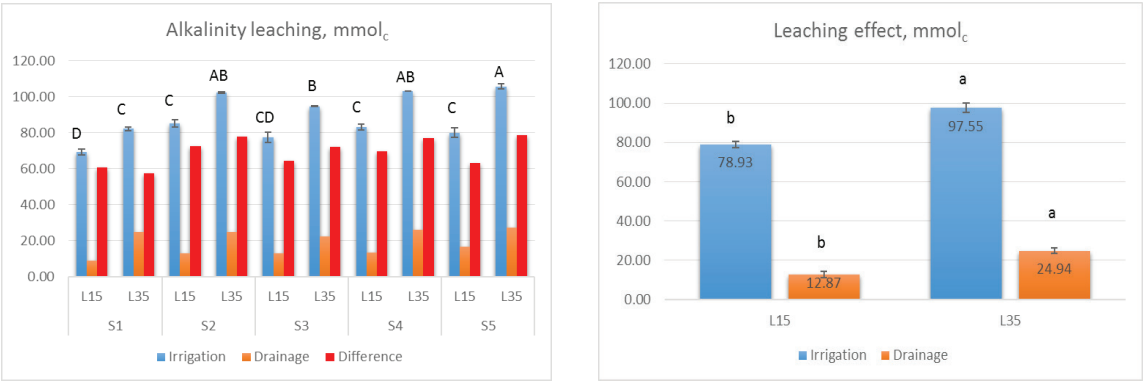


Figure 7- Irrigation and drainage water alkalinity variations related with treatments ($mmol_c$)

fraction in all treatments. Alkalinity that washed away was lower than the amount carried to the profiles with irrigation, and this result is compatible with Yurtseven et al (2014). Hence, there was alkalinity accumulation in the profiles. Naturally, a higher leaching ratio causes higher alkalinity and leaching. As leaching ratio increased from 15% to 35%, alkalinity load increased 24% in irrigation water and 94% in drainage water. Accumulation in L_{15} and L_{35} treatments were respectively 66.1 and 72.6 $mmol_c$ in average. CO_3^{-2}+ HCO_3^- added with irrigation were approximately 4.7 times to that leached away with drainage.

3.2.4. Sodium load

Sodium loads showed interaction in irrigation water, while in drainage water only leaching fractions effected significantly (Figure 8). As leaching ratio increased from 15% to 35%, irrigation water Na^+ load increased by 28%, and drainage water Na^+ load increased by 78%. In all treatments it has been seen that sodium leached out from the soil profile were higher than added with irrigation. Total leached out sodium were 825 $mmol_c$ for $L_{15,}$ and 1713 $mmol_c$ for L_{35}. Na^+ leached away with drainage water were approximately 3.3 times the total amount of loaded.

3.2.5. Ca^{+2} load

Calcium was one of the ion that were added to all irrigation waters except S_1. It has been seen that Ca^{+2} accumulated in all treatments. The highest level Ca^{+2} loads were detected at S_3 and S_5 treatments for irrigation waters due to the adding Ca^{+2} at highest level. Interaction is statistically important for irrigation treatments (Figure 9). Small variations

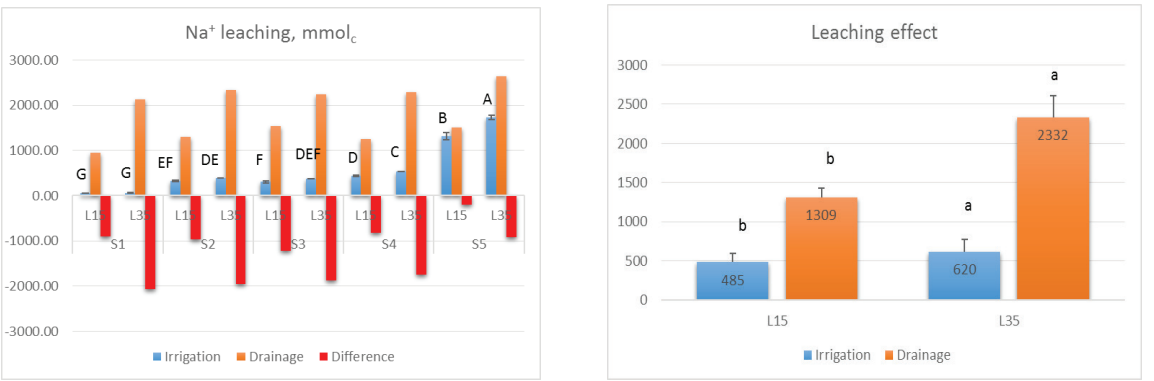


Figure 8- Na^+ load variation as $mmol_c$ of Irrigation and drainage water related with the treatments

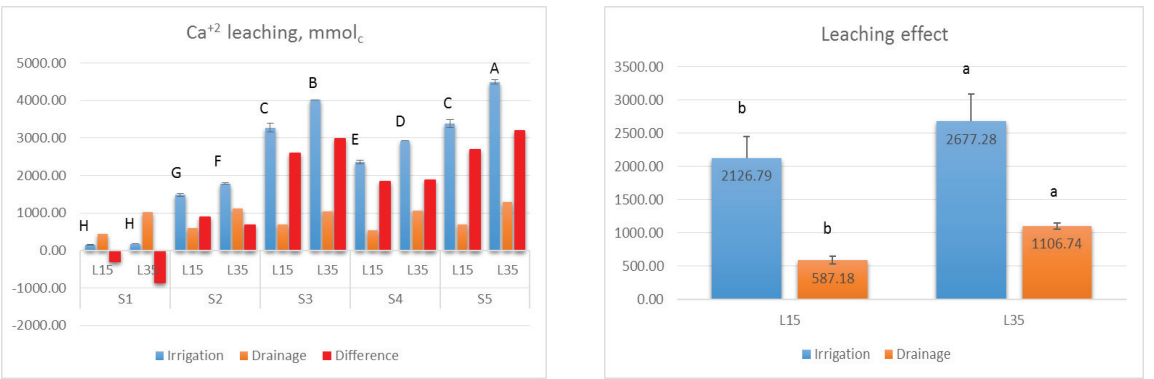


Figure 9- Ca^{+2} load variation of irrigation and drainage water as $mmol_c$ related with the treatments

were detected for leaching Ca^{+2} with the drainage water but higher at L_{35} level than L_{15} for leaching treatments and the difference is 88.4%. However for the irrigation waters the same difference is 26%. Ca^{+2} loads that were carried with water were approximately 2.8 times the total amount of Ca^{+2} leached away with drainage.

3.2.6. Mg^{+2} load

Since no extra Mg^{+2} were added to the soil with irrigation water, Mg^{+2} was the ion that washed away the most. In all treatments, level of leaching was higher than the level of initially added Mg^{+2} (Yurtseven et al 2014). While irrigation water Mg^{+2} loads showed interaction effect, leaching fractions showed an important effect on drainage water Mg^{+2} loads. For the leaching fractions in L_{35} Mg^{+2} loads increased by 24.5% in irrigation, while Mg^{+2} leached away with drainage increased by 87% (Figure 10). Mg^{+2} leached away with drainage were approximately 18 times the initial Mg^{+2} carried by irrigation water.

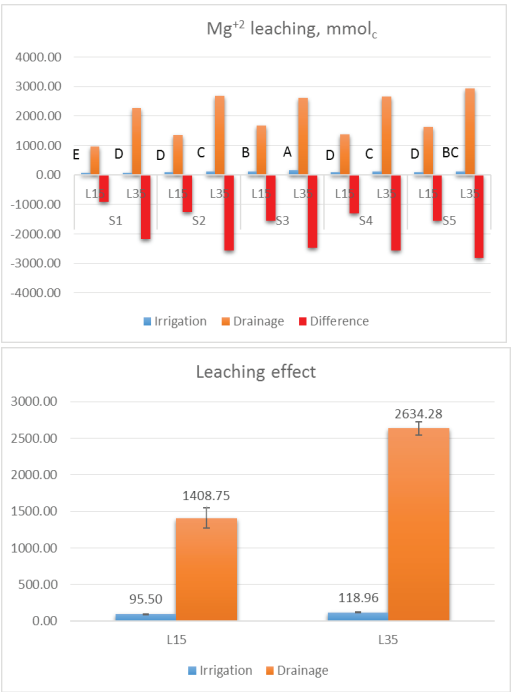


Figure 10- Mg^{+2} load variations as $mmol_c$ due to the experimental treatments

4. Conclusions

In this experimental conditions with SL soil, it can be said that drainage water became more saline with 15% of leaching fraction than with 35%. It means that the salinity could be leached out easily, with this percentage (15%) of leaching water, as Ayers & Westcot (1988) stated, under drip irrigation. It is concluded that, under effective leaching conditions, drainage water concentration can be increased due to the fact that leachable salts in the soil contributed easily to the leaching water. So drainage water became more concentrated, and drainage water salinity was concluded to be highest in treatment with most soluble salts added (S_3).

High level of chloride salts, i.e. NaCl and $CaCl_2$, having a high solubility led to high levels of salinity (S_3) in drainage water. Gypsum (S_4, S_5) has limited effect to drainage water salinity.

When it is considered the salt load; in almost all treatments drainage water salt load was high and was not affected by salinity treatments but rather by the increase in leaching fraction. The higher the leaching fraction caused the higher the drainage water salt load. The salt leached from the lyzimeters at almost the same for the treatments, varying with the leaching fractions. Although no salt were added to the S_1 treatment, it has seen that the most salt leached from the soil of S_1. This is because of the effects of added salt to the other treatments. Added salts caused that relatively the less leaching has been occured. Consequently more salt were leached with the higher level of leaching fraction.

Since chloride is easily move in the soil with water, more Cl^- added treatments in irrigation water caused more concentrated drainage water. Also increasing leaching ratio caused to increase drainage water Cl^- load.

Sulphate, leached to all treatments and leaching SO_4^{-2} increased with increasing leaching fraction. However, since sulphate has limited solubility and motion with water, there was no difference in drainage water between various salinity levels as it was for chloride.

Alkalinity (CO_3^{-2}+HCO_3^{-}) values lead to accumulation in the profiles under the effect of leaching fractions.

The analysis of Na^{+}, Ca^{+2} and Mg^{+2} loads concluded that while Na^{+} and Mg^{+2} showed leaching, Ca^{+2} showed accumulation. All three ions concluded in increased total leached ion levels due to the increase in leaching fractions.

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The Effects of Hot and Cold Water Treatment on Quality Parameters and Enzymatic Activity in Chestnut

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ABSTRACT

This study was carried out in order to limit the biochemical and enzymatic changes occurred after harvested in a variety of (*Castanea sativa* Mill. cv. 'Sarıaşlama') chestnuts to prolong the stored period by reduce the loss of quality. Before samples were stored, hot (46±2 °C, 45 minutes) and cold water (15±2 °C, 8 days) treatments were performed. After the hot and cold water treatments the fruits were stored in normal (NA) and controlled atmosphere (CA). In CA storage the fruits were kept in three different atmosphere combinations: (10% CO_2, 2% O_2; 15% CO_2, 2% O_2; 20% CO_2, 2% O_2). In both storage methods, chestnuts were stored for 5 months in 0±1 °C temperature and 90±5% relative humidity conditions. During the storage, parameters such as weight loss (%), content of relative water (%), polyphenol oxidase (PPO) (units mg^{-1} protein), soluble solids (°brix), starch (mg mL^{-1}), total sugar (mg mL^{-1}), vitamin C (mg 100 mL^{-1}), macro (potassium, phosphorus, calcium, sodium) and micro (iron, magnesium) elements (mg 100 g^{-1}) were examined in the fruit samples taken. Maximum weight loss was examined in the fruit kept in NA whereas total sugar and starch showed less of a change in CA in this present study. PPO enzymatic activity can successfully control with hot water treatment in 15% CO_2, 2% O_2 gas combination. When soluble solids was examined, a smaller degree of change was observed in the fruit stored in CA. Promising results were achieved with the fruit that was stored using hot water treatment and 15% CO_2, 2% O_2 combination in this study, which was conducted on the 'Sarıaşlama'chestnut.

Keywords: Chestnut; Cold water treatment; Controlled atmosphere; Hot water treatment; Polyphenol oxidase; Quality

1. Introduction

Chestnut (*Castanea sativa* Mill.) is a kind of fruit that is grown in the coastal regions of Turkey. It has been a part of the culture since ancient times and its cultivation and breeding is at an advanced level, especially in different parts of the Marmara Region. 59,789 tonnes of chestnuts are grown on average and 15,417 tonnes are exported (Soylu 2004b; FAO 2012).

Although chestnuts are achene fruit, unlike walnuts, hazelnuts etc., it is a fruit that is rich in terms of carbohydrates, especially starch, but poor in terms of oil (1.5-2.0%), and protein (2.5-3.0%) (Dassler & Heitmann 1991; Holland et al 1992; Ozturk et al 2010). In the fruit, reducing sugars like glucose and fructose are scarce, whereas sucrose is more plentiful (Holland et al 1992). The level of

water is high in chestnuts (45-50%) while it is low in hard-shelled oily fruits (5-10%) (Jaynes 1979; Holland et al 1992; Ozturk et al 2010). Chestnuts contain high levels of potassium and magnesium, as well as vitamin B, C, E and folic acid (Ozturk et al 2010). The pericarp in chestnuts wraps the seed in a ligninased, dry, hardened and browned layers of different tones. The pericarp is dry, crispy and grain-rich in structure. The embryo forms the part that is consumed along with the bilamellate, wavy-indented carpel (Dassler & Heitmann 1991).

Fresh, bright, vibrant, plump chestnut fruit is edible (Ryall & Pentzer 1982). Fruit stored by drying is used in the candy industry. For this reason, chestnuts are stored fresh or dried. However, studies conducted about chestnut storage are not at an adequate level.

Dried chestnuts are stored easier and last longer. While chestnuts with water levels reduced to 10% at 4.5 °C can be stored for only one year, fruit with a 50% water level at 4.5 °C can only be stored for 8 weeks (Westwood 1978; Cetin & Akbudak 2012). The aim of fresh storage is to prevent water loss and molding. There is an embryo in the fruit ready to grow under appropriate conditions. It is essential that it remains fresh during the storage period but that its growth remains suppressed. Dead embryos cause bad smell in the fruit (Ryall & Pentzer 1982). The growth of the embryo, especially offshoot end's coming out of the fruit damages the quality of the fruit (TSE 1982).

Chestnuts are considered a fresh fruit as they contain 45-50% water. For this reason, it should have a storage temperature of 0 °C, or even -1 °C and 85-90% humidity level. In order to decrease water loss, polyethylene (PE) bags and tin-cans with holes are used (Woodroof 1967; Troyan et al 1975; Ryall & Pentzer 1982; Cetin & Akbudak 2012). For this reason, antitranspirant materials were tried in the Bursa region chestnuts (Ayfer et al 1989). Fresh chestnuts can be kept for 4-5 months under appropriate conditions (Westwood 1978; Ryall & Pentzer 1982; Ayfer et al 1989; Bilginer & Serdar 1997; Karacalı 2004). In order to prevent molding, various fungicides were experimented with the chestnuts. Succesful results were seen with tiabendazol (Ayfer et al 1989).

This study intends to determine the preservability, hot and cold water treatments, storage duration, and the quality loss under different storage conditions of the 'Sarıaşlama' variety of chestnut from the Bursa region.

2. Material and Methods

2.1. Material

This research was conducted at Uludag University, Faculty of Agriculture, Department of Horticulture, Cold Storage Research and Treatment Unit and Postharvest Physiology Laboratory. 'Sarıaşlama' chestnut, harvested in September 2013 from a private single-estate field in the Cumalıkızık Village of Bursa. 'Sarıaşlama', is an Anatolian based fruit supplied by a selection process. Its tree grows broadly at a moderate level. It is high yielding and its fruit is medium sized, wide and oval shaped. The fruit shell is medium-thick, typical chestnut colour and bright. The flesh of the fruit is coloured and very good quality. This early and edible type can also be used in candied chestnut production (Soylu 2004a).

2.2. Methods

Chestnuts harvested at commercial maturity abouth 50 kg were brought to Uludag University, Faculty of Agriculture, Department of Horticulture, Cold Storage Research and Treatment Unit a couple of hours after classification and the treatments below were carried out within the scope of experiments.

Normal atmosphere (NA) storage: Chestnuts were stored in both sides perforated plastic box, at 0±1 °C temperature and 90±5% relative humidity in NA with only adjustments made for temperature and humidity.

Hot water treatment+NA: The fruits were stored after waiting 45 minutes in 3 units of 45-48 °C hot water per 1 unit of chestnut.

Cold water treatment+NA: The fruits were stored after waiting 8 days in 3 units of cold water

at 15 °C per 1 unit of chestnuts which was changed every two days (Jermini et al 2006).

Controlled atmosphere (CA) Storage: In this process, the fruits were stored in both sides perforated plastic box, at 0±1 °C temperature and 90±5% humidity level, at 10% CO_2, 2% O_2; 15% CO_2, 2% O_2 and 20% CO_2, 2% O_2 atmosphere combinations (Jermini et al 2006; Wang et al 2008).

Hot water treatment+CA: Fruits were stored at 10% CO_2, 2% O_2; 15% CO_2, 2% O_2 and 20% CO_2, 2% O_2 CA combinations after waiting 45 minutes in 3 units of water at 45-48 °C hot water per 1 unit of chestnuts (Jermini et al 2006).

Cold water treatment+CA: Fruits were stored at 10% CO_2, 2% O_2; 15% CO_2, 2% O_2 and 20% CO_2, 2% O_2 CA combinations after waiting 8 days in 3 units of water at 15 °C per 1 unit of chestnuts that was changed every two days (Jermini et al 2006).

In the study, the fruit samples that were taken during the storage process were examined according to the quality parameters below.

Determination weight loss: Weight loss was calculated in percents (%) by weighing the wet weight of each month's samples' with a high-precision scale (precision of 0.01 g, Radwag PS 3600/C/1, Radom, Poland).

Determination content of relative water: The content of relative water was calculated in percents (%) each month after samples were measured by a with a high-precision scale (precision of 0.01 g, kept in a vacuum furnace at 80 °C (Binder, ED 53 E2, Germany) for 24 hours and measured again.

Determination Polyphenol Oxidase (PPO): PPO activity was assayed according to the method of Jiang (1999), by measuring the oxidation of 4-methylcatechol. The increase in absorbance at 410 nm was automatically recorded for 3 min, using a spectrophotometer (Thermo Spectronic, Nicolet evolution 100, England). The enzym activity was expressed as units per mg protein (units mg^{-1} protein).

Total soluble protein was determined according to Bradford (1976) using bovine serum albimin as the standart.

Determination soluble solids: Fruits grinding with the help of a blender (Moulinex, DJ750, France) and ground using a mortar and a pestle (HM Mellert, M8 S, Germany) were homogenized by adding pure water until took on the consistency of mud. The soluble solids content of the solution obtained was calculated in percents (°brix) with the help of a refractometer (0-32 °brix) (Atago, R 500, Atago Co., Ltd, Tokyo, Japan).

Determination starch: The analysis of the samples was calculated (mg mL^{-1}) at the Marmara Research Centre of the Scientific and Technological Research Council of Turkey via the polarimetric kit method (TSE 2000; EC 2009).

Determination total sugar: The total amount of sugar in the fruit was calculated **(**mg mL^{-1}**)**, after diluted samples went through various processes and measured with a spectrophotometer (Thermo Spectronic, Nicolet evolution 100, England) at a wavelength of 600 nm.

Determination vitamin C: 350 mL of a 0.04% oxalic acid solution was poured into 50 g of fruit puree and it was stirred for 2 minutes and then filtered with vatman paper. 1 mL was taken from the filtered puree and 9 mL of 2,6 Dichlorophenol Indophenole dye solution was addedand it was read at 520 nm absorbance (Hısıl & Otles 1989). Vitamin C in chestnut samples were calculated as mg 100 mL^{-1}.

Determination macro (potassium, phosphorus, calcium, sodium) and micro (iron, magnesium) elements: The macro and micro element determination of the samples was conducted at Yalova Atatürk Horticultural Central Research Institute by the wet decomposition method was calculated with sulfuric acid and hydrogen peroxide (Lott et al 1956; Cottenie 1980; Kacar & Inal 2008). Macro and micro elements in chestnut samples were calculated as mg 100 g^{-1}.

2.3. Statistical analysis

The study was designed to be replicated 3 times with 150 g of fruit for each one according to random blocks test pattern and the results obtained were evaluated using Minitab-14 (2004). The evaluation of the differences between the results was determined with LSD test ($P<0.05$).

3. Results and Discussion

In this study, depending on the storage period of the chestnuts, it was observed that there were some weight losses. While these losses were between 40.21 and 39.45% at NA conditions, highest loss was 7.90% at 10% CO_2, 2% O_2 cold water treatment and the lowest loss was 3.67 and 4.00% at 15% CO_2, 2% O_2 control and 20% CO_2, 2% O_2 hot water treatment (Table 1). In addition, it was also found that there were some significant differences in terms of weight losses among the treatments. According to our results, CA had positive effects on the storage time of the fruits. The effects of atmospheric composition on the physical and chemical structure of the fruit were investigated in this study. As a result of our experiments, it was found that the practical way to reduce water loss without changing the physical and chemical structure of the fruit is to store the fruit in CA. In Ryall & Pentzer (1982), the weight loss was 6-7% between treatments and the weight loss during the storage period was 15-18%. However our studies were similar to Cecchinia et al (2011) and Cetin & Akbudak (2014) studies and the loss rate between treatments in CA were 3.67-7.90%, and the weight loss during the storage period remained within a range of 3.67-40.21%.

While more biochemical changes occurred under NA conditions, there were fewer changes in CA. Under NA slowing the metabolic activity is acheved by reducing the ambient temperature, and under CA, in addition to a decrease in temperature, metabolic activities were slowed down even further by altering the gas compositions of the environment. Metabolic activities increased water consumption. For example, breaking off one mole of glucose from a starch chain is carried out with the consumption of 1 mole of water. There are many other metabolic activities that cause water loss (Table 1). While the water percentage was initially 47.21%, at the end of the storage it varied between 15.17% and 23.28% under NA, between 34.88% and 40.21% in CA in our study. In Tzortzakis & Metzidakis (2012) study the rate of water loss rate in chestnut fruits was 3.57% after 90 day storage period under CA conditions, whereas in our study it was 7.00% at the end of 150 days under CA conditions. This shows that there is a similarity between the relative water loss results of the two studies.

Figure 1 shows changes in PPO activity of chestnut treated with hot and cold water in NA and CA atmosphere conditions in low-temperature for 150 days. PPO activity decreased from 1167.70 units mg^{-1} protein to 507.36 units mg^{-1} protein during storage at NA conditions at 0±1 °C (Figure 1). In contrast, it changed more prominently at CA, by decreasing to 124.28 units mg^{-1} protein in hot water treated gas combination at 15% CO_2, 2% O_2 (Figure 1) this suggests that CA conditions can control enzymatic browning successfully. Xu (2005) were stored fresh chestnuts at 4 °C in a refrigerator, fully covered with pre-sterilized wet sand (sieved through diameter 2 mm, 40% humidity) in boxes. The changes of the PPO specific activity during 6 months low-temperature storage decreased dramatically 1180 units mg^{-1} protein to 340 units mg^{-1} protein for 4 °C at the end of storage. Jiang et al (2004) reported similar effect of low-temperature storage on the chestnut. This might also indicate heavy loss of PPO activity during the separation process for chestnuts stored in NA much more than CA for 150 days at low temperature.

The soluble solids level change in the fruit stored under CA and NA increased during the storage period (Table 1). This increase can be explained by the conversion of some starch to glucose depending on the intrinsic biochemical changes in the fruit. The soluble solids of the fruit stored at various atmospheric compositions under CA did not show significant changes during the storage periods. The soluble solids changes under NA and CA were found to be significant. While the soluble solids

Table 1- Changes in weight loss, content of relative water, the soluble solids, total sugar and vitamin C during the storage of the 'Sarıaşlama' type of chestnut under NA and CA

Storage time (day)	*Treatment 1*	*Treatment 2*	*Weigt loss (%)*	*Content of relative water (%)*	*Soluble solids (°brix)*	*Total sugar (mg mL^{-1})*	*Vitamin C (mg 100 mL^{-1})*
0	NA[a]	Control	0.00	47.21	5.50	8.10	10.60
		Hot water	0.00	47.21	5.50	7.62	10.91
		Cold water	0.00	47.21	5.50	5.70	11.63
	LSD		-	-	-	-	-
30	NA	Control	9.81 a[b]	31.36 b	5.13 b	8.89 a	27.74 a
		Hot water	11.68 a	34.16 b	5.40 a	8.18 a	25.61 a
		Cold water	12.64 a	36.13 b	5.40 a	8.42 a	27.52 a
	10:2	Control	4.36 b	45.50 a	5.37 a	7.39 b	11.94 b
		Hot water	3.88 b	46.52 a	5.47 a	7.18 b	12.38 b
		Cold water	3.03 b	46.01 a	5.47 a	7.62 b	11.84 b
	15:2	Control	1.52 b	45.43 a	5.37 a	7.52 b	11.69 b
		Hot water	1.22 b	46.00 a	5.47 a	7.48 b	11.31 b
		Cold water	1.25 b	46.37 a	5.47 a	7.45 b	11.91 b
	20:2	Control	1.70 b	45.30 a	5.33 a	7.10 b	11.56 b
		Hot water	0.74 b	46.41 a	5.47 a	7.13 b	12.54 b
		Cold water	0.47 b	46.09 a	5.40 a	7.77 ab	10.76 b
	LSD		5.34	4.38	0.16	0.63	2.39
60	NA	Control	27.61 a	28.76 b	5.63 b	9.20 a	16.84 a
		Hot water	27.84 a	32.17 b	6.07 a	9.22 a	15.83 a
		Cold water	32.80 a	34.29 b	6.00 a	9.48 a	15.68 a
	10:2	Control	5.31 b	43.79 a	6.00 a	8.89 ab	9.35 b
		Hot water	4.99 b	44.10 a	6.10 a	8.28 b	8.89 b
		Cold water	4.58 b	45.24 a	6.07 a	8.23 b	8.51 b
	15:2	Control	3.73 b	43.74 a	6.00 a	7.90 bc	5.25 c
		Hot water	2.17 b	46.01 a	6.13 a	7.32 c	4.44 c
		Cold water	2.44 b	46.01 a	6.10 a	7.22 c	4.66 c
	20:2	Control	2.99 b	43.96 a	6.03 a	8.76 ab	8.60 b
		Hot water	2.03 b	45.36 a	6.13 a	8.34 b	7.97 b
		Cold water	4.08 b	45.32 a	6.13 a	8.63 ab	7.54 b
	LSD		8.45	5.27	0.18	0.95	1.25
90	NA	Control	38.37 a	25.02 d	6.17 b	10.93 a	1.55 b
		Hot water	38.04 a	27.98 d	6.50 a	10.84 a	1.68 b
		Cold water	38.98 a	32.87 c	6.67 a	10.45 a	1.80 b
	10:2	Control	5.59 b	39.83 a	6.03 b	9.87 b	4.67 a
		Hot water	6.72 b	42.10 a	6.10 b	9.52 b	3.47 a
		Cold water	5.61 b	42.17 a	6.07 b	9.58 b	3.73 a
	15:2	Control	4.32 b	41.45 a	6.03 b	9.66 b	0.78 c
		Hot water	2.61 b	43.81 a	6.10 b	8.53 c	1.48 b
		Cold water	4.65 b	42.76 a	6.07 b	8.70 c	1.24 bc
	20:2	Control	3.32 b	40.79 a	6.03 b	9.72 b	0.36 c
		Hot water	3.14 b	42.55 a	6.10 b	8.67 c	0.77 c
		Cold water	5.77 b	42.91 a	6.03 b	8.83 c	1.95 b
	LSD		6.83	4.16	0.25	0.51	1.28

Table 1 (Continue)- Changes in weight loss, content of relative water, the soluble solids, total sugar and vitamin C during the storage of the 'Sarıaşlama' type of chestnut under NA and CA

Storage time (day)	*Treatment 1*	*Treatment 2*	*Weight loss (%)*	*Content of relative water (%)*	*Soluble solids (°brix)*	*Total sugar (mg mL^{-1})*	*Vitamin C (mg 100 mL^{-1})*
120	NA	Control	39.27 a	20.20 d	8.10 a	11.83 a	1.27 a
		Hot water	39.07 a	24.30 d	7.17 b	11.12 a	1.04 a
		Cold water	39.65 a	26.00 d	7.33 b	11.48 a	1.38 a
	10:2	Control	5.94 b	37.90 a	6.83 c	10.65 b	4.78 b
		Hot water	7.52 b	39.69 a	7.00 c	10.33 b	5.89 b
		Cold water	7.46 b	39.82 a	6.93 c	10.47 b	4.70 b
	15:2	Control	5.00 b	39.20 a	6.90 c	10.14 b	4.21 b
		Hot water	3.06 b	41.57 a	6.50 d	9.17 c	3.84 b
		Cold water	6.72 b	41.76 a	6.90 c	9.56 c	4.78 b
	20:2	Control	4.45 b	38.90 a	6.93 c	10.59 b	4.56 b
		Hot water	3.80 b	41.65 a	7.00 c	10.26 b	3.84 b
		Cold water	6.58 b	40.73 a	6.97 c	10.33 b	3.77 b
	LSD		8.78	4.69	0.18	0.83	1.87
150	NA	Control	39.93 a	15.17 e	8.43 a	12.66 a	5.59 ab
		Hot water	39.45 a	19.88 d	8.53 a	12.37 a	7.51 a
		Cold water	40.21 a	23.28 d	8.37 a	12.48 a	7.58 a
	10:2	Control	6.34 b	34.88 b	7.90 b	11.83 ab	6.73 a
		Hot water	7.85 b	36.92 a	7.63 b	11.36 b	6.29 a
		Cold water	7.90 b	37.24 a	7.83 b	11.42 b	7.32 a
	15:2	Control	5.97 b	37.60 a	7.73 b	11.05 b	4.49 b
		Hot water	3.67 b	40.17 a	7.23 c	10.23 c	2.59 d
		Cold water	7.44 b	40.21 a	7.35 c	10.25 c	3.60 c
	20:2	Control	4.82 b	36.42 a	7.87 b	11.70 ab	4.66 b
		Hot water	4.00 b	38.53 a	7.71 b	11.00 b	3.47 c
		Cold water	6.95 b	38.14 a	7.69 b	11.33 b	3.65 c
	LSD		8.89	3.80	0.26	0.45	1.35

[a]NA, normal atmosphere; [b]There is a 5% difference between the averages represented by different letters in the same column

rates increased from 5.50 °brix to 8.63 °brix at the end of 5 months of storage under NA, it increased from 5.50 °brix to 7.23 °brix under CA. Kim et al (2006) found the rate of increase in the soluble solids to be 8.00 °brix in the study they conducted on the different varieties of chestnuts grown in Korea during a 16 week storage period. The fact that the storage time was longer and the rate of increase of the brix is low indicates the importance of the findings of this study.

Changes in the proportion of starch in the study are shown in Figure 2. Our results showed that the difference between the treatments is significant. The changes in the starch proportion in 15% CO_2, 2% O_2 and 20% CO_2, 2% O_2 treatments were lower than other treatments. The main reason for the decrease in the starch levels can be the breakdown of starch into sugar through enzymatic activities. Sugar molecules enter the Kreps cycle and get into a reaction with the oxygen in the environment and turn into carbondioxide. Figure 2 shows that the percentages of starch in 15% CO_2, 2% O_2 and 20% CO_2, 2% O_2 treatments were the highest. This is because the CO_2 levels were high in these treatments. The respiration

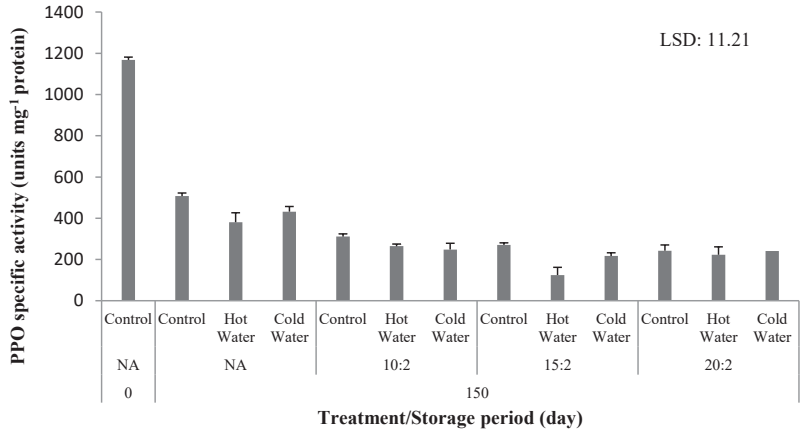

Figure 1- The PPO specific activity (units mg^{-1} protein) changes in the 'Sarıaşlama' type of chestnut at the beginning, during and at the end of the storage periods under NA and CA

rate in the hot water treatment was lower than other treatments. Tzortzakis & Metzidakis (2012) reported a 25% decrease in the rate of starch in the research, conducted on the storage conditions of chestnuts under CA conditions. In our study, decrease in the starch under CA conditions was 17.9% at the end of the 150 day storage period. This show that starch substitution in our study was relatively low.

The total sugar amount in the chestnuts is one of the important quality parameters. Changes in the total amount of sugar in the fruits were significantly effected by the storage process and gas mixtures (Table 1). Chestnut cultivar, 'Sarıaşlama' showed a linear increase in total sugar during the storage period. Under CA, 15% CO_2, 2% O_2 and 20% CO_2, 2% O_2 treatments were identified as better ones. These results were similar to those of Bounous et al (2000).

Vitamin C content had significant differences under NA and CA conditions. Under NA control group, the Vitamin C content rose from 10.60 mg 100 mL^{-1} to 27.74 mg 100 mL^{-1} in the first month,

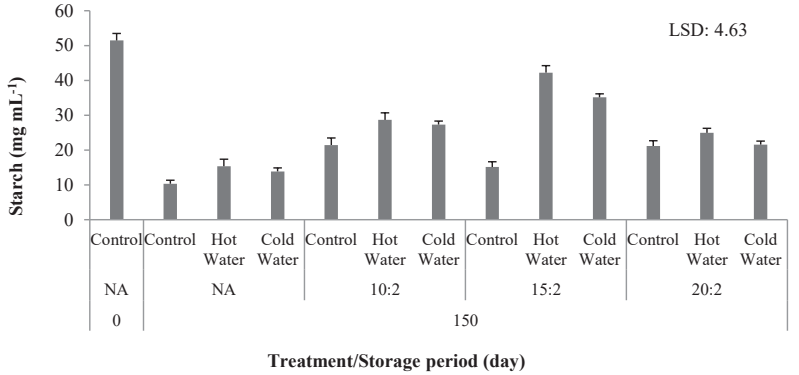

Figure 2- Starch percentages of the fruit at the beginning and at the end of the storage periods of the 'Sarıaşlama' type of chestnut under NA and CA

in the second month, began to fall and at the end of the fourth month fell to 1.27 mg 100 mL^{-1}. Then, it rose again to 5.59 mg 100 mL^{-1} at the end of the fifth month. Under CA, while the Vitamin C content consistently fell from 10.60 mg 100 mL^{-1} to 0.36 mg 100 mL^{-1} but there was an increase in the fourth month. Our results were consistent with the results of Kalt et al (1999) on juicy fruits (Table 1).

Macro and micro element contents at the beginning and the end of the storage period are shown in Figure 3. There were significant differences between the treatments. The amount of potassium increased from 760.12 mg 100 g^{-1} to 810.56 mg 100 g^{-1}, phosphate ions from 138.40 to 146.79 mg 100 g^{-1}, and calcium from 59.63 mg 100 g^{-1} to 60.00 mg 100 g^{-1}in hot water 15% CO_2, 2% O_2 gas combinations. In a similar study conducted by Ho Jin (2012), there was an increase in the amount of potassium, from 263.00 mg 100 g^{-1} to 420.60 mg 100 g^{-1}, and in phosphate ions, from 45.80 mg 100 g^{-1} to 69.60 mg 100 g^{-1}.

As a result, best results were obtained from this study conducted on the 'Sarıaşlama' type of chestnut using hot water treatment and 15% CO_2, 2% O_2 gas combinations in terms of the duration of storage of the stored chestnuts and fruit quality.

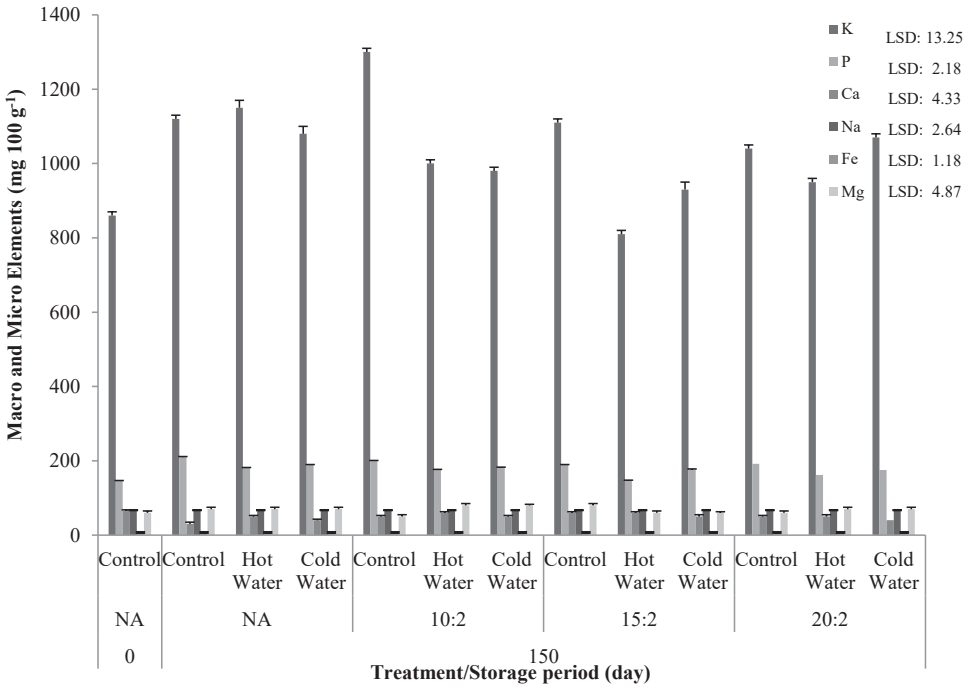

Figure 3- The macro and micro elements in the 'Sarıaşlama' type of chestnut at the beginning, during and at the end of the storage periods under NA and CA

4. Conclusions

The results show that CA conditions at gives on some quality parameters better results than NA conditions in storage of 'Sarıaşlama' chestnuts. The hot water treatment in 15% CO_2, 2% O_2 gas combination at 0±1 °C was effective for the inhibition of PPO activities. In conclusion, hot water treatment with 15% CO_2, 2% O_2 gas combination at 0±1 °C storage temperature could be suggested for reducing surface browning and biochemical changes in chestnut for 150 days.

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Populations of Exotic × Locally Adapted Germplasm - A Potential Source of Inbred Lines for Superior Indigenous Maize Hybrids

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ABSTRACT

Experimental indigenous maize hybrids were evaluated in a series of three maize experiments during 2011, 2012 and 2013 to identify superior hybrids for commercial cultivation. Parental inbred lines of these hybrids were obtained from various indigenous populations, including a new improved maize population PSEV-3 which developed by the crossing of a locally adapted variety to an exotic hybrid obtained from CIMMYT and improved through S_1 progeny recurrent selection. Two popular commercial hybrids namely, Babar (Public sector hybrid) and one Pioneer hybrid i.e., P-3025 were included as check genotypes. Results revealed that experimental PESV-3 derived hybrids revealed better performance by comparing with three check hybrids for grain yield (11.35 vs. 8.13 t ha^{-1}, 10.67 vs. 9.60 t ha^{-1}, and 11.69 vs. 11.20 t ha^{-1}), thousand grain weight (372 vs. 338 g, 370 vs. 322 g, and 416 vs. 396 g), shelling % (87 vs. 86.2%, 86 vs. 85%, and 87 vs. 90%) and days to flowering (66.3 vs. 73.6 days, 64 vs. 67 days, and 69 vs. 68 days), respectively in three experiments. Three most superior hybrid combinations developed through exotic × locally adapted germplasm were found too much responsive and suggested their further testing through on-farm trials before releasing as commercial hybrids.

Keywords: Commercial hybrid; Exotic germplasm; Indigenous maize hybrid; Improved maize population; *Zea mays* L

1. Introduction

Maize (*Zea mays* L.) is the highest tonnage crop in the world, followed by paddy rice and wheat (FAO 2016). During 2016, maize was cultivated on an area of 181 million ha in the world with production of 1060 million tons. In Pakistan, maize is the fourth largest grown crop after wheat, cotton, and rice. In Pakistan, during 2016-2017, maize was cultivated on 1.334 million ha, and production was 6.13 million tons with an average yield of 4.595 tons ha^{-1} (PBS 2016; 2017). In Khyber Pakhtunkhwa province of Pakistan, maize occupies the second position as a summer cereal, after wheat, in the crop husbandry (Khan et al 2004). Besides its use as staple food by the farming community, it is an important source of both green and dry fodder for

livestock for much of the province, especially the mountainous areas (Khan et al 2003). A modest increase of 2% in the average yield ha^{-1} in Khyber Pakhtunkhwa province compared to 100% increase in the average yield of maize in Punjab province of Pakistan in the last 10 years, primarily due to use of maize hybrids especially single crosses, has been observed. In Khyber Pakhtunkhwa, normally open-pollinated maize varieties are being used which have low yield potential compared to hybrids. This yield gap between the average and the achievable potential in the Khyber Pakhtunkhwa province could be easily filled with the cultivation of single cross hybrids by the farmers on a commercial basis (Khan et al 2011). Farmers have gradually shifted towards hybrid maize from traditionally open-pollinated varieties (OPVs) as maize has increased production from 3750 to 10000 kg ha^{-1}. The corn crop is now highly lucrative for farmers as it has higher per hectare yield than other crops in the area (The Nation 2014).

The introgression of some useful genes from exotic maize germplasm into locally adapted germplasm is an effective way to broaden the genetic base of local maize germplasm and to extract new superior inbred lines for hybrid maize development (Kauffman et al 1982; Albrecht & Dudley 1987; Abadassi & Herve 2000; Fan et al 2008b). Most maize breeders, with experience of exotic germplasm, are of the opinion that inbred lines or hybrids are more promising source materials than populations with no history of inbreeding (Goodman 1992; 1999).

The primary reasons to use exotic germplasm are a) that increased genetic diversity provides a safeguard against unpredictable biological and environmental hazards (Goodman 1992; Michelini & Hallauer 1993), b) that exotic germplasm is a source of genes for specific traits including aflatoxin resistance, drought tolerance, and husk coverage (Albrecht & Dudley 1987; Betran et al 2006), and c) that exotic germplasm is a source of favorable alleles for increased yield and enhanced heterosis (Albrecht & Dudley 1987; Tallury & Goodman 1999).

Most of the plant breeders in the world agree that exotic germplasm is a source of desirable traits; however, several challenges limit their use. Tropical inbreds are poorly adapted to temperate environments expressing excessive lodging, poor cold tolerance, poor floral synchronization, disease susceptibility, late maturity, high grain moisture and slow dry down, barrenness in high plant densities, photoperiod response, tall stature, large tassel size, and high ear placement (Goodman et al 1990; Tallury & Goodman 1999; Lewis & Goodman 2003). The use of exotic germplasm is also limited by lack of information about as to how exotic germplasm combines with adapted germplasm and the resulting loss of heterotic patterns (Hallauer 1978; Echandi & Hallauer 1996). Moreover, close linkage of favorable alleles with unfavorable alleles is still another challenge for the breeders working with exotic germplasm (Echandi & Hallauer 1996).

To increase genetic diversity, breeders can use tropical and subtropical collections as a source of new genes (Echandi & Hallauer 1996; Goodman et al 2000). Germplasm from CIMMYT, OPVs and inbred lines derived from these populations, have shown to possess a vast genetic variation since these populations were a mixture of subtropical, tropical mid-altitude and highland maize populations and pools (Xia et al 2005) and appeared as the best sources of genetic diversification across the world (Fan et al 2002; Fan et al 2003a; Fan et al 2003b; Fan et al 2008a; Fan et al 2008b; Aguiar et al 2008; Nelson & Goodman 2008).

In general, using a range of germplasm sources seems to be necessary for sustaining long-term breeding progress in maize and for avoiding an increase in the susceptibility to stresses (Reif et al 2010). Exotic or adapted, but not improved, germplasm can be used to increase the genetic base of maize breeding germplasm (Romay et al 2011). The adapted, non-improved germplasm needs to be improved before it can be included in hybrid breeding programs because of its poor agronomic performance, particularly low yield and high lodging.

Therefore, the purpose of the present research was to improve the existing population(s) for use in the extraction of superior inbred lines for high yielding maize hybrids. Specific objectives of the study were to a) evaluate the experimental (indigenous) maize hybrids for grain yield and other agronomic traits, and b) identify superior hybrid combinations for further testing on farmer's fields before releasing for commercial cultivation.

2. Material and Methods

2.1. Germplasm improvement and inbred line development

A new breeding population, PSEV-3 (CHSW × Azam), was developed by crossing the exotic subtropical material from CIMMYT with an adapted local open pollinated maize variety in spring 1999 at the Cereal Crops Research Institute (CCRI), Pirsabak-Nowshera, Pakistan. Azam is a white kernel adapted open pollinated maize variety possessing temperate and subtropical germplasm for earliness, adaptability and grain yield. The CHSW was an experimental CIMMYT hybrid of subtropical origin having a white kernel, resistance to foliar and stalk rot diseases, stay-green and good resistance to lodging. The population was improved through two cycles of half-sib recurrent selection alternating with homogenization after each cycle through half sib recombination block. The objective was to incorporate the desirable traits of the hybrid into the adapted local variety. This population (PSEV-3) was then subjected to S_1 progeny intra-population recurrent selection for further improvement in grain yield and other agronomic traits for variety development and to extract inbred lines for development of superior maize hybrids. A number of inbred lines were derived from this population by using an early-generation testing program for inbred line development (Table 1). Selected S_1 lines were advanced to S_2 and subsequently to S_3 and S_4 generations through selfing procedure in the breeding nursery. Superior S_4 lines were selected and advanced to S_5 after replicated yield trial (data not shown). Superior S_5 lines were further advanced to S_6 through sibbing (Fehr et al 1987).

2.2. Evaluation trials

The plant material in the present study comprised of various hybrids (single crosses, double crosses, three-way crosses, and inbred-population crosses) among inbred lines derived from various source populations, including that developed from crossing exotic × adapted local germplasm, PSEV-3 (Table 1). PSEV-3 (ES), Jalal and Sarhad White (SW) were among the three cultivars used in the inbred-variety crosses. Jalal and Sarhad White have been developed from subtropical germplasm and are now under commercial cultivation in the area, whereas PSEV-3 (ES) is an experimental earlier maturing variety derived from PSEV-3. Two popular commercial hybrids well known to farmers and breeders, namely, Babar (public sector hybrid) and one Pioneer hybrid i.e., P-3025 were included as checks. These hybrids were evaluated in three separate preliminary yield trials during 2011, 2012 and 2013 in a randomized complete block design with four replications at COMSATS Abbottabad, Pakistan. A plot size of four rows each of 5 m long, and 20 and 75 cm spacing between plant to plant and row to row, respectively were used in each plot. Management, fertilization, pest and weed control were carried out according to local practices in the area.

2.3. Traits measurement and statistical analysis

Data were recorded for grain yield, 1000-grain weight, shelling percentage, plant population, cobs ha^{-1}, plant height, ear height and days to 50% silking. Data were analyzed using analysis of variance appropriate to the design, using MSTAT-C computer program (Steel et al 1997). Means for various traits among genotypes were compared using the least significant differences (LSD) test.

3. Results

Mean comparison indicated significant differences among 22 hybrids for various traits in the first experiment during 2011 (Table 2). Grain yield

Table 1- Inbred lines, source population and description of source population

Sample no	*Inbred line*	*Source population*	*Population description*
1	USDA-17	Not known	Not known
2	FRW-8	FRHW-22 (F2)-5	FRHW-22 (F2)-5, a single cross hybrid- used as a female parent of an indigenous commercial double cross hybrid, Babar
3	FRW-2	FRHW-20 (F2)-4	FRHW-20 (F2)-4, a single cross hybrid- used as a male parent of an indigenous commercial double cross hybrid, Babar
4	FRW-3	FRHW-22 (F2)-8	FRHW-22 (F2)-8, a single cross hybrid- used as a female parent of an indigenous commercial double cross hybrid, Babar
5	FRW-6	FRHW-22 (F2)-4-7	FRHW-22 (F2)-4-7, a single cross hybrid- used as a female parent of an indigenous commercial double cross hybrid, Babar
6	PK-9	FRHW-20 (F2)-4	FRHW-20 (F2)-4, a single cross hybrid- used as a male parent of an indigenous commercial double cross hybrid, Babar
7	PSEV-3-4-0-7	PSEV-3	PSEV-3, an improved population derived from crossing of subtropical exotic germplasm (CHSW) to an adapted local commercial variety Azam
8	PSEV-3-5-4-2-7-5-1	PSEV-3	PSEV-3, an improved population derived from crossing of subtropical exotic germplasm (CHSW) to an adapted local commercial variety Azam
9	PSEV-3-4-3-8-5-4	PSEV-3	PSEV-3, an improved population derived from crossing of subtropical exotic germplasm (CHSW) to an adapted local commercial variety Azam
10	PSEV-3-2-3-2-0-2-1	PSEV-3	PSEV-3, an improved population derived from crossing of subtropical exotic germplasm (CHSW) to an adapted local commercial variety Azam
11	SWAJ-4-9-2	Sarhad White	Sarhad White, an improved late maturing local commercial variety-derived from subtropical germplasm from CIMMYT.
12	SHS-2-17	Shaheen	Shaheen, an improved early maturing local commercial variety-derived from temperate germplasm.
13	SHS-2-19	Shaheen	-do-
14	SHS-2-26	Shaheen	-do-
15	SHS-2-62	Shaheen	-do-
16.	SHS-2-115	Shaheen	-do-
17	SHS-2-117	Shaheen	-do-
18	SHS-2-129	Shaheen	-do-
19	SHS-2-131	Shaheen	-do-
20	SHS-2-155	Shaheen	-do-
21	SHS-2-164	Shaheen	-do-
22	SHS-2-173	Shaheen	-do-
23	SHS-2-174	Shaheen	-do-
24	SHS-2-189	Shaheen	-do-

variations ranged from 3.38 t ha^{-1} for SW × USDA-17 (single cross hybrid) to 11.35 t ha^{-1} for (FRW-3 × FRW-6) × PSEV-3-4-0-7 (a modified single cross). FRW-3 × PSEV-3-4-0-7 (a single cross hybrid) was the second highest hybrid with a grain yield of 10.92 t ha^{-1}. Both these high yielding hybrids i.e., FRW-3 × PSEV-3-4-0-7 and (FRW-3 × FRW-6) × PSEV-3-4-0-7 possessed the highest shelling % of 87.0 and 87.9, 1000 grain weight of 368 g and 372 g, and a reasonable time to maturity of 66.3 and 68.3 days, respectively. Except for (FRW-3 × FRW-6) × SHS-2-26, with a grain yield of 9.20 t ha^{-1}, all other hybrids including the two commercial hybrids (Babar and P-3025) were found to have a grain yield and 1000-grain weight significantly lower compared to two experimental hybrids involving PSEV-3-4-0-7 as the male parent in the cross.

Table 2- Means of 21 experimental and three check maize hybrids for various traits during 2011

Hybrids	*Grain yield (t ha^{-1})*	*1000-grain weight (g)*	*Shell. (%)*	*Plant pop. ha^{-1} (000)*	*Cobs ha^{-1} (000)*	*Plant height (cm)*	*Ear height (cm)*	*50% silking (days)*
SW × USDA-17	3.38	301	82.1	69.3	50.4	229	100	61.5
SW × SHS-2-174	7.87	331	83.1	67.9	60.8	239	90	63.5
(FRW-2 × FRW-8) × SHS-2-129	7.73	314	85.1	62.4	58.8	245	115	64.6
SW × SHS-2-17	5.40	277	83.2	62.4	49.6	232	97	64.0
FRW-3 × PSEV-3-4-0-7	10.92	372	87.9	63.9	55.7	271	133	68.3
(FRW-3 × FRW-6) × PSEV-3-4-0-7	11.35	368	87.0	65.6	68.0	269	139	66.3
SHS-2-174 × FRW-2	5.59	358	86.5	30.2	30.7	250	103	66.0
(FRW-2 × FRW-8) × SHS-2-19	6.16	298	85.0	70.1	54.2	235	109	62.4
SHS-2-131 × PSEV-3(ES)	6.21	304	82.2	68.5	64.9	222	88	61.0
SW × SHS-2-62	4.03	298	85.8	48.6	38.9	202	68	59.6
SW × SHS-2-164	7.37	326	80.5	70.0	66.4	228	102	63.0
SHS-2-189 × SW	6.57	298	83.4	48.6	54.2	234	92	64.0
FRW-2 × SHS-2-19	3.97	304	84.8	45.5	26.6	222	99	61.3
SHS-2-73 × SW	4.90	324	84.0	42.5	39.3	217	78	56.3
(FRW-3 × FRW-6) × SHS-2-131	7.70	284	87.5	67.0	69.5	221	104	61.0
(FRW-3 × FRW-6) × SHS-2-26	9.20	349	84.5	70.0	66.4	231	110	61.6
(FRW-3 × FRW-6) × SHS-2-115	7.14	294	86.3	68.5	68.0	228	92	59.3
(FRW-3 × FRW-6) × SHS-2-174	5.50	332	86.9	76.2	57.3	201	85	66.0
SHS-2-155 × SW	4.58	247	84.8	40.9	43.5	214	95	62.0
SHS-2-174 × SW	6.83	298	85.2	62.4	54.7	244	96	63.6
(FRW-2 × FRW-8) × SHS-2-19	5.89	308	86.6	68.5	52.7	229	107	63.3
FRW-2 × FRW-8	8.17	300	84.8	63.9	54.2	237	110	71.6
Babar (check-1)	8.37	302	86.4	71.6	64.9	221	96	67.0
P-3025 (check-2)	8.13	338	86.4	54.7	67.3	251	108	73.6
$LSD_{0.05}$	3.08	45	2.32	10.7	17.5	23.1	24.8	3.96
CV (%)	26.37	8.76	1.67	14.6	22.3	5.98	14.5	3.75

During 2012, significant differences were observed among 10 hybrids after mean comparison for various traits in the second experiment (Table 3). Grain yield of hybrids ranged from as low as 8.20 t ha^{-1} for commercial hybrid (Babar) to as high as 10.67 t ha^{-1} for (FRW-3 × FRW-6) × PSEV-3-4-0-7. Again FRW-3 × PSEV-3-4-0-7 appeared as the second highest yielding cross in the trial with a grain yield of 10.63 t ha^{-1}. These two hybrids, having PSEV-3-4-0-7 as the male parent, were significantly different from all other hybrids including the commercial hybrids for grain yield and 1000-grain weight whereas no such differences could be seen for shelling %. However, these hybrids appeared to have a comparable number of days to mid silking with local commercial check, Babar (64.5 days) and significantly lower number of days to 50% silking (64.0 and 64.8 days for (FRW-3 × FRW-6) × PSEV-3-4-0-7 and FRW-3 × PSEV-3-4-0-7, respectively) compared to that of commercial exotic hybrid (P-3025) with 67.0 days.

During 2013, results revealed that significant differences were noted among 22 maize hybrids for all traits including grain yield in the third experiment (Table 4). Grain yield variation ranged from the lowest of 4.32 t ha^{-1} for FRW-6 × FRW-3 to the highest of 11.69 t ha^{-1} for (FRW-3 × FRW-6) × PSEV-3-4-0-7, whereas days to mid silking ranged from as lowest as 61 days for SHS-2-131-6 × FRW-2 to as highest as 75 days for PSEV-3-2-3-2-0-2-1 × FRW-2. Similar variations were also observed for other traits i.e., 1000-grain weight varied from 265 g (FRW-6 × FRW-3) to 416 g (FRW-3 × FRW-6) × PSEV-3-4-0-7, shelling % from the lowest of 79% (PSEV-3-2-3-2-0-2-1 × FRW-2) to the highest of 90% (P-3025) and plant height from 210 (FRW-6 × FRW-3) to 272 cm (PK-9 × PSEV-3-5-4-2-7-5-1). Two experimental hybrids i.e., (FRW-3 × FRW-6) × PSEV-3-4-0-7 (11.69 t ha^{-1}) and PSEV-3-5-4-2-7-5-1× FRW-2 (11.47 t ha^{-1}), had higher grain yield than both commercial check hybrids and were significantly different only from check-1 (Babar).

4. Discussion

On the average, 73% hybrids of those which had one parental inbred line derived from the PSEV-3 population in their pedigree were high yielding and significantly different from commercial check-1, while only 9% of the other hybrids had grain yield superiority over the local commercial check. A

Table 3- Means of the 10 experimental and two check maize hybrids for various traits during 2012

Hybrids	*Grain yield (t ha^{-1})*	*1000-grain weight (g)*	*Shell. (%)*	*Plant pop. ha^{-1} (000)*	*Cobs ha^{-1} (000)*	*Plant height (cm)*	*Ear height (cm)*	*50% Silking (days)*
Jalal × FRW-2	9.57	326	84	73.9	67.9	260	124	66.0
PK-9 × Jalal	9.60	325	82	65.6	63.3	270	123	70.0
FRW-3 × PSEV-3-4-0-7	10.63	370	86	70.9	72.3	247	110	64.8
SHS-2-26 × FRW-2	8.93	326	84	64.6	61.6	239	114	61.0
PSEV-3(ES) × FRW-2	8.95	320	83	45.3	42.6	271	123	68.2
SHS-2-173-6 × PSEV-3(ES)	8.14	332	85	72.9	66.6	230	113	58.8
SHS-2-117-3 × PSEV-3(ES)	9.19	315	85	71.6	72.9	248	100	59.2
(FRW-3 × FRW-6) × PSEV-3-4-0-7	10.67	356	85	71.3	70.6	258	128	64.0
SHS-2-131-6 × FRW-2	9.40	337	82	75.3	74.6	245	111	61.0
SHS-2-174-5 × PSEV-3(ES)	9.16	319	84	69.9	65.6	251	102	64.8
Babar (Check-1)	8.20	300	84	64.6	60.6	235	104	64.5
P-3025 (Check-2)	9.60	322	85	73.3	68.6	265	115	67.0
$LSD_{0.05}$	1.02	22.1	1.4	7.3	8.8	19.1	14.57	1.6
CV (%)	7.9	4.7	1.2	7.4	9.4	5.3	8.9	1.8

Table 4- Means of 22 experimental and two check maize hybrids for various traits during 2013

Hybrids	*Grain yield (t ha^{-1})*	*1000-grain weight (g)*	*Shell. (%)*	*Plant pop. ha^{-1} (000)*	*Cobs ha^{-1}(000)*	*Plant height (cm)*	*Ear height (cm)*	*50% Silking (days)*
PSEV-3-5-4-2-7-5-1 × FRW-2	11.47	324	84	72.9	73.9	280	132	71
SHS-2-131-6 × FRW-2	7.72	319	83	61.4	58.3	228	85	61
FRW-6 × PSEV-3-4-0-7	9.31	380	86	69.7	63.5	247	108	66
FRW-3 × PSEV-3-4-0-7	9.33	387	86	70.8	63.5	218	120	68
FRW-6 × FRW-3	4.32	265	84	61.4	53.1	210	110	67
SHS-26-2 × FRW-2	7.83	334	84	68.7	64.5	220	102	65
SHS2-131-6 × PSEV-3-5-4-2-7-5-1	8.24	365	86	71.8	69.7	233	96	63
SHS-2-131-6 × PSEV-3-4-0-7	8.40	382	83	55.2	58.3	265	113	62
RMW8 × FRW-2	7.31	304	83	70.8	53.1	247	102	69
SHS-2-131-6 × FRW-2	8.91	317	83	69.7	69.7	245	112	59
PSEV-3-4-3-8-5-4 × FRW-2	10.41	376	85	72.9	64.5	248	107	70
PK9 × RMW8	6.23	336	81	71.8	55.2	251	105	73
PK-9 × PSEV-3-5-4-2-7-5-1	9.24	340	84	60.4	57.2	272	112	72
PSEV-3-4-3-8-2-5-4 × FRW-2	8.67	344	84	68.7	62.5	241	95	73
PSEV-3-2-3-2-0-2-1 × FRW-2	9.83	386	79	75.0	65.6	257	92	75
PK-9 × SWAJ-4-9-2	7.26	301	84	66.6	58.3	236	102	71
SWAJ-4-9-2 × FRW-2	7.63	343	84	60.4	54.1	237	112	69
PK-9 × PSEV-3-4-0-7	10.87	363	85	67.7	67.7	268	117	73
(FRW-3 × FRW-6) × PSEV3-45-4-3-7	6.75	352	85	65.6	44.7	247	96	70
(FRW3 × FRW6) × PSEV-3-5-4-2-7-5-1	7.82	349	86	72.9	54.1	255	108	70
(FRW-3 × FRW-6) × SWAJ-4-9-2	6.14	267	84	70.8	67.7	243	100	66
(FRW-3 × FRW-6) × PSEV-3-4-0-7	11.69	416	87	70.8	67.7	271	130	69
Babar (Check-1)	7.25	300	84	67.7	64.5	231	105	66
P-3025 (Check-2)	11.02	396	90	66.6	64.5	270	115	68
$LSD_{0.05}$	1.59	28.43	1.7	7.6	10.2	27.0	20.61	2.7
CV (%)	14.1	5.9	1.5	8.0	11.8	7.8	13.6	2.7

similar trend of an increased superiority of hybrids with lines from the PSEV-3 was also seen for 1000-grain weight and shelling percentage where 100% of these hybrids had significantly higher 1000-grain weight compared to local check-1 as against only 5% for the other hybrids not having such lines in their pedigrees. Majority of the high yielding experimental hybrids having parental lines from PSEV-3 and also the Pioneer check hybrid (P-3025) were generally taller in stature with high ear placement and more days to flowering compared to other experimental hybrids and local commercial check hybrid (Babar). Present results corroborate the findings of previous studies on the use of exotic germplasm as a source of favorable alleles for increased grain yield and other desirable traits in maize breeding programs (Hallauer & Carena 2009).

The higher grain yield and grain yield components (such as 1000-grain weight and shelling %) of our hybrids with one parental inbred line derived from PSEV-3, especially (FRW-3 × FRW-6) × PSEV-3-4-0-7, PSEV-3-5-4-2-7-5-1 × FRW-2 and FRW-3 × PSEV-3-4-0-7, which could be attributed to favorable alleles for increased grain yield and heterosis (Albrecht & Dudley 1987; Tallury & Goodman 1999), and might have accumulated in the elite parental lines during pre-breeding component

of the hybrid development (Carena 2005; Carena et al 2009).

The genetic diversity of PSEV-3 (CHSW × Azam) has probably allowed the extraction of a number of elite inbred lines for high grain yield and other desirable traits since germplasm from CIMMYT possess a vast genetic variation (Xia et al 2005) and found as the best sources of genetic diversification across the world (Fan et al 2002; Fan et al 2003a; Fan et al 2003b; Fan et al 2008a; Fan et al 2008b; Aguiar et al 2008; Nelson & Goodman 2008). The higher shelling percentage for the experimental hybrids with higher grain yield could be the result of a positive association between the two traits in the selection for high grain yield during inbred line development (Kadubiee & Kurianta 2004; Rafique et al 2004). Moreover, tall stature, high ear placement and late flowering of these hybrids appeared to be due to close linkage of favorable alleles with unfavorable alleles for such traits while working with exotic germplasm (Echandi & Hallauer 1996).

5. Conclusions

Results of the present study suggested that exotic maize germplasm from CIMMYT has been a useful source of many desirable traits including grain yield for incorporation into locally adapted germplasm. The improved population derived from such crosses led to the development of many elite inbred lines for use in the development of superior indigenous maize hybrids. Two single crosses, (FRW-3 × PSEV-3-4-0-7) and PSEV-3-5-4-2-7-5-1 × FRW-2, and one modified single cross (FRW-3 × FRW-6) × PSEV-3-4-0-7 appeared as the potential candidates for release as commercial hybrids, after evaluation on farmers' field.

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Effects of Chiral 3-Dichloroacetyl Oxazolidine on Glutathione S-Transferase and Antioxidant Enzymes Activity in Maize Treated with Acetochlor

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ABSTRACT

The objective of this paper was to investigate the protective effect of three potential herbicide safeners (3-dichloroacetyl oxazolidine and its two optical isomers) on detoxifying to chloroacetanilide herbicide acetochlor in maize. In this study, physiological and biochemical tests were conducted under laboratory condition in 2015. All safeners increased the expression levels of herbicide detoxifying enzymes, including glutathione S-transferases (GST), catalase (CAT) and peroxidase (POD) to reduce chloroacetanilide herbicide phytotoxicity in maize seedlings. Our results suggest that the R-isomer of R-29148 can induce glutathione (GSH) expression, GST activity, and affinity for the 1-chloro-2,4-dinitrobenzene (CDNB) substrate in maize, which can protect maize from injury by chloroacetanilide herbicide acetochlor. Further information on the chiral safener role in antioxidative enzymes activation was obtained from CAT and POD activity to overcome oxidative stress caused by the herbicide.

Keywords: Herbicide safener; Chiral 3-dichloroacetyl oxazolidine; Biological activity; GST activity; Acetochlor

1. Introduction

Acetochlor is a kind of selective herbicide before sprout. It is principally used for control of most annual grasses and certain broadleaf weed species of corn, cotton, cabbage, citrus, and peanut crops. Acetochlor is absorbed mainly by germinating plant shoots, and secondly by roots. It appears to inhibit geranylgeranyl pyrophosphate (GGPP) cyclisation enzyme synthesis in susceptible plants (Braswell et al 2016). However, studies showed that crops injury from acetochlor was greater in wet soil conditions within a two week period after application (Bouchonnet et al 2011; Hausman et al 2013). Corn injury from acetochlor was often associated with the phenomenon that leaves couldn't pull free from the whorl and form a "ladder" like plant. General symptoms included stunted plants with abnormally thick, short roots or twisted shoots and dark green leaves, also (Jursik et al 2011; Braswell et al 2016).

Herbicide safener was a widely used agrochemical with the unique ability to selectively protect crop plants from herbicide damage and

improve the selectivity of herbicide (Elmore et al 2016). Herbicide safeners are particularly effective in protecting monocot crops by increasing herbicide detoxification (Mhlanga & Chauhan 2016; Bartucca et al 2017). The current studies suggested herbicide safener appeared to induce a set of enzymes and improve herbicide metabolism (Stoilkova & Yonova 2010; Buono & Ioli 2011). It was found that the ability of safeners to protect maize from herbicide damage was related to the induction of glutathione-s-transferase activity (Fu et al 2011; Li et al 2017). Moreover, the researches had suggested that the detoxification ability of safener was involved in the level of glutathione conjugation in plant (Jo et al 2011; Ye et al 2016). However, the effects of chloroacetanilide safeners on the herbicide detoxification pathway were rarely reported. Safeners naphtbalic anhydride and dichlormid could increase crop tolerance to herbicide acetochlor by increasing the content of GSH and enhancing the activity of GST (Kraehmer et al 2014). It was also found that dichloromethyl-dioxolane safener protected maize by enhancing the activity of GST on catalyze glutathione conjugation in the metabolic detoxification of chloroacetanilide herbicide acetochlor (Rezaei et al 2013).

Studies indicated that some 3-dichloroacetyl-substituted oxazolidines with a chiral center often have different biological activities (Sriharsha & Shashikanth 2006; Zhao et al 2015). One of the most widely used dichloroacetamide safeners in maize was 3-(dichloroacetyl)-2,2,5-trimethyl-1,3-oxazolidine (R-29148). It could protect corn effectively by enhancing the expression of GST enzymes, which involved in herbicide detoxification (Li et al 2017). The activity of several antioxidative system enzymes, such as CAT and POD, was responsible for alleviating the oxidative stress generated by herbicides (Martins et al 2011; Rajasekar et al 2015; Sytykiewicz 2015). However, there is no clear mechanism for explaining the principle of action of the chiral safeners (Jablonkai 2013). Chiral R-29148 and 3-dichloroacetyl substituted oxazolidines were successfully synthesized in our previous research (Gao et al 2012). Therefore, this study was concerned with the possible mechanism of one chiral center safeners to alleviate toxicity of acetochlor to maize. Enzyme activities of GSH, GST, POD, and CAT in maize, which treated with safener racemic R-29148 and its chiral isomers, were investigated. It was hypothesized that three potential herbicide safeners could effectively protect maize against herbicide injury.

2. Material and Methods

The experiments were carried out with maize seeds Dongnong 253 (*Zea mays* L.). Racemic R-29148, R-isomer, and S-isomer were synthesized in our laboratory, and their purity levels were greater than 99.0% (Table 1). Acetochlor emulsifiabale concentrate (50%) was provided by Zhongshi Pharmaceutical Co., Ltd (Shandong, China). Acetolachlor standards were purchased from Aladdin Reagent Co., Ltd. And 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB) and GSH were purchased from Sigma (Shanghai, China). Methanol (99.9%) was provided by Dikma (Beijing, China).

Table 1- Chemical name of safeners

Safener	*Chemical name*
R-29148	3-(dichloroacetyl)-2,2,5-trimethyl-1,3-oxazolidine
R-isomer	(R)-3-(dichloroacetyl)-2,2,5-trimethyl-1,3-oxazolidine
S-isomer	(S)-3-(dichloroacetyl)-2,2,5-trimethyl-1,3-oxazolidine

Safeners were applied as a seed coating, and maize seeds were soaked in safener solution (0, 1, 5, 10, 25, 50, 100 mg L^{-1}) for 12 h at 26.5 ℃ before sowing. The non-treated control seeds were immersed in distilled water under the same conditions. After soaking, the seeds were germinated at 26.5 ℃ for 24 h in a growth chamber with a 12/12 photoperiod. Next, the seeds were directly sown in paper-cups (8 cm × 12 cm) containing 150 mL quartz sand prewashed with 10% (v v^{-1}) hydrochloric acid solution and sterilized in 5%

(w v^{-1}) sodium hypochlorite solution, 6 seeds per cup. The treatments were performed by adding 60 mL of acetochlor solution (10 mg L^{-1}) to the sand quartz, each cup with water holding capacity at 60%. The control was treated with water. Seedlings were grown at 26.5 ℃ under a 12 h photoperiod using artificial light (relative humidity 75%). Plant material was harvested 8 d after the treatment began. For completely randomized designs, each treatment was replicated thrice. After the treatments, maize shoots and roots were collected and rinsed with water, and then dried through blotting. We determined the shoot and root length as well as fresh weight (FW). The maize growth index recovery rates were calculated to determine the optimal safener concentration. The growth index recovery rates were calculated by Equation (1).

In addition, the shoots and roots were frozen in liquid nitrogen and stored at -80 ℃ for enzymatic assays (GSH, GST, POD, and CAT). The experiment was carried out with three replicates.

GSH level assay: GSH level was measured in accordance with Ismaiel & Papenbrock (2014). The maize tissue was homogenized in 5% (w v^{-1}) sulfosalicyclic acid and the homogenates were centrifuged at 15000 × g for 20 min at 4 °C. GSH levels in maize roots and shoots were measured using spectrophotometry at 412 nm with the DTNB reagent and calculated through a comparison with the known concentration.

GST enzyme extraction and assay *in vivo*: The extraction and assay of GST was performed as described by Buono & Ioli (2011). To measure the

$$\text{Recovery rate (\%)} = \frac{\text{Treated with compounds and acetochlor - Treated with acetochlor}}{\text{Contrast - Treated with acetochlor}} \quad (1)$$

GST activity, 200 mg frozen maize seedling tissue was ground into powder under liquid nitrogen and homogenized in 1 mL of QB buffer (potassium phosphate buffer 100 mM pH 7.8, with EDTA 1 mM and polyvinylpyrrolidone at 5% w v^{-1}) at 4 °C. The homogenate was centrifuged at 15000 × g for 20 min at 4 °C. The final assay mixture consisted of 50 mM phosphate buffer (pH 6.5), 1 mM CDNB, 1 mM GSH, and 0.5 mM EDTA. The reaction began by adding the root extract. The reaction mixture was measured through spectrophotometry at 340 nm for 180 s (60 s intervals). GST activity was expressed as the quantity of herbicide consumed by GSH catalyzed by GST per unit time per mg of enzyme (nmol s^{-1} mg^{-1} protein).

GST activity assay *in vitro*: To determine the GST activity *in vitro* against acetochlor in this study, HPLC assays were performed to determine the GST activity towards the herbicide acetochlor as substrate in accordance with Scarponi et al (2006). The GST enzyme extraction was added to GSH and an acetochlor standard solution. The reaction mixture was incubated for 2 h. The reaction was stopped by adding 10 µL 3.6 M HCl, and the mixture was extracted with methanol and injected into an HPLC. The GST activity was measured by comparing the initial and residual concentrations of acetochlor. GST activity was expressed as the quantity of acetochlor consumed per minute per milligram of enzyme (nmol min^{-1} mg^{-1} protein).

Kinetic parameters of GST assay: The kinetic parameters constants V_{max} and K_M were determined using a linear regression analysis of $1/V$ vs. $1/S$ according to double reciprocal plots (Scarponi et al 2006). The GST activity was determined over a range of 1-chloro-2,4-dinitrobenzene (CDNB) concentration (1.0-32.0 mM) at a single GSH concentration of 5 mM.

POD enzyme extraction and assay: To investigate the effect of safener to target enzyme, POD activity was determined as described a modified method from Rajasekar et al (2015) with certain modifications. The final assay mixture consisted of 1 mL 50 mM sodium phosphate buffer

(pH 7.0), 2 mL 0.3% of hydrogen peroxide and 0.95 mL 0.2% guaiacol. The reaction was started by addition of 0.01 mL enzyme extract to reaction mixtures. Then, the POD enzymatic activity was measured through spectrophotometry at 470 nm for 5 min. The peroxidase activity was expressed as mmol min^{-1} g^{-1} FW.

CAT enzyme extraction and assay: CAT enzymatic activity was determined following the procedures described in Hemanth Kumar et al (2016). The reaction mixtures (1.9 mL H_2O, 0.1 mL enzyme extract and 1 mL 0.3% (v v^{-1}) hydrogen peroxide) were measured by spectrophotometry at 240 nm through monitoring the decrease in H_2O_2 for 3 min. The catalase activity was expressed as µmol H_2O_2 min^{-1} g^{-1} FW.

The data were analyzed using SPSS version 16.0 software. The least significant difference was applied to assess differences between the treatments using the grouped mean and Duncan multiple range test at a 95% confidence level ($P<0.05$). Data were expressed as mean±standard deviation (n= 3).

3. Results and Discussion

3.1. Growth index of maize

In inhibited growth experiments of the maize, the acetochlor showed a severe shoot and root growth retardation. The treated maize growth index inhibition rate based on the plant height, fresh weight of shoot, root length, and root fresh weight decreased by 42, 27, 36 and 24%, respectively. To determine a suitable treatment regime, a range of concentrations of safeners were tested for their ability to decrease the injury caused by acetochlor. The protective effects of three safeners at different concentrations were recorded for R-29148 at the concentration 25 mg L^{-1}, R-isomer at the concentration 5 mg L^{-1}, and S-isomer at the concentration 50 mg L^{-1}. The results showed that all the three safeners significantly decreased the inhibition by the acetochlor herbicide, and the order of protective ability of three chiral safeners was as follows: R-isomer > R-29148 > S-isomer. The maize growth indicator recovery rates ranged from 54 to 139% as shown in Figure 1, respectively.

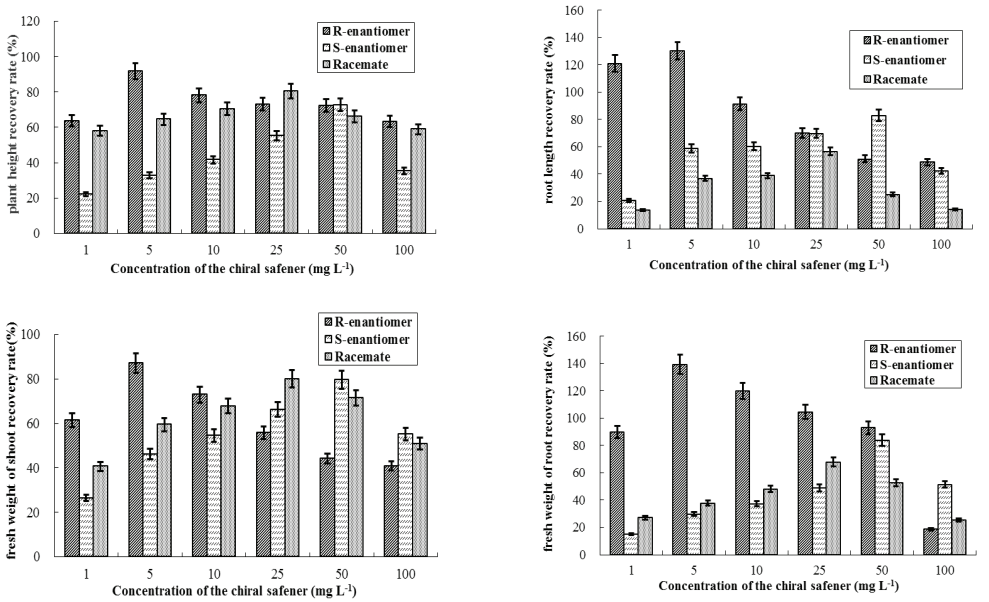

Figure 1- Recovery rate of growth indexes of maize affected by acetochlor and safeners

3.2. GSH level

In the maize root and shoot tissue, increases of GSH level after pretreatment with safeners were greater than with the acetochlor treatment (Table 2). Similarly, GSH level in the seedling tissue with the R-isomer-acetochlor treatment was greater than with the acetochlor treatment in maize. After pretreatment with the R-enantiomer the GSH contents markedly increased by 86 and 53% in the root and shoot respectively. Our results suggested that the enhanced GSH level in the maize seedlings may be related to the protective activity of herbicide safener.

Table 2- Effect of safeners and acetochlor on GSH level

Treatment	*GSH level in root ($\mu g\ g^{-1}$)*	*GSH level in shoot ($\mu g\ g^{-1}$)*
Control	4.043±0.331 d	10.044±0.171 d
Acetochlor	4.391±0.282 d	12.354±0.234 c
R-isomer+Acetochlor	8.192±0.149 a	18.868±0.178 a
S-isomer+Acetochlor	5.446±0.473 b	10.068±0.429 d
R-29148+Acetochlor	4.810±0.356 c	15.519±0.322 b

3.3. GST activity

The GST activity in vivo of maize seedling root treated by racemic R-29148 or R-isomer combined with acetochlor showed significantly increases (Table 3). The data indicated that the safener could enhance the GST activity, and the enhanced GST activity facilitated maize seedling survival at low acetochlor concentrations. When acetochlor was added as the substrate instead of CDNB, as expected, the in vitro activity of GST was also enhanced due to the chiral safeners (Table 3). The results of GST activity in maize indicated that different protect effects of safener in the maize root were due to the different levels of GST enzyme activity toward CDNB or acetochlor substrate. The R-isomer effectively promoted the GST activity among the three safeners.

3.4. Kinetic parameters of GST

The kinetic parameters tests of maize GST were carried out by using enzymatic extracts from maize roots (Table 4). The V_{max} of this process decreased, while the K_M increased under treatment with acetochlor. V_{max} increased by 60 and 18% after treated by R-isomer and racemic R-29148 compared with the untreated control, and the K_M decreased by 24 and 7%, respectively. The results in Table 4 showed that obvious influence of R-enantiomer to induction and dynamics of GST activity.

Table 4- Effect of safeners and acetochlor to kinetic parameters of GST

Treatment	*V_{max} (nmol min^{-1} mg^{-1} protein)*	*K_m (mmol L^{-1})*
Control	14.87±0.030 c	0.51±0.042 b
Acetochlor	6.69±0.044 e	0.56±0.027 a
R-isomer	23.82±0.023 a	0.39±0.019 d
S-isomer	12.73±0.067 d	0.50±0.043 b
R-29148	17.54±0.035 b	0.48±0.037 c

3.5. POD and CAT activity

The activities of POD and CAT were involved in metabolizing the oxidative stress due to high herbicide doses and protecting plants from the stress generated by herbicide. The effect of

Table 3- Effect of safeners and acetochlor on GST

Treatment	*GST activity in vivo (nmol s^{-1} mg^{-1} protein)*	*Treatment*	*GST activity in vitro (nmol min^{-1} mg^{-1} protein)*
Control	7.67±0.32 d	Control	68.17±2.65 c
Acetochlor	8.39±0.44 c	Acetochlor	-
R-isomer+Acetochlor	20.68±0.65 a	R-isomer	117.88±5.11 a
S-isomer+Acetochlor	8.17±0.38 c	S-isomer	34.60±2.39 d
R-29148+Acetochlor	11.68±0.49 b	R-29148	99.05±4.07 b

safeners and acetochlor on POD and CAT activity were determined to investigate the protective effectiveness of chiral safeners (Table 5). Compared with the control, POD activity in the maize seedling roots exhibited a significant increase. In addition, an extreme decrease in POD activity was observed after treatment with the S-isomer and racemic R-29148 compared with the acetochlor treatment alone. Upon treatment with the R-isomer, POD enzyme activity decreased from 2388 to 1834.

CAT was involved in metabolizing the oxidative stress due to high herbicide doses and then protecting plants from the stress generated by herbicide. In this case, CAT activity increased to 9.00 after the acetochlor treatment compared with the untreated control. The data in Table 5 showed that the CAT activity decreased to 7.60, 2.30 and 4.60 after treatment with three safeners, respectively.

Table 5- Effect of safeners and acetochlor on CAT and POD activity

Treatment	*CAT Activity (μmol min^{-1} g^{-1} FW)*	*POD Activity (mmol min^{-1} g^{-1} FW)*
Control	2.09±0.03 d	1135±3.25 e
Acetochlor	9.00±0.05 a	2388±4.87 b
R-isomer+Acetochlor	7.60±0.03 b	1834±1.62 d
S-isomer+Acetochlor	2.30±0.01 d	1988±2.21 c
R-29148+Acetochlor	4.60±0.02 c	2807±2.58 a

Marked acceleration of glutathione conjugation responsible for herbicide resistance in plant had been well-documented (Ismaiel & Papenbrock 2014). Safeners can stimulate GST activity and effectively detoxify by enzyme-catalyzed conjugation of GSH with the acetochlor herbicide (Scarponi et al 2006; Jablonkai 2013). This meant that the detoxification ability of safener could be decided by the degree of glutathione conjugation in maize to a certain extent. Overall, the R-isomer could protect maize from chloroacetanilide herbicide injury with enhanced GSH content and stimulated GST activity to promote glutathione conjugation with acetochlor in the maize seedlings. Compared with treated by acetochlor, dynamics of GST activity toward CDNB in safener-treatment increased significantly (Ye et al 2016). The data showed that the R-isomer significantly altered the kinetic parameter V_{max} and K_M.

Reports show that POD and CAT were involved in herbicide tolerance and two antioxidant enzymes activity increase during herbicide exposure (Rajasekar et al 2015; Sytykiewicz 2015). Our results further suggested that the activities of POD and CAT in maize were decreased by treated with the R-isomer, which indicates resistance to oxidative stress in which the chiral safener played a certain role in maize. This mechanism could be an important pathway for chiral safener detoxification in maize.

4. Conclusions

Based on data obtained in this study, it can be concluded that the effects of racemic R-29148 and its chiral isomers on growth and enzymes activity of maize could protect maize against injury from chloroacetanilide herbicides acetochlor. We investigated the changes of GST, CAT and POD activity after treatment with a safener. The maize growth level and GST activity were significantly inhibited by acetochlor, which could be tempered by adding the R-isomer. The results also suggested that the R-isomer can affect POD and CAT activity, which detoxified the plant from the effects of the acetochlor. Moreover, further studies are still needed to determine the exact mechanism of chiral safener to protect maize from injury by chloroacetanilide herbicide.

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Acknowledgements

Acknowledgements should be a brief statement at the end of the text and may include source of financial support. The contract number should be provided.

References

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Journal Articles

Doymaz I (2003). Drying kinetics of white mulberry. *Journal of Food Engineering* **61**(3): 341-346

Basunia M A & Abe T (2001). Thin-layer solar drying characteristics of rough rice under natural convection. *Journal of Food Engineering* **47**(4): 295-301

Lawrence K C, Funk D B & Windham W R (2001). Dielectric moisture sensor for cereal grains and soybeans. *Transactions of the ASAE* **44**(6): 1691-1696

Akpinar E, Midilli A & Biçer Y (2003a). Single layer drying behavior of potato slices in a convective cyclone dryer and mathematical modeling. *Energy Conversion and Management* **44**(10): 1689-1705

Books

Mohsenin N N (1970). Physical Properties of Plant and Animal Materials. Gordon and Breach Science Publishers, New York

Book Chapter

Rizvi S S H (1986). Thermodynamic properties of foods in dehydration. In: M A Rao & S S H Rizvi (Eds.), *Engineering Properties of Foods*, Marcel Dekker, New York, pp. 190-193

Publications of Institutions / Standard Books

ASAE (2002). Standards S352.2, 2002, Moisture measurement - unground grain and seeds. ASAE, St. Joseph, MI

Internet Sources

FAO (2013). Classifications and standards. Retrieved in April, 12, 2011 from http://www.fao.org/economic/ess/ess-standards/en/

Thesis and Dissertations

Berbert P A (1995). On-line density-independent moisture content measurement of hard winter wheat using the capacitance method. PhD Thesis, Crandfield University (Unpublished), UK

Conference Proceedings (Full papers)

Yağcıoğlu A, Değirmencioğlu A & Cağatay F (1999). Drying characteristics of laurel leaves under different drying conditions. In: *Proceedings of the 7th International Congress on Agricultural Mechanization and Energy*, 26-27 May, Adana, pp. 565-569

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