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Callus induction and embryogenesis from Crocus sativus L. corm explants culture
Photo: Nasser ZARE

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In Vitro Indirect Somatic Embryogenesis and Secondary Metabolites Production in the Saffron: Emphasis on Ultrasound and Plant Growth Regulators

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ABSTRACT

In the present study, the effects of ultrasound and plant growth regulators on in vitro callogenesis and secondary metabolites production in saffron calli were investigated. Accordingly, the saffron corms surface sterilized, sonicated and cultured on different concentrations of plant growth regulators (0.5, 1, 2 and 4 mg L^{-1} 2,4-D or NAA in combination with 0.5 and 1 mg L^{-1} Kin or BAP). The percentage of callus induction, callus yield (fresh weight) and embryogenic callus formation were recorded and secondary metabolites of calli were measured by UV/VIS spectrophotometer three months after culture. The results indicated that sonication of the saffron corm explants significantly increased the in vitro callus induction and growth. So, the highest callus induction (100%) and yield (4.68 g) was achieved with sonicated explants cultured on MS medium supplemented with 2 mg L^{-1} 1-naphthaleneacetic acid (NAA) and 0.5 mg L^{-1} kinetin (Kin). Somatic embryogenesis was significantly influenced by plant growth regulator regimes and the MS medium supplemented with 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) plus 0.5 mg L^{-1} 6-benzylaminopurine (BAP) and 0.5 mg L^{-1} NAA plus 0.5 mg L^{-1} Kin exhibited the highest percentage (75 and 72, respectively) of somatic embryogenesis. Secondary metabolite content of the callus cells was significantly different among the plant growth regulator regimes and the highest production of picrocrocin and safranal were occurred on the medium containing 0.5 mg L^{-1} 2,4-D plus 0.5 mg L^{-1} BAP and 1 mg L^{-1} NAA plus 1 mg L^{-1} BAP.

Keywords: *Crocus sativus* L; Saffron secondary metabolites; Sonication; Tissue culture

1. Introduction

Saffron, *C. sativus* L. from the genus Crocus and Iridaceae family, is one of the most important medicinal plants and valuable products of Iran. Saffron is currently being cultivated in many countries mainly Iran, Morocco, India, Greece, Spain and Italy (Ghorbani 2008). In the past years, researchers showed that the crocetin, crocin, safranal and picrocrocin, the most valuable secondary metabolites in the saffron, have anticancer properties, mainly on papilloma, squamous cell carcinoma, leukemia and soft tissue sarcoma (Chryssanthi et al 2007). Various carotenoids and unique compounds called apocarotenoids found in Saffron. This apocarotenoids are produced via oxidative tailoring of carotenoids (Auldridge et al

2006). Apocarotenoids are synthesized in a number of plants such as Maize, Tomato, Arabidopsis, Crocus and etc., but Crocus species is the only plants which synthesize crocin, picrocrocin and safranal in significant quantities (Liao et al 1999). The saffron apocarotenoids are formed by zeaxanthin cleavage followed by specific glycosylation steps (Rubio et al 2008).

Plant cell and tissue culture techniques offer effective approaches for improvement and propagation of plants such as saffron (Castellar & Iborra 1997). Recent fields of application of plant cell culture are the large scale production of secondary metabolites and other molecules of pharmaceutical interest, such as heterologous proteins from cell suspension cultures (Wilson & Roberts 2012). Plant cell growth and production of secondary metabolites affected by many factors in vitro, e.g. plant growth regulators (PGRs), which are one of the most important agents influence cell growth, differentiation and secondary metabolites formation in plants (Liang et al 1991). On the other hands, optimization of medium components and culture conditions is the critical factor in the callus induction, cell suspension culture establishment and growth, and metabolite synthesis in the plant cells.

Low energy ultrasound (US) acts as a physical stress, which influence the biological, biochemical and developmental processes of the plant cells (Teixeira da Silva & Dobranszki 2014). Cavitation and acoustic micro streaming produced by low-frequency ultrasound cause the modifications in the cellular ultrastructure, cell membrane permeability, enzyme permanency and finally cell development progress (Ward et al 2000; Guzman et al 2001). Regarding the wide pharmaceutical properties of saffron secondary metabolites and low productivity of dried saffron pistils (approximately 6-10 kg h^{-1}), in vitro plant cell culture techniques provide an attractive and reliable alternative source for production of crocin and its derivatives. Therefore, the main objectives of this study were: (1) set up an efficient protocol for callus induction and somatic embryogenesis by applying the PGRs and ultrasound treatment, (2) assessment of main secondary metabolites production in saffron callus under different regimes of PGRs.

2. Material and Methods

2.1. Plant material and sterilization

C. sativus L. corms were prepared from the Ghaen, South Khorasan Province, Iran (33°43′N 59°11′E) in June 2014. Corms of 1-2 cm in diameter were used as explant. The external sheath of corms which microorganisms ubiquitously found on them, were removed. Corms were thoroughly washed under running tap water for 30 min and surface sterilized using the following procedure; Corms treated by ethanol (70% v v^{-1}) for 90 s, washed one times with sterile distilled water (SDW), then immersed in commercial sodium hypochlorite (5% w v^{-1}) plus one drop of Tween-20 per 100 mL for 15 min. Corms were then thoroughly rinsed 3-4 times with SDW and cultured on the MS (Murashige & Skoog 1962) medium supplemented with different combinations of PGRs. All the disinfection steps were carried out in aseptic conditions in a laminar air flow cabinet.

2.2. Callus induction

In order to preparation of leaf explants, corms were cultured on the Hoagland solution for 10 days and then, new and fresh developed leaves were surface sterilized, and were cut into small fragments (0.5-1 cm).

To prepare corm explants, the sterilized corms were cut into two or more (depending on their size) equal parts with meristematic tissues attached to them. The leaf and corm explants were cultured on agar-solidified (0.8% w v^{-1}, Merck, Darmstadt, Germany) MS media supplemented with different concentrations of plant growth regulators (0.5, 1, 2 and 4 mg L^{-1} of 2,4-D and NAA in combination with 0.5 and 1 mg L^{-1} of Kin and BAP) and sucrose (30 g L^{-1}).

For assessing the effects of ultrasonic waves on in vitro callogenesis of saffron, the sterilized explants were sonicated for 5 min at 25 ºC in a bath sonicator (Bandelin electronic®, Germany) at 35 kHz and then cultured on MS medium supplemented with

aforementioned PGRs. Sonication of the explants were repeated every two weeks for 1 min, so that the cultured jars dipped in bath sonicator. Unsonicated explants were used as control.

Cultures were maintained in a growth room at 25±1 ºC, 16 h photoperiod, 700-800 lux of cool-white light intensity and were subcultured at monthly intervals. The percentages of callus induction and embryogenic callus formation, and callus yield (fresh weight) were recorded after three months.

2.3. Extraction and determination of secondary metabolites

Secondary metabolites of saffron callus were extracted by methanol (Lage & Cantrell 2009). Briefly, approximately 600-800 mg of callus samples were extracted with 8 mL of methanol, sonicated for 1 h and then stored overnight at 4 °C. The next day, the samples were sonicated again for an hour, and brought to initial volume with methanol. Samples were passed through 0.22 µm filter (Albet labscience, 0.22 µm pore size) using a sintered glass funnel and under vacuum. In order to prevent degradation of saffron secondary metabolites, the whole process was carried out in darkness and at room temperature.

The main secondary metabolites of saffron callus were determined according to ISO 3632 trade standard (ISO/TS 3632-2, 2003) method at 440, 330 and 257 (nm) wavelengths corresponding to the maximum absorbance of the coloring strength (crocin), the aromatic strength (safranal) and the bitterness (picrocrocin), respectively. The absorbance readings obtained using a UV/VIS spectrophotometer (SmartSpec Plus spectrophotometer, Bio-Rad, Hercules, CA, USA) and quartz cell of 1 cm path-length. The extracts were diluted 4-6 times in order to minimize derivations in Beer-Lambert's law (Lakowicz 2006). Value of each compound was calculated (for crocin, safranal and picrocrocin) using Equation 1 and 2 (ISO/TS 3632-2 2003):

$$E_{1cm}^{1\%} = \frac{D \times 10000}{m(100 - H)} \quad (1)$$

$$H = \frac{Initial\ mass - Constant\ mass}{Initial\ mass} \times 100 \quad (2)$$

Where; D is the absorbance at 257 nm (for the picrocrocin), 330 nm (for the safranal) and 440 nm (for the crocin); m is the mass of the callus (g) and H is the moisture and volatile content of the sample, expressed as a mass fraction. For determination of moisture and volatile contents, samples were maintained uncovered in an oven (103 ºC) for 16 h. The moisture and volatile matter content are calculated as a percentage of the initial sample using the Equation (2) (ISO/TS 3632-2 2003). The reported values are the average values of three replicates.

2.4. Statistical analysis

All data were analysed by one-way ANOVA followed by Duncan's multiple range test or least significant difference (LSD) mean comparisons. The experimental design was a factorial based on CRD with three replicates and 8-9 explants per replicate. All analyses were performed using SPSS ver. 16 (SPSS Inc, Chicago, IL.) and SAS Ver. 9 (SAS Institute, Cary, NC, USA) softwares and the graphs were produced using Microsoft Office Excel 2010. All values were presented as mean ± SE (Standard Error) with significance at $P \leq 0.05$.

3. Results and Discussion

3.1. Callus induction and somatic embryogenesis

Callus induction and formation was not occurred from the leaf explants (Figure 1a). In contrast, callus initiation and formation was occurred from buds and cut edges of the corm explants within two months (Figure 1b). The nonembryogenic calli was characterized by its yellowish color (Figure 1c). Data analysis indicated that percentage of callus induction and callus yield (fresh weight) were significantly ($P \leq 0.01$) influenced by PGRs and ultrasound treatments. Frequency of calli initiation ranged from 25% to 100%, depending on plant growth regulator combination and ultrasound treatment (Figure 2 and 3). In the treatments without ultrasound, the highest percentage of callus induction (100%) and callus yield (4.3 g per explant) was achieved on MS medium supplemented with 2 mg L^{-1} NAA and 0.5 mg L^{-1} Kin, which was significantly higher than those

Figure 1- Callus induction and embryogenesis from *Crocus sativus* L. corm explants culture on MS medium supplemented with 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} Kin; a, leaf explants; b, corm explants; c, Nonembryogenic callus; d-f, somatic embryos at different stages of development (ME, matured embryo; GS, globular stage; HS, heart stage).

of other PGRs combination. While, the corm explants cultured on MS medium supplemented with 1 mg L^{-1} NAA plus 1 mg L^{-1} BAP or Kin and 4 mg L^{-1} 2,4-D plus 0.5 mg L^{-1} Kin exhibited lowest percentage of callus induction and callus yield in without sonication condition (Figures 2 and 3). In the study of Vahedi et al (2015) callus formation from saffron was successfully achieved by using a combination of 2.4-D and Kinetin hormones, which agreed with studies reported by Chaloushi et al (2007), Karamian & Ranjbar (2010) and Dalila et al (2013). They observed the highest frequency of callus induction (44%) on MS medium supplemented with 2.4-D (2 mg L^{-1}) and Kinetin (0.5 mg L^{-1}), culture from apical meristem. Darvishi et al (2006) also reported that a media containing NAA and BAP induced non-embryogenic calli; in contrast media containing 2.4-D and BAP had the best effect on induction of embryogenic calli in saffron.

As shown in Figures 2 and 3, stimulatory effects of ultrasound were different and varied in different PGRs treatments. Sonication of the explants were led to 2.0, 1.42 and 1.38-fold increase in callus induction on MS medium containing 1 mg L^{-1} NAA + 1 mg L^{-1} BAP, 1 mg L^{-1} NAA + 1 mg L^{-1} Kin and 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP, respectively. While, sonication of explants did not influenced the callus induction on MS medium supplemented with 2 mg L^{-1} NAA + 0.5 mg L^{-1} Kin, which was exhibited 100% callus induction in the absence of ultrasound treatment. In contrast, sonication of explants reduced the callus induction on MS medium supplemented with 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kin (Figure 2).

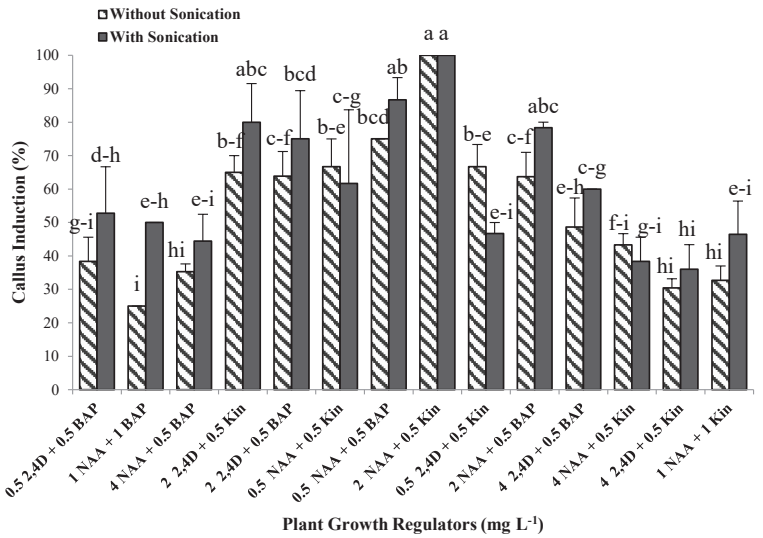

Figure 2- The effect of ultrasound on the callus induction from corm explants of *Crocus sativus* L. Each value represents the mean±SE of three replicates. Values with different letters are statistically different at P≤0.05

It has been shown in the past studies that ultrasound could be stimulate development in several plant species depending on its frequency and exposure time applied. Wang et al (1998) showed that growth rate of carrot (*Daucus carota* L.) cells in suspension culture was enhanced by about 5% while exposed to ultrasound (28 kHz) for 2 s, but exposure to US for 40 s reduced the growth rate by 57% relative to the control. The same results also were obtained in Liu et al (2003a) study. Liu et al (2003a) applied ultrasonic stimulation to rice (*Oryza sativa* L.) 'Nipponbare' cell culture with the same frequency (28 kHz). Results showed that cell growth was enhanced after exposure to 2 and 5 s. But cell growth and proliferation was inhibited when exposure time increased (30-120 s). In other study investigated by Wang et al (2004), Chrysanthemum callus sonicated with a frequency of 1.4 kHz twice daily for two 0.5 h periods. After 20 days, callus growth accelerated (unquantified) and differentiation of the shoots (28% higher shoot forming index than the control) stimulated. Low frequency ultrasound may vary the permeability of cell membranes (Rokhina et al 2009), subsequently may change the activity and the conformation of membrane-bound enzymes (Wang et al 2002; Liu et al 2003b) and thus may improve the transport processes in cells. Acoustic cavitation (the growth and collapse of pre-existing microbubbles under the influence of an ultrasonic field in liquids) is another consequence of ultrasound, which may influence plant metabolism (Rokhina et al 2009).

The growth regulators especially auxins and cytokinins play critical role in the control of plant cells division, growth and development, and a combination of auxins and cytokinins is mostly needed for reinitiate cell proliferation in tissue culture (Van Staden et al 2008). Plant species and genotypes show various in vitro growth responses to different types and concentrations of auxins and cytokinins (Guo et al 2009). As presented in Figure 3 (without sonication), although all combinations of PGRs combination successfully induced callogenesis from corms explants, however, various combinations of PGRs showed significantly different percentage of callus induction and callus yield. Generally, our findings suggested that combination of 2 mg L^{-1} NAA or 2,4-D with 0.5 mg L^{-1} Kin or BAP were more favorable for callus induction and growth. Castellar & Iborra (1997) reported that MS medium supplemented with 10 mg L^{-1} NAA and 5 mg L^{-1} BA was more favorable for callus induction

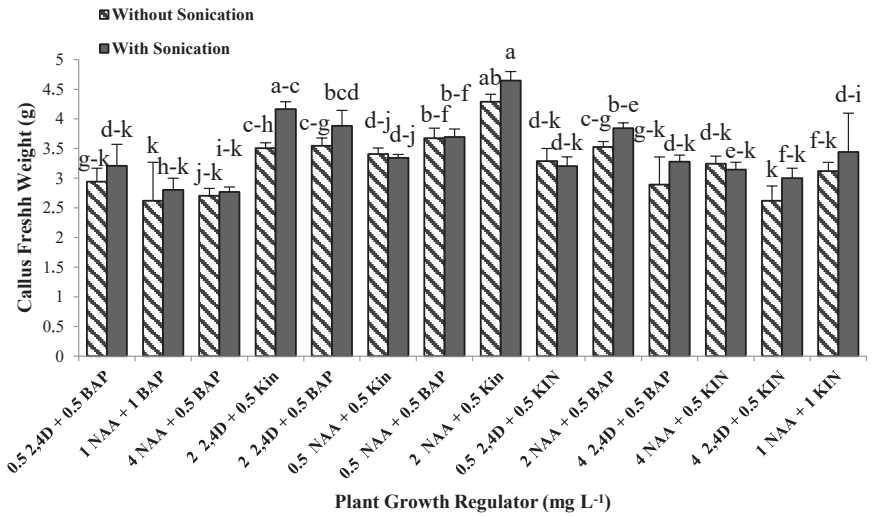

Figure 3- The effect of ultrasound on the callus fresh weight from corm explants of *Crocus sativus* L. Each value represents the mean±SE of three replicates. Values with different letters are statistically different at P≤0.05

and undifferentiated growth, while the MS medium containing 1 mg L^{-1} BAP and 1 mg L^{-1} NAA was the most appropriate for stigma differentiation. But, our results indicated that increasing NAA or 2,4-D levels to relatively high concentration (4 mg L^{-1}) led to reduced callus induction and growth. Recently, Verma et al (2016) reported the friable callus initiation in five Turkish *Crocus* species (*Crocus specious* ssp. *Specious*, *Crocus oliveri* ssp. *Oliveri*, *Crocus pestalozzae*, *Crocus abantensis*, *Crocus paschei*) within 20 days on the medium supplemented with 4 mg L^{-1} TDZ+4 mg L^{-1} NAA and 5% (w v^{-1}) sucrose. These inconsistencies could be explained by differences in the genotype of plant materials used in various studies, which can strongly affect the in vitro response of plant cell to PGRs (Venkatachalam & Jiayabalan 1997).

Our results demonstrated the sonication of corm explants stimulate the percentage of callus induction and callus yield of *C. sativus* L. even up to two-fold of without ultrasound treatments. But, the stimulus effects of ultrasound were varied depending on PGRs combination (Figures 2 and 3). The effectiveness of sonication depends upon the intensity, ultrasound frequency and exposure period (Rokhina et al 2009). The low-energy ultrasound (US), as a physical stimulus initiate a range of biochemical processes in the plant cells. These effects mainly rise from mechanical and cavitation properties of ultrasound irradiation, which significantly stimulate protein synthesis, cellular metabolic activity of enzymes in plant cells and protoplasts. Additionally, ultrasound cause transient pore formation on the cell wall and membrane and in enhanced plasma-membrane permeability that facilitate mass transfer and uptake of molecules from the medium and release of intracellular products by the cells (Dong et al 2002). The ultrasound treatment at 300 W for 5 min improved the conversion frequencies of protocorm like bodies (PLBs) to shoot in the *Dendrobium officinale* (Wei et al 2012). In Zare et al (2014) study, the combination of ultrasound (10 s) and L-tyrosine feeding (2 mM) significantly increased the production of thebaine in comparison to individual utilisation of 2 mM L-tyrosine and ultrasound (10 s).

Analysis of somatic embryogenesis (Figures 1d-f) data revealed significant differences ($P \leq 0.01$) between different PGRs. As shown in Figure 4, in the MS medium supplemented with 0.5 mg L^{-1} 2,4-D plus 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA plus 0.5 mg L^{-1} Kin, the most of explants and calli (75% and 72.22%, respectively) were produced somatic embryos, which was significantly higher than those of other treatments. Contrariwise, the explants cultured on medium containing higher level of auxin (4 mg L^{-1} 2,4-D or NAA) exhibited the lower somatic embryogenesis response compared to others. According to the past studies, decreasing concentration or complete removal of exogenous auxins has been reported to be essential for embryo maturation and further development (Sharifi et al 2012). Selection of embryogenic regions and transferring them into growth regulator free media caused to notable growth of embryogenic calli. It was recommended that division of the pre-embryogenic cells and their development into embryos is only resumed at lower auxin concentrations (Karamian & Ebrahimzadeh 2001). These findings are in line with our results that the highest somatic embryos were obtained in low concentrations of auxins and contrariwise the lowest percentage of somatic embryogenesis (8.33%) was obtained on MS medium supplemented with high level of auxin (4 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kin) (Figure 4). Somatic embryogenesis provides an effective approach for large-scale plant micropropagation, production of transgenic plants, artificial seed production and cryopreservation (Kita et al 2007). As shown in Figure 4, the effect of type of auxins (NAA or 2,4-D) and cytokinins (BAP or Kinetin) on somatic embryogenesis (%) were not significant, but concentration of both auxins and cytokinins meaningfully influenced the somatic embryogenesis of saffron corm explants. So that the efficiency of low and moderate concentrations of auxins (NAA or 2,4-D) in combination with BAP or Kin were significantly high and the maximum values of somatic embryogenesis belonged to the MS medium supplemented with 0.5 mg L^{-1} NAA or 2,4-D plus 0.5 mg L^{-1} BAP or Kin. It has been shown that expression of somatic embryogenesis influenced by different factors including plant species and

cultivar, medium composition (especially by types and concentrations of PGRs), environmental and physiological conditions of the donor plant (Bajaj 2013). Karamian (2005) were obtained the best embryogenesis rates in the presence of 4 mg L^{-1} kinetin and 1 mg L^{-1} 2,4-D, as approximately 30% of calli formed on this medium produced somatic embryos.

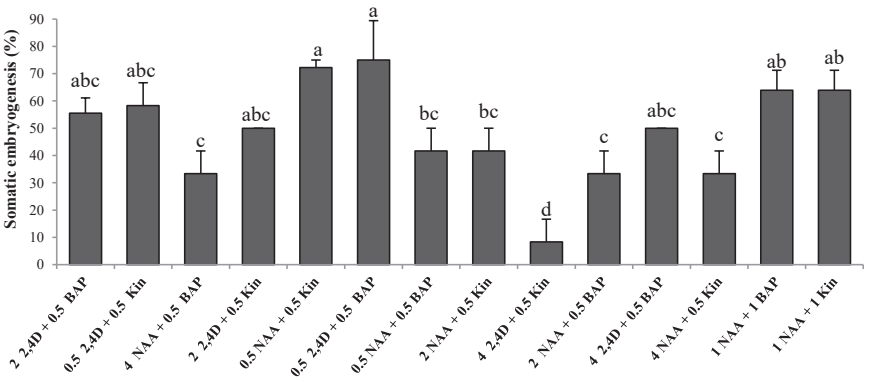

Figure 4- Effect of various concentrations of PGRs on percentage of *Crocus sativus* L. explants showing somatic embryogenesis response. Each value represents the mean±SE of three replicates; values with different letters are statistically different at P≤0.05

3.2. Secondary metabolites

The effects of different concentration of auxins (2,4-D and NAA) and cytokinins (BAP and Kin) on picrocrocin, safranal and crocin production were shown in the Figure 5. As shown in the Figures 2 and 3, although the combination of NAA (2 mg L^{-1}) and Kin or BAP (0.5 mg L^{-1}) provided optimal condition for callus growth and proliferation, the highest picrocrocin production and accumulation was obtained from the calli grown on MS medium supplemented with 0.5 mg L^{-1} 2,4-D plus 0.5 mg L^{-1} BAP. Safranal content of the calli grown on MS medium supplemented with 1 mg L^{-1} NAA and 1 mg L^{-1} BAP or 1 mg L^{-1} Kin were significantly higher than those of others. As shown in Figure 5, picrocrocin and safranal production and accumulation in the saffron calli decreased significantly with increasing the concentration of NAA from 1 mg L^{-1} to 2 and 4 mg L^{-1}. The lowest content of the crocin was obtained in the MS medium supplemented with 2 mg L^{-1} NAA and 0.5 mg L^{-1} BAP. It has been shown that production of secondary metabolites in the plant cells influenced by many environmental factors (such as temperature, humidity, CO_2, photoperiod and light intensity) and medium composition (mainly by PGRs, type and concentration of carbohydrate and nitrogen source) (Akula & Ravishankar 2011). In recent years, various studies have been conducted to establish practical approaches to enhance the yield of secondary metabolites under in vitro conditions. In contrast, there are very few reports on the effects of culture conditions and PGRs on the secondary metabolites (including crocin, picrocrocin and safranal) production on the saffron callus cultures. In the present study, the experimental data supported the speculation that the amount of safranal, crocin and picrocrocin in the saffron calli are dependent on type and concentration of PGRs. So that, our data revealed that the low or moderate levels of PGRs are optimal for the production and accumulation of secondary metabolite in the saffron callus (Figure 5). In Liu et al (2002) studies, best results for promotion of saffron callus growth and crocin biosynthesis achieved by NAA and IAA application, respectively. Their findings showed that IAA (4 mg L^{-1}), GA_3 (2 mg L^{-1}) and uniconazole (S-07) (1.25

mg L^{-1}) remarkably enhanced the crocin content in calli. They also found that NAA (2 mg L^{-1}) promoted the growth of saffron callus but had no advantage and may inhibit crocin synthesis whereas uniconazole (1.25 mg L^{-1}) had the opposite effect and the growth of calli with S-07 was significantly inhibited. In contrast, GA_3 promoted both growth and synthesis. Plant growth regulator plays important role in secondary metabolites accumulation in a plant cell and tissue culture studies (15). It has been that auxin and cytokinin individually or in combination significantly could alter both the growth and secondary metabolite accumulation in cells cultures (Dicosmo & Towers 1984). Sahai & Shuler (1984) showed that NAA or IAA enhanced the production of nicotine in suspension culture of *N. tabacum.* Similarly results obtained in Coste et al (2011) study in which investigate the effects of plant growth regulators (BA, Kin, 2iP, TDZ and NAA) and two elicitors (jasmonic acid and salicylic acid) on accumulation of hypericins (hypericin and pseudohypericin) and hyperforin in shoot cultures of *Hypericum hirsutum* and *H. maculatum*. Their findings revealed that culture of shoots on MS medium supplemented with BA (0.4 mg L^{-1}) or Kin (0.4 mg L^{-1}) enhanced production of hypericins and hyperforin in *H. maculatum* and *H. hirsutum*, respectively. Carmona et al (2007) identified the flavonoid fraction in saffron spice using of LC-DAD/MS/MS ESI. They also analysed is there if differences in the flavonoid contents of samples from different geographical origins that could be used as biomarkers for the determination of saffron origin? They found five kaempferol derivatives in which kaempferol 3-sophoroside contents of the saffron samples could clearly separate different geographical origins.

4. Conclusions

In conclusion, present study showed that ultrasound together with PGRs extremely affected the saffron callus and cell growth in solidified MS. Based on the results, it seems that high concentration of auxins inhibit both cell growth and secondary metabolite production in saffron calli. The results suggested that maximum callus growth and valuable secondary metabolites production in the saffron in vitro cultures could be achieved in the moderate concentrations of PGRs.

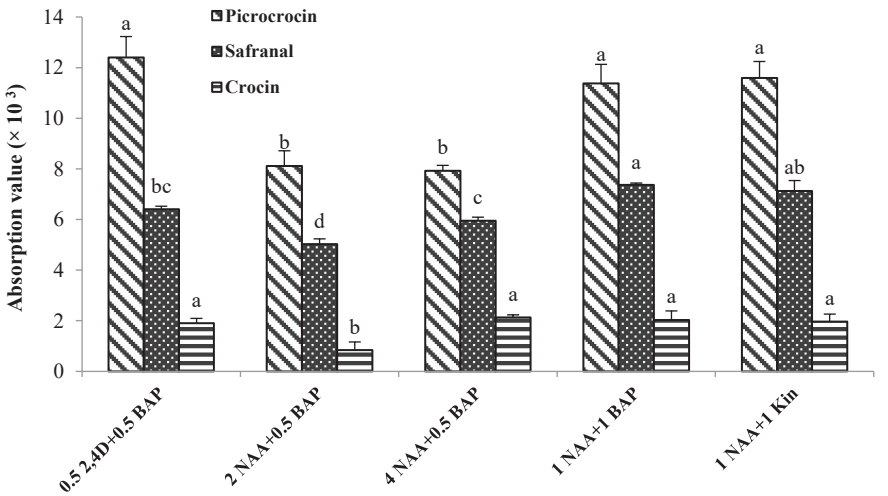

Figure 5- Comparison of $E^{1\%}_{1cm}$ values for picrocrocin, safranal and crocin under different treatment extracted by ISO/TS 3632-2 procedure in *Crocus sativus* L. Each value represents the mean±SE of three replicates. Values with different letters are statistically different at P≤0.05

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Introducing Different Cherry Cultivars to Inner and Crossover Areas

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ABSTRACT

This study was carried out to evaluate quality, yield and phenology of some sweet cherry cultivars with different maturation periods in Isparta-Egirdir conditions. The study was carried out between 2000 and 2011, on Veysel, Précoce Bernard, Star, Venus, Mechlain Haimer, Summit, Techlovan, Fercer Arcina, Sylvia, Noire de Meched, Oktavia, Belge, 0900 Ziraat, Kordia, and Ferbolus. Venus, Bernard, Techlovan and Star cultivars were identified as the earliest blooming. Oktavia, Belge, Kordia and 0900 Ziraat cultivars were found to be the latest blooming cultivars. Generally; Veysel, Bernard and Star come to harvest maturity first. Venus, Mechlain Haimer, Summit, Techlovan, Fercer Arcina, Sylvia were found to be middle season cultivars, while N. De Meched, Oktavia, Belge, 0900 Ziraat, Kordia and Ferbolus were found to be the latest. Veysel early cherry cultivar were determined to be the most productive one, while late cultivar N. De Meched and mid-season cultivar Techlovan were also found to be very efficient. In terms of fruit sizes; early sweet cherry cultivar P. Bernard, mid-season cultivar Summit and late season cultivar 0900 Ziraat gave the biggest fruits.

Keywords: Sweet cherry; *Prunus avium*; Fruit quality; Yield; Phenology

1. Introduction

Sweet cherry is among the most consumed fruits in the world. It has its own attractiveness, taste, aroma, flavor, and size. Besides, it is tastefully and easily eaten by everyone. For these reasons, it is a fruit the consumer insists on and readily consumes in both domestic and foreign markets. Therefore, it is one of the luxurious fruits that can find buyers at high prices in the market. For sweet cherry, demand is higher than supply almost every year. Consumers volunteer to pay higher prices for sweet cherry fruit (O'Rourke 2007). Despite all this, climate is the most important factor limiting sweet cherry production (Webster & Loney 1996). All of these make cherry growing more advantageous than many other fruit species.

Sweet cherry production of the world is 2,294,455 tons. The most important sweet cherry producer countries are Turkey (494,325 tons), USA (301,225 tons), Iran (200,000 tons), Italy (131,175 tons) and Uzbekistan (100,000 tons) (FAO 2015). Turkey, which is the leading country

in the production of sweet cherries, exports about 10% of its production every year. The plans for the future are focused on increasing sweet cherry export. Ecological suitability affects the quality of production positively and provides a competitive advantage.

Sweet cherry has a wide range of consumption. However, as in the whole world, supply in Turkey is not sufficient even for fresh consumption, so the amount left for processing is very low and production is generally directed towards fresh consumption. Sweet cherries are grown in almost every region. However, commercial production is in Izmir, Afyon, Denizli, Manisa, Isparta, Konya, Bursa, Canakkale, Amasya. These provinces make up about 57.7% of the production (TUIK 2012). Sweet cherry is more attractive to the market when the fruit cultivar is less. For this reason domestic and foreign market buyers are able to pay high premium prices for quality sweet cherries. Highly priced sweet cherries make production attractive. As a result, sweet cherry production in Turkey expands and goes beyond the production areas mentioned above day by day without any basic study. Ekinci et al (2007) reported that positive developments experienced especially in recent years led to a rapid increase in the production of sweet cherries, 0900 Ziraat, among other cultivars exported, stood out with its superior quality characteristics, but the quantity of quality products was not sufficient in spite of the high production potential. Ozturk et al (2010) pointed out that the most important problem of the industry is the inadequacy of raw material supply and quality since the first day of Turkey's sweet cherry export. Similarly, Webster & Looney (1996) and O'Rourke (2007) reported that the world is inadequate in terms of raw material supply in the sweet cherry industry and that future supply and quality of raw materials in the global sweet cherry industry will be important competition criteria. Although Turkey is one of the germplasm, the production of sweet cherry cultivars with high resistant transportation, which can meet the demands of foreign market other than 0900 Ziraat cultivar, has not been widespread. The choice of alternative cultivars for sweet cherry production in a region is determined by the effect of regional conditions. The productivity and quality of cultivars can vary from region to region. Climatic conditions particularly affect blooming and ripening season cultivars. In addition to its superior quality features, it also requires to focus on alternative cultivars, such as irregular production, inefficiency and other negative features, prolonging the supply season and diversifying the product.

For this purpose, it is very important to determine the morphological, phenological and pomological characteristics of domestic and foreign sweet cherry cultivars in different regions, to determine the cultivars that have high yield and quality, are in good agreement with the domestic and foreign market demands, and are well adapted to the regional conditions and other basic studies. In this study, adaptation ability of some important sweet cherry cultivars in Isparta-Egirdir conditions was evaluated; sweet cherry cultivars having different maturity periods and economical value were determined.

2. Material and Methods

2.1. Orchard layout and plant materials

The experiment was carried out in the field of Egirdir Fruit Research Institute (37° 49'12.95"N; 30° 52'13.73"D; 921 m altitude) in the years 2000-2011.

Sweet cherry cultivars grafted onto Mazzard (*P. avium* L.) seedling rootstock were planted spaced 6x5 m, in soil conditions characterized by loamy, calcareous (12% total lime), alkaline (pH 8.34). Trees were trained to a central leader and pruned in late winter and standard cultural practices. The orchard were irrigated with drip irrigation, fertilization applications were made with fertigation.

In the study Veysel, Précoce Bernard (S_3S_9), Star, Venus, Mechlain Haimer, Summit, Techlovan (S_1S_3), Fercer Arcina (S_2S_6), Sylvia (S_1S_4), Noire de Meched, Oktavia, Belge (S_3S_4), 0900 Ziraat, Kordia, Ferbolus sweet cherry cultivars were used.

2.2. Determination of phenological stages of cultivars

The phenological observations and yields in this study include data between 2006 and 2011 following juvenility. Phenological observations of the cultivars were made following the period of juvenility. Phenological observation dates were recorded as bud burst, first bloom, full bloom, bloom end and harvest date. Bud burst and petal fall dates were noted according to Chapman & Catlin (1976). The time of 70% and 5% of blooms opened was noted as respectively full and first bloom dates. Fadón et al (2015) characterized the phenology of some sweet cherry varieties and adapted to 97 numerical BBCH codes, and framed flower development within the growth stages. According to researchers, phenological stages (BBCH scale) were defined as follows: bud burst-stage 53; first bloom-stage 61; full bloom-stage 65; and petal fall-stage 69. Harvest time, however, was recorded as the date when the cultivars reached harvest maturity according to stage 87 reported by Fadón et al (2015). Harvest times were grouped as early, mid-season and late.

2.3. Determination the yields of the cultivars

The yield of the cultivars was taken as yield (kg) per tree per year following juvenility. Average yield per tree, cumulative yield and cumulative yields per unit area (kg ha^{-1}) were calculated. In the last year of the experiment, the cumulative yield of the trunk section area was determined (kg cm^{-2}).

2.4. Determination of some fruit characteristics of cultivars

In the last two years of the experiment (2010-2011), pomological analyzes of the cultivars were carried out and the differences among the cultivars were determined.

Pomological analyzes were performed with 20 fruits sampled each time. Fruit weight (g), width (mm), length (mm), stone weight (g), stalk length (mm) and stalk weight (g) were measured using standard methods. Fruits were measured by soluble solid content (SSC, %-refractometer), total acidity (TA, titrated with 0.1N NaOH; %), fruit juice pH, fruit firmness (with hand penetrometer using 4.5 mm tip).

2.5. Statistical analysis

The experimental design was a randomized blocks, 5 replicates using a single tree. Statistical analyses were performed using the JMP statistical software package (vers 8; SAS Inst. Inc., Cary, NC, USA). Mean separation was performed using LSD's multiple range test at $P<0.01$ level.

3. Results and Discussion

3.1. Determination of phenological stages of cultivars

When the first blooming dates were compared, it was determined that the cultivars of Venus, Bernard, Techlovan and Star were the earliest blooming. Overall, it was observed that Oktavia, Belge, Kordia and 0900 Ziraat cultivars were in the late-blooming group. It can be said that cherry cultivars generally bloomed between 3rd week and 4th week of April in Egirdir ecology in terms of full blooming dates. The blooming end dates of cultivars were generally between the end of April and the beginning of May. A distribution parallel to the first blooming and full blooming dates was observed among the cultivars (Figure 1).

The blooming time varies depending on the cultivar and climate. In this respect, determination of the time and duration of blooming of the cultivars depending on the region and cultivar is a very important issue for the production of sweet cherries, which is a problem of incompatible which is especially required to use a pollinator cultivar. The cultivars in our study are able to pollinate each other. Because the blooming duration of the cultivars coincides with each other in groups. However, an early blooming cultivar cannot be recommended as pollinator for a late blooming cultivar. In our study, Oktavia (S_1S_3), Summit (S_1S_2) and Kordia (S_3S_6) cultivars can be used as pollinator to each other since the blooming time with 0900 Ziraat cultivar is at the same time with each other. As a matter of fact, Sarısu et al (2016a)

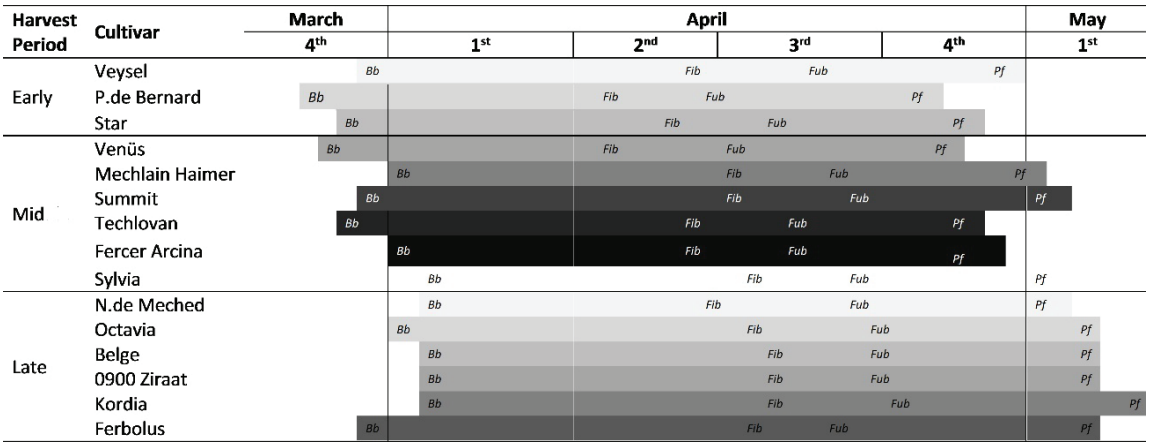


Figure 1- Blooming period of sweet cherry cultivars in average six years. *Bb*, bud burst; *Fib*, first bloom; *Fub*, full bloom; *Pf*, petal fall

reported that Kordia (23.28%) and Summit (22.28%) and Oktavia (12.59%) gave very good results in the 0900 Ziraat as pollinator and found that the pollinating capacities of these cultivars were generally high. However, since N. de Meched and 0900 Ziraat, which are good efficiency in the study, are in the same incompatible group (22nd incompatible group-S_3S_{12}) (Schuster 2012) they cannot pollinate each other. Similarly, although the blooming times of the Venus and Star cultivars coincide, they cannot be pollinate with each other because they are in the second group (S_1S_3) incompatible (Schuster 2012) group. For this reason, as many researchers have noted (Choi & Andersen 2001; Wünsch & Hormoza 2004; Beyhan & Karakaş 2009; Ipek et al 2011; Schuster 2012), cultivars should be preferred, in which both blooming times and incompatible groups are appropriate when setting up a sweet cherry orchard.

In the study, the harvesting times of sweet cherry cultivars are shown in Figure 2, and the blooming and handling times (visual) are given in Figure 3. It was seen that the cultivars in the experiment generally provided a harvest advantage from the beginning of June until the beginning of July in Egirdir ecology. Generally, the cultivars of Veysel, Bernard and Star come to the first harvest maturity. As mid-season cultivars, Venus, Mechlain Haimer, Summit, Techlovan, Fercer Arcina, Sylvia, the latest cultivars of the harvest maturity are N. de Meched, Oktavia, Belge, 0900 Ziraat, Kordia, Ferbolus. When the harvest dates are considered; for Egirdir ecology, the cultivars in the experiment did not maintain continuity in the 2nd week of harvest during the 4 week harvest period. During the first, third and fourth weeks, the harvesting period could be closed with different cultivars (Figure 2). It is very important that the sweet cherry fruits maturity on the tree almost at the same time and therefore the harvesting process should be completed in a short time without losing much time. In addition, the sweet cherry harvest is very difficult and requires lots of labor. For these reasons, if the sweet cherry orchard has to be established in large areas, it is economically advantageous for growers that the harvesting times of the appropriate cultivars are different from each other. For example, in our study, establishing sweet cherry orchard in large areas with two types of combination Mechlain Haimer and Summit, Techlovan and Fercer Arcina, 0900 Ziraat and Kordia will not be economically viable for this region. The different harvesting times of the cultivars will contribute to the diversification of cultivars in the production regions.

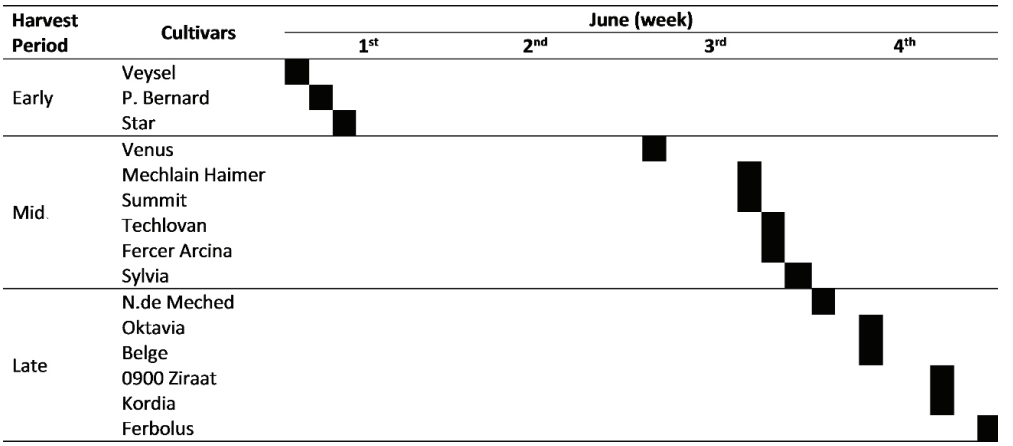


Figure 2- Harvest period of sweet cherry cultivars in average six years

3.2. Determination of yields of cultivars

The yields per tree and cumulative yields of cultivars following 6 years juvenility are given Table 1.

As a result of the analysis of variance in terms of cumulative yield, the differences between the cultivars were found significant (P<0.01). It was determined that the most productive cultivars of the earliest cultivars of Veysel among all cultivars (147.86 kg^{-1}) and P. Bernard cultivar, which is also an early cultivars, has the lowest yield cultivar (35.96 kg^{-1}). N. de Meched, which is a latest cultivar, and Techlovan

Table 1- Yield (kg $tree^{-1}$) and cumulative yield of sweet cheery (2006-2011)

Cultivars	*Yield (kg $tree^{-1}$)*						
	2006	*2007*	*2008*	*2009*	*2010*	*2011*	*Cumulative*
Veysel	1.54	4.20	5.22	26.15	63.95	46.80	147.86a**
P.de Bernard	0.58	1.46	5.53	9.28	10.94	8.17	35.96de
Star	0.80	2.12	5.41	10.40	18.48	38.96	76.17cd
Venüs	1.11	5.10	0.50	6.20	16.00	7.20	36.11de
Mechlain Haimer	1.60	6.32	2.33	14.18	54.23	53.33	131.99ab
Summit		2.03	0.64	7.60	9.50	8.76	28.53de
Techlovan	6.53	13.97	3.28	18.00	39.27	52.67	147.64a
Fercer Arcina	0.78	1.08	0.50	5.00	3.00	9.25	19.61e
Sylvia		2.35	3.00	14.40	25.70	19.53	64.98c-e
N. de Meched	1.64	10.34	8.39	27.12	44.80	55.00	147.29a
Oktavia	0.84	3.78	6.13	21.92	25.06	35.75	93.48bc
Belge	0.70	4.88	5.13	14.25	20.02	19.05	64.03c-e
0900 Ziraat	1.48	11.55	8.99	15.50	26.00	16.20	79.72b-d
Kordia	3.63	3.40	2.49	19.81	17.50	20.35	67.18c-e
Ferbolus	1.20	2.58	8.85	27.81	41.80	33.60	115.835a-c

**, means within cultivars with the same letter are not significantly different by LSD's Multiple Range Test at P<0.01

Figure 3- Phenological periods of sweet cherry

cultivar, which are mid-season cultivars, appear to be second and third among all cultivars in efficiency (Table 1). The middle season cultivar Fercer Arcina was identified as the least efficient cultivar after P. Bernard cultivar. In the late season cultivars, 0900 cultivars were the lowest yield (79.72 kg^{-1}). In our study, we used 0900 Ziraat and Kordia, besides some other foreign cultivars which not much information have been given about the productivity of them. For this reason, our findings show originality in terms of yield efficiencies. Our findings are similar to some other studies for Kordia and 0900 Ziraat cultivars (Lichev et al 2004; Stehr 2008; Grzyb & Rozpara 2009; Sarısu et al 2016b).

The yield per cross section area ($P<0.01$) and trunk cross sections ($P<0.05$) of the experimented sweet cherry cultivars were found to be statistically significant (Table 2). The largest cross-sectional area was observed in Ferbolus (567.02 cm^2) while Kordia cultivar (316.28 cm^2) had lowest cross-sectional area. While the highest

Table 2- Yield efficiencies of sweet cherry cultivars (2016)

Cultivars	*Trunk section area (cm^2)*	*Yield per unit cross-sectional area ($kg\ cm^{-2}$)*	*Cumulative yield per unit area ($t\ ha^{-1}$)*
Veysel	378.78b-e*	0.40ab**	48.79a**
Précoce Bernard	533.30a-c	0.07fg	11.87e-g
Star	361.76c-e	0.21c-e	25.13c-f
Venus	466.52a-e	0.10e-g	11.92e-g
Mechlain Haimer	542.73ab	0.26cd	43.56ab
Summit	390.41b-e	0.08fg	9.42fg
Techlovan	358.99de	0.47a	48.72a
Fercer Arcina	471.14a-e	0.04g	6.47g
Sylvia	459.99a-e	0.25cd	38.64a-c
Noire de Meched	450.12a-e	0.30bc	48.61a
Oktavia	514.59a-d	0.18d-f	30.85b-d
Belge	441.90a-e	0.18d-f	21.13d-g
0900 Ziraat	335.49e	0.25cd	26.31c-e
Kordia	316.28e	0.22c-e	22.17d-g
Ferbolus	567.02a	0.21c-e	38.23a-c

*, means within cultivars with the same letter are not significantly different by LSD's Multiple Range Test at $P<0.05$; **, means within cultivars with the same letter are not significantly different by LSD's Multiple Range Test at $P<0.01$

the unit area cumulative yield are 48.79 t ha^{-1} with Veysel and 48.72 t ha^{-1} with Techlovan, these cultivar were included in the same group in the analysis of variance. The cumulative yield per unit cross-sectional area was at most Techlovan (0.47 kg cm^2) then Veysel (0.40 kg cm^2) and in the statistical analysis, the Techlovan cultivar is different from the Veysel cultivar. The Fercer Arcina has the lowest both in unit area cumulative yield and cumulative yield per unit cross-sectional area (Table 2).

3.3. Determination of some fruit characteristics of cultivars

The most important criteria that are mentioned as fruit quality factors in cherries are; fruit weight, fruit shape, fruit firmness, stalks remain green for a long time, taste, fruit color, SSC and TA (Kader 1983; Younce & Davis 1985; Drake & Fellman 1987; Fischer et al 1996). In this respect, when we studied some fruit quality properties of fruits, Sweet cherry fruit size were found to be statistically significantly at the sweet cherry cultivars at different harvesting periods ($P<0.05$) (Figure 4). Among the early cherry cultivars in terms of fruit sizes, P. Bernard cultivar showed the best results. P. Bernard was the largest cultivar in the early with fruit weight (7.39 g) and fruit diameter (24.67 mm). Fruit size increased in mid and late season cultivars. Among the mid-season cultivars, the largest fruit was obtained Summit (11.21 g) cultivar. Also, Fercer Arcina and Sylvia of the same period cultivars were large enough to be ignored with 9.34 g and 9.25 g fruit weights respectively.

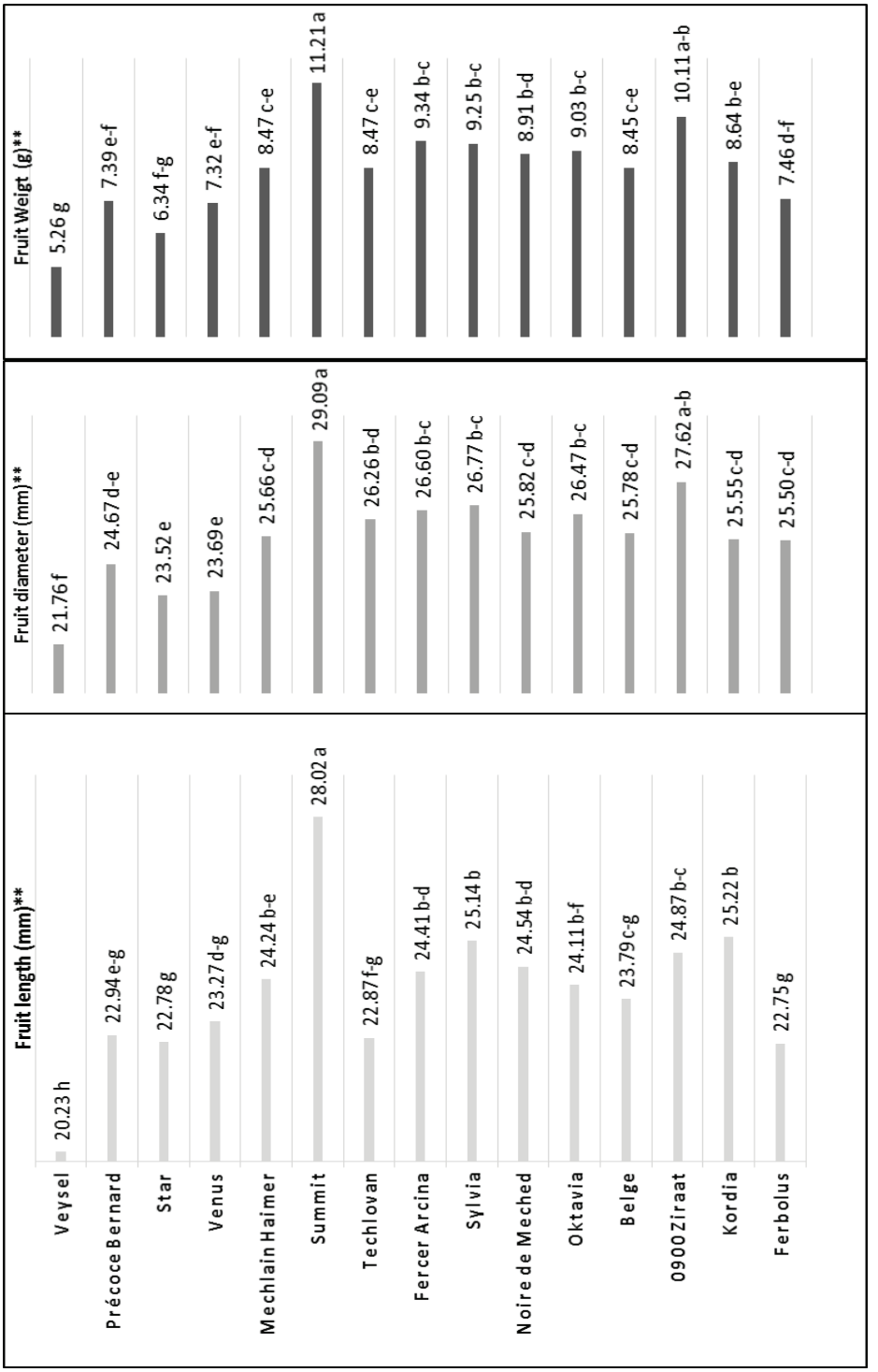
The most important export cultivar of Turkey among the late period cultivars was found to be 0900 Ziraat which gave statistically significant fruit weight (10.11 g) ($P<0.01$). Oktavia (9.03 g) and Belge (8.91 g) were also found to be important cultivars in terms of size (Figure 4). Kappel et al (1996) giving the model of 'ideal' of sweet cherry cultivar, state that it should have the weight of fruit 11-12 g. Crisosto et al (2003) stated that the size of the fruit determines the crop, quality and acceptance of the cultivar by the customers. In our study also, ideal weight cultivars were Summit and 0900 Ziraat.

Sweet cherry cultivars stone, fruit stalk and some fruit chemical characteristic were given Table 3. Stone weights were found statistically significant at sweet cherries ($P<0.05$). The bigger stone weight

Table 3- Fruit stone, fruit stalk and some chemical characteristic of sweet cherry cultivars

Harvest period	*Cultivars*	*Stone*	*Fruit stalk*		*Some chemical characteristic*		
		Weight (g)	*Weight (g)*	*Length (mm)*	*pH*	*SSC (%)*	*TA (%)*
Early	Veysel	0.273d**	0.107e*	45.32ef**	3.77	14.17	0.71e-g**
	Précoce Bernard	0.447b	0.135a-e	48.72b-e	3.82	12.77	0.55g
	Star	0.547a	0.126c-e	41.06f	3.71	12.52	0.58g
Mid	Venus	0.349cd	0.117de	32.84g	3.75	14.72	0.70e-g
	Mechlain Haimer	0.463ab	0.149a-d	52.67a-d	3.52	13.59	0.93a-c
	Summit	0.454b	0.140a-e	41.32f	3.94	14.03	0.85b-f
	Techlovan	0.387bc	0.115de	32.19g	3.79	14.63	1.04a
	Fercer Arcina	0.453b	0.161a-c	45.64d-f	3.61	14.95	0.89a-d
	Sylvia	0.443b	0.145a-e	46.92c-f	3.79	15.18	0.81c-f
Late	Noire de Meched	0.456b	0.168ab	55.91a	4.01	14.34	0.72d-g
	Oktavia	0.417bc	0.152a-d	54.34ab	3.96	13.04	0.81c-f
	Belge	0.414bc	0.129b-e	52.29a-e	3.96	13.69	0.83b-f
	0900 Ziraat	0.466ab	0.174a	55.94a	4.04	15.47	0.69f-g
	Kordia	0.417bc	0.163a-c	53.74a-c	3.95	14.40	0.87a-e
	Ferbolus	0.434bc	0.135a-e	47.22b-f	3.87	15.50	0.99ab

*, means within cultivars with the same letter are not significantly different by LSD's Multiple Range Test at $P<0.05$; **, means within cultivars with the same letter are not significantly different by LSD's Multiple Range Test at $P<0.01$



**, means within column with the same letter are not significantly different by LSD's Multiple Range Test at P<0.01

Figure 4- Fruit size of sweet cherry cultivars

was determined Star (0.547 g), 0900 Ziraat (0.466 g) and Meclain Haimer (0.463 g) cultivars. It was determined to have the smallest stone (0.273 g) parallel to the fruit size of the Veysel cultivar. Fruit stalk length is an important characteristic for post-harvest strength and ease of harvest. Longer stalk is better than shorter one because of easier picking and lesser tendency to decay and cracking of the fruit (Stojanovic et al 2012). According to Schick & Toivonen (2000) short and green stalk reminds buyers on freshness and juiciness of the fruit. In our study, fruit stalk length ($P<0.01$) and fruit stalk weight ($P<0.05$) were found statistically significant among the sweet cherry cultivars. Mechlain Haimer, Noir de Meched, Oktavia, Document, 0900 Ziraat and Kordia cultivars were statistically analyzed by variance analysis ($P<0.01$). And these cultivars are 5 cm long and have the longest stalk cultivars with fruit stalk. Venus (32.84 mm) and Techlovan (32.19 mm) are the shortest stalk cultivars.

Although there was no statistical difference between pH and SSC values among the cultivars in the study, TA was found to be statistically significant ($P<0.01$) (Table 3). Meclain Haimer, Techlovan, Fercer Arcina, Kordia and Ferbolus cultivars have high SSC. In general, early cultivars were found to have lower SSC. The original fruit colors and SSC were taken into consideration when the harvest time of the cultivars was decided. The SSC of the cultivars generally ranged between 12-16% (Table 3). Main factor of fruit quality, is the content of soluble solids (Crisosto et al 2003). It depends on many factors, and mostly on the cultivar (Gonçalves et al 2006), rootstock (Usenik et al 2010) and stages of fruit ripeness (Drake & Elfving 2002). According to Kappel et al (1996) the 'ideal' of sweet cherry cultivars would be the one having the content of soluble solids between 17% and 19%. The differences between our results and results of other authors can be explained by the influence of different rootstock, soil and climate conditions, cultural practices, and stage of maturity (Drake & Elfving, 2002; Crisosto et al 2003).

4. Conclusions

In conclusion, in Isparta-Egirdir ecology, as a result of this study carried out with different cherry cultivars, Mechlain Haimer, Techloven and Sylvia midseason cultivars, Oktavia, N. De Meched, Kordia, 0900 and Belge late season cultivars were found to be advisable. The Early Veysel cultivar is not recommended for this region because of its low fruit quality, although it gave the highest yield.

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An Approach to Color Change and Quality Relation in Roughages

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ABSTRACT

In this work, it was aimed to associate CIELAB Scale (L*, a*, b*) with some quality parameters of roughages such as pH, crude protein (CP) and relative feed value (RFV). To this end, five different applications; haylages unwrapped on 25th, 40th and 55th days of fermentation, traditional silage unwrapped 55th days of fermentation and degraded haylage were examined for vetch-triticale (*Vicia sativa* L. - *Triticasecale wittmack*) and caramba-berseem clover (*Lolium multiflorum* cv Caramba-*Trifolium alexandrinum* L.) mixtures in the study. The tests were conducted according to randomized block design with three replications for both forage mixtures. The ΔL and Δb color values and pH, CP, RFV were significantly correlated in the vetch-triticale mixture. The regression coefficients (R^2) for ΔL and Δb were 0.68 and 0.79 for pH, 0.40 and 0.38 for CP, 0.63 and 0.70 for RFV, respectively. In the mixture of the caramba - berseem clover, the regression coefficients for ΔL and Δb were found to be 0.64 and 0.85 for pH, 0.14 and 0.12 for CP, 0.28 and 0.37 for RFV respectively. In both mixtures, the level of relation between Δa* color value and quality parameters remained very low. Also, it was obtained that the best roughage in terms of RFV was haylages opened after 55th days. Decreasing the L*, b* color values by 50% on average after fermentation can be regarded as an indication of roughage degradation and RFV reduction. From this point view, it can be possible to determine the quality change by following the colors before and after fermentation of roughage.

Keywords: Silage; Color change; Degradation; Quality; Roughage

1. Introduction

Roughages is one of the largest expenses for livestock production and generally supplied as grass, hay and silage. Recently, a large proportion of forages for livestock feeding is conserved as silage due to its several advantages. These advantages include less field and harvest losses, many crop options, high mechanization of harvesting, storage and feeding, less likelihood of weather damage during harvesting and low loss of nutrient. However, the silage quality is quite important for a profitable livestock farming.

Silage can be classified as good quality by physical characteristics like taste, smell, color and by chemical characteristics such as dry matter (DM) content, pH value, crude protein (CP), acid detergent fiber (ADF), and neutral detergent fiber (NDF). Good quality silage has vinegar smell, on acidic taste and a firm soft texture, leaves not

easily rubbed, light greenish or greenish brown in color (Uygur 2009). From the point of chemical characteristics, DM content of traditional silage varied in between 70%-85%. However, this value is around 40%-60% for haylage (bale silage). The pH value for good quality silage ranges between 3.8 and 4.0, however it can be higher (4.3-5.1) for haylage. Also, it is desirable that the CP value is as high as possible and the ADF and NDF values are low for pointing the silage as good quality.

Many researches have targeted to evaluate silage quality. In these studies, generally, it was aimed to determine the effects of storage period on chemical composition. It was reported that the pH value of silage decreased in long-term storage periods (Müller et al 2007; Shinners et al 2009; Weinberg & Chen 2013).

There are many studies on changing pH, ADF, NDF, and RFV with fermentation duration in the literature. Shinners et al (2009) determined that the pH, ADF and NDF values of the alfalfa silage incubated for 154 and 364 days were decreased from 5.9 to 5.13, from 34.4% to 29.4% and from 47.1% to 38.3%, respectively. Borreani et al (2008) found an opposite relationship for corn silage. The pH, ADF and NDF values of the corn silage storaged for 154 and 364 days were increased from 3.74 to 3.80, from 22.95% to 26.2% and from 44.17% to 44.92%, respectively. However, they emphasized that the CP value decreased from 8.17% to 7.55%. Müller et al (2007) were found that the CP value of meadow grass storaged for 2 and 14 months reduced from 4.88 to 4.50. In another work done by Weber & Kaiser (2006), it was obtained that the pH value of the sugar beet pulp silage has not changed for 14 and 183 days storage period. Moreover, Lee et al (2000) have determined that CP values decreased (from 10.03% to 3.04%) and ADF values increased (from 37.2% to 42.92%) according to 40, 50, 60, 65 days storage period for different forage species.

The extent of the effects of air ingress on bale silage is greatly important. The effect can be measure via color change. Color changing is one of the important parameters for quality of roughages (Anonymous 1987). Also, it was reported that storage conditions affect color change in haylages and color values of the bale which is storaged at protected areas were better than the others storage conditions (Toruk et al 2009; Toruk & Gonulol 2011). Silage color gives information about fermentation. Color evaluation of silages is considered as a physical criterion in literature and scored according to color change. In this context, relating color change to the quality parameters which are determined according to chemical analyzes is an important issue and there is limited studies focused on obtaining the relation between roughage quality and color change in the literature. The quality of roughage is foremost parameter for purchasing and adding to the feeding ration. DM, pH, CP and RFV can be listed as most important quality parameters. Although there are a lot of methods for determination of quality, it is another necessity for farmers to use fastest methods. Because, chemical analysis are costly and take times. In this case, affects the livestock economy negatively by increasing the cost of roughage. However, there are methods free from chemical analysis, but the results of these methods can change relatively depends on the person who makes decision. The significant difficulties occur in determining quality of forage for farmers due to there is no standard for the estimation of roughage quality. From this point of view, the objective of this current work was to associate CIELAB Scale (L*, a*, b*) with some quality parameters of roughages such as pH, CP and RFV. Thus, practical methods can be developed to estimate the quality of forage using color change.

2. Material and Methods

Two roughages mixtures namely, vetch - triticale (*Vicia sativa* L. - *Triticasecale wittmack*) and caramba - berseem clover (*Lolium multiflorum* cv Caramba - *Trifolium alexandrinum* L.) were used as plant material in the experiment. Mixture rates were 70% and 30% for vetch-triticale and 50% and 50% for caramba-berseem clover, respectively. Plants were harvested at the end of the flowering stage. Materials have been storaged as haylage and traditional silage. Haylage bales were wrapped with

0.025 mm plastic film in white color as four layers. Haylage bales were opened at the days of 25, 40 and 55 and the color changing was compared with traditional and degraded silage in terms of pH, CP and RFV.

The silage samples color was examined by using Minolta CR-100 Chromameter color measurement device. In the CIE 1976 (L*, a*, b*) color space, abbreviated CIELAB, the lightness coefficient, L*, ranges from black = 0 to white = 100 and is roughly analogous to the Munsell value scale times 10. For any measured color of lightness, L_i*, the coordinates (a*, b*) locate the color on a rectangular-coordinate grid perpendicular to the L* axis at L_i*. The color at the grid origin (a* = 0, b* = 0) is achromatic (gray). On the horizontal axis, positive a* indicates o hue of red-purple; negative a*, of bluish-green. On the vertical axis, positive b* indicates yellow and negative b* blue (Figure 1) (McGuire 1992).

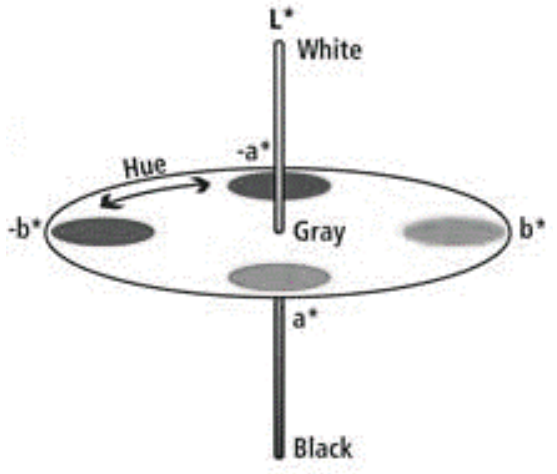

Figure 1- CIELAB color space

For determination of color changing, material color was measured before storage and after unwrapped and calculated by subtracting second (2) measurement from first one (1) (Snell et al 2002).

$$\Delta L = L_1^* - L_2^* \quad (1)$$
$$\Delta a = a_1^* - a_2^* \quad (2)$$
$$\Delta b = b_1^* - b_2^* \quad (3)$$

The quality of the material was evaluated in terms of pH, CP, ADF, NDF and RFV. Nitrogen (N) content was measured using the Kjeldahl Method. The pH values of plants were obtained as reported by Chen et al (1994). The CP was calculated as N × 6.25 (AOAC 1990). The NDF and ADF were determined as suggested Van Soest et al (1991) by using ANKOM fiber analyzer. The RFV was calculated by using equation given below (Mayouf & Arbouche 2014).

$$RFV = \left(88.9 - (0.779 x ADF\%)\right) x ((120/NDF\%)/1.29) \quad (4)$$

To investigate the effect of color changing on quality parameters, the tests were conducted according to randomized block design with three replications for both forage mixtures. Five different applications were tested in the study (Table 1). The Jump 7 statistical program was used in evaluating the data and groupings were made according to LSD (Yurtsever 1984).

Table 1- Applications tested in the study

Abbreviation	*Applications*
H1	Haylage, unwrapped on the 25th day of the fermentation
H2	Haylage, unwrapped on the 40th day of the fermentation
H3	Haylage, unwrapped on the 55th day of the fermentation
TS	Traditional silage, opened on the 55th day of the fermentation
DH	Degraded haylage

3. Results and Discussion

A review of Table 2 shows that the color values of both mixtures did not differ statically before wrapping but they were found significant at 1% level after opening.

It is desirable that the color values after opening as silage quality indicators do not differ much from the initial color values. Table 3 presents the color changing values according to applications. When the color values are examined after both mixtures

Table 2- Coefficients of variation for according to mixtures

Parameters	*Vetch-Triticale*			*Caramba-Berseem Clover*		
	P value	$LSD_{(0.05)}$	*CV(%)*	*P value*	$LSD_{(0.05)}$	*CV(%)*
L_1^*	0.3526^{ns}	-	5.08	0.2893^{ns}	-	2.41
a_1^*	0.9211^{ns}	-	13.0	0.3040^{ns}	-	8.06
b_1^*	0.1507^{ns}	-	7.37	0.8241^{ns}	-	8.73
L_2^*	$<0.0001^{**}$	4.20	5.34	0.0010^{**}	6.15	7.79
a_2^*	0.0047^{**}	1.45	23.24	$<.0001^{**}$	0.53	6.52
b_2^*	$<0.0001^{**}$	1.94	4.80	0.0001^{**}	2.81	7.44

*, **, ^{ns} significant at the levels of 5%, 1%, and not significant respectively

are opened, it was determined that the L* value before wrapping was 52.7 for the vetch-triticale mixture and 50.2 for the caramba-berseem clover mixture on average for silage and haylage (H1, H2, H3 and TS). It can be seen that the best color values in terms of L* are obtained from the H3 application in the study. It was determined that the L* values decreased in both mixtures when comparing H3 and TS applications. This value decreased from 47.70 to 43.56 in the case of the vetch-triticale mixture and from 42.50 to 49.85 in the case of the caramba-berseem clover mixture. This is an indication that the storage conditions affect the L* value. After opening, these values decreased 45.0 and 44.5 in average for the vetch-triticale mixture and the caramba-berseem clover mixture, respectively. The L* value was found to be 28.5 for the vetch-triticale mixture and 31.4 for the caramba-berseem clover mixture, on average, for the degraded haylage (DH) samples. It was found that the average L* value of the degraded roughage decreased by 45.6% in the vetch-triticale mixture and by 37.6% in the mixture of caramba-berseem clover according to the initial L* value. That means, the degraded samples lost their brightness compared to the other roughage samples. When the b* (yellow-blue) value before wrapping was examined, it was determined that this value was 23.3 in the vetch - triticale mixture and 21.0 in the caramba-berseem clover mixture. After opening the silage and haylage, this value increased slightly, reaching 23.9 for the vetch-triticale mixture and 21.8 for the caramba-berseem clover mixture. This situation can be interpreted as an indication that the b* color value is not affected by the storage duration. In the degraded specimens, b* decreased by an average of 50% and it was found to be 12.1 and 13.1 for the vetch-triticale mixture and the caramba-berseem clover mixture, respectively. The a* (red-green) value was found as 11.4 for the vetch-triticale mixture and 11.9 in the caramba-berseem clover before wrapping, then decreased to 4.1 and 4.7 after opening, respectively. In deteriorated samples, this value decreased to 2.2 and 2.9, respectively. Snell et al (2003) found a relation between applications and color values of L* and a*. Ball et al (2017) also emphasized that a bright green color can be an indicator of storage conditions which shows good protection during storage. However, statistical mean value comparison regarding the b* values did not result in any significant difference. This study also gave similar results.

Table 4 illustrates that in the vetch-triticale mixture, all applications significant at 1% level on pH, ADF, NDF, CP and RFV. In the case of the mixture of caramba-berseem clover, the applications have a significant effect on pH, ADF, CP at 1% level, but at 5% level on RFV and no effect on NDF.

It was determined that the pH value was highest in deteriorated haylage and the lowest in traditional silage. In both mixtures, it was obtained that the best application among haylages was roughage which was left for 55 days fermentation (H3). In terms of

Table 3- Color changing values according to applications

Mixture		*H1*	*H2*	*H3*	*TS*	*DH*
Vetch- triticale	L_1^*	52.70	50.05	54.85	52.43	53.46
	a_1^*	11.35	11.40	12.00	11.40	10.86
	b_1^*	24.55	24.65	23.85	22.83	21.03
	L_2^*	46.30^{ab}	42.60^{b}	47.70^{a}	43.56^{ab}	28.56^{c}
	a_2^*	6.05^{a}	2.65^{c}	3.50^{bc}	4.43^{ab}	2.26^{c}
	b_2^*	24.75^{a}	22.10^{b}	24.75^{a}	24.00^{ab}	12.10^{c}
Caramba-Berseem clover	L_1^*	50.25	50.30	49.70	51.53	49.25
	a_1^*	10.90	11.65	12.50	12.46	12.10
	b_1^*	20.60	21.90	20.60	21.63	20.60
	L_2^*	39.35^{c}	47.55^{ab}	49.50^{a}	41.83^{bc}	31.40^{d}
	a_2^*	4.70^{b}	4.10^{c}	4.50^{bc}	5.56^{a}	2.90^{d}
	b_2^*	19.35^{b}	22.05^{ab}	24.65^{a}	21.26^{b}	13.10^{c}

Means in each column with the same letters are not significantly different (P<0.05)

Table 4- Coefficients of variation pH, ADF, NDF, CP and RFV according to mixtures

Parameters	*Vetch-Triticale*			*Caramba-Berseem Clover*		
	P value	$LSD_{(0.05)}$	*CV(%)*	*P value*	$LSD_{(0.05)}$	*CV(%)*
pH	$<.0001^{**}$	0.12	1.34	$<0.0001^{**}$	0.41	4.26
ADF	0.0003^{**}	4.11	4.58	0.0071^{**}	6.54	7.63
NDF	0.0014^{**}	5.67	5.34	0.3322^{ns}	-	4.74
CP	0.0005^{**}	0.89	5.73	0.0031^{**}	0.93	6.39
RFV	0.0012^{**}	14.55	8.25	0.0372^{*}	15.05	8.65

*,**, ^{ns} significant at the levels of 5% , 1%, and not significant respectively

ADF, the H3 application has the lowest value in both mixtures, and this value was highest in the degraded haylage. Shinners et al (2009) also obtained similar results in their studies. It can be said that the fermentation period has the positive effect on ADF and pH. This may be explained by the increase in the amount of sugar used by Lactic Acid Bacteria in the length of time. When the HP values were compared according to the applications, it was determined that the HP ratio in the caramba-berseem clover was not affected statistically by the fermentation period and remained in the same group. However, the CP ratio for the conventional silage remained low compared to haylages. In the vetch-triticale mixture, the CP value was slightly increased with the fermentation time. In both mixtures, the CP value was lower in the degraded silage than the other applications (Table 5).

The relationship between color values (ΔL, Δa, Δb) and quality parameters (pH, CP and RFV) for both mixtures was determined and graphically presented in Figure 2, 3 and 4. It was determined that ΔL and Δb color values and pH, CP, RFV were significantly correlated in the vetch-triticale mixture. The regression coefficients (R^2) for ΔL and Δb were 0.68 and 0.79 for pH and 0.63 and 0.70 for RFV, respectively. However, for CP these coefficients were calculated lower as 0.40 and

Table 5- Quality values according to applications

Parameters		*H1*	*H2*	*H3*	*TS*	*HD*
Vetch-triticale	pH	5.05^{b}	4.79^{c}	4.49^{d}	3.46^{e}	7.18^{a}
	ADF(%)	46.70b^{c}	49.50^{b}	41.35^{d}	44.06^{cd}	56.36^{a}
	NDF(%)	56.40^{b}	54.60^{b}	52.35^{b}	51.36^{b}	67.03^{a}
	CP(%)	7.90^{bc}	8.80^{b}	10.16^{a}	8.34^{bc}	7.01^{c}
	RFV	93.14^{b}	92.59^{b}	108.34^{a}	106.83^{ab}	67.21^{c}
Caramba-Berseem clover	pH	5.18^{b}	4.96^{bc}	4.25^{d}	4.55^{cd}	6.60^{a}
	ADF(%)	42.30^{b}	43.50^{b}	42.10^{b}	45.16^{b}	55.60^{a}
	NDF(%)	57.50	56.45	56.00	59.53	60.15
	CP(%)	8.62^{a}	8.47^{a}	8.07^{a}	6.94^{b}	6.60^{b}
	RFV	97.30^{a}	97.42^{a}	100.16^{a}	90.52^{ab}	76.38^{b}

Means in each column with the same letters are not significantly different (P<0.05)

0.38, respectively. In the mixture of the caramba-berseem clover, the regression coefficients for ΔL and Δb were found to be 0.64 and 0.85 for pH, 0.14 and 0.12 for CP, 0.28 and 0.37 for RFV respectively. In both mixtures, it was obtained that R^2 which was calculated to determine the level of relation between Δa color value and quality parameters remained very low. It was found that the values of ΔL and Δb were significantly related to pH in both mixtures, while the value of L* before wrapping decreased gradually after opening, while pH value increased and RFV decreased. The similar situation was obtained for b* color value. It has been determined that the best roughage in terms of RFV was obtained from haylages opened after 55 days (H3). In this period the ΔL and Δb values for the vetch-triticale mixture were 7.15-0.9 and 0.2-4.05 for the mixture of caramba-berseem clover, respectively. This shows that when the difference between the values of L* and b* before and after fermentation approaches the value of 0, RFV increases.

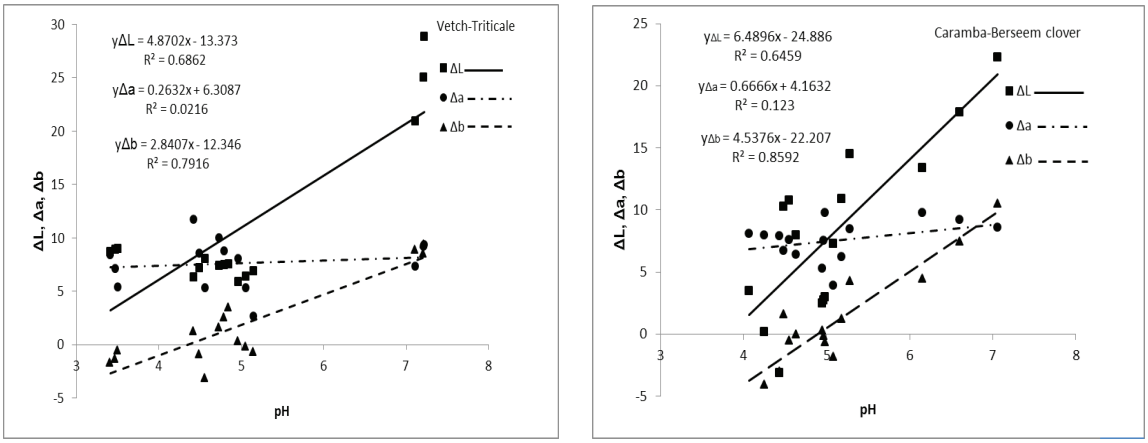

Figure 2- Relationship between color changes and pH

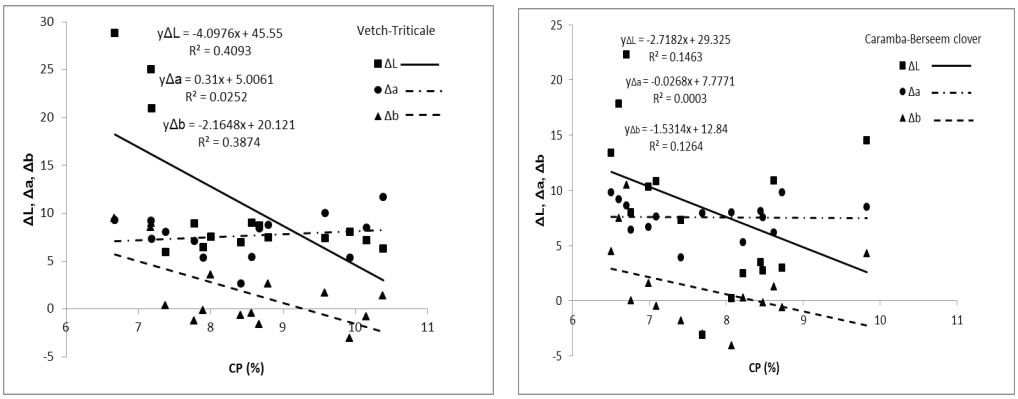

Figure 3- Relationship between color changes and CP

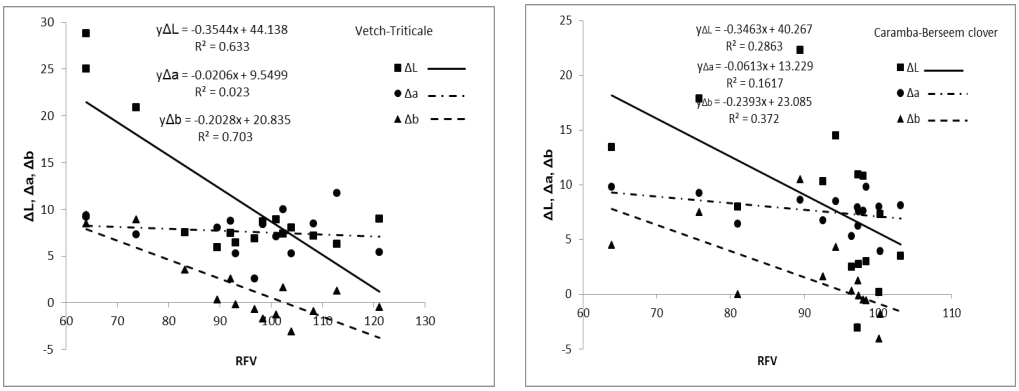

Figure 4- Relationship between color changes and RFV

4. Conclusions

In the study, some quality criteria were tried to be associated with the change of L*, a*, b* color values in roughage. It has been determined that these values have an acceptable correlation with pH and RFV in both mixtures. Decreasing the L*, b* color values by 50% on average after fermentation can be regarded as an indication of roughage degradation and RFV reduction. From this point view, it can be possible to determine the quality change by following the colors before and after fermentation of roughage. It can be said that this study is important because of showing possibility to have information about the quality of roughages by following the color change. Further, similar studies can be performed on other roughage to obtain color change scales, which can be used for quality estimation.

Abbreviations and Symbols	
CIELAB (L*, a*, b*)	Color space
pH	Potential of hydrogen
CP	Crude protein, %
RFV	Relative feed value
ΔL	Color change of L*
Δb	Color change of b*
Δa	Color change of a*
L*	Lightness
a*	Red-green
b*	Yellow-blue
DM	Dry matter, %
ADF	Acid detergent fiber, %
NDF	Neutral detergent fiber, %
N	Nitrogen
CV	Coefficient of variation, %
P	Probability
LSD	Least significant difference

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Anatomical Analysis of Graft Compatibility in Some Almond Scion-Rootstock Combinations

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ABSTRACT

In this study, graft compatibility between almond cultivar Lauranne and almond seedling and Rootpac R, Rootpac 90, Rootpac 70 and Rootpac 40 clonal rootstocks was anatomically investigated. The anatomical analysis of scion/rootstock combinations was performed by taking cross sections for 30 days and 12 months after T-budding in June, 2017. It was determined that, 30 days after grafting, the callus cells developed but cambial continuity has not occurred between the rootstock and scion tissues in all scion/rootstock combinations. 12 months after grafting, cambial relation was established, vascular differentiation was observed, regular parenchymatic tissue properties and scleroid (petrosal cell) cells and sclerenchyma bundles were seen in the graft union. There was no problem in terms of rootstock- scion compatibility in Lauranne almond cultivar which was grafted on almond seedling and some Rootpac clonal rootstocks.

Keywords: *Prunus dulcis*; Grafting; Almond seedling; Rootpac clonal rootstocks

1. Introduction

Fruits have gained more interest in recently due to high nutritional value, phytochemical content and human health effects. Fruit has been recognized as a good source of vitamins and minerals, and for their role in preventing vitamin C and vitamin A deficiencies (Kamiloğlu et al 2009; Tosun et al 2009; Zorenc et al 2016; Çalışkan et al 2017). Almond has a high nutritional value, and is used as raw material in many sectors including food, cosmetics and pharmaceutical industry (Gebauer et al 2016; Taş & Gökmen 2017). Cultivation and trade of the almond around the world is significant.

The most important factor limiting the cultivation of almond in the world is special ecological demands. In addition to this, factors such as high lime rate of soil and some soil-based diseases also play a limiting role in almond cultivation. Problems arising from different soil types, climatic conditions, diseases and damages that restrict cultivation can be eliminated by using suitable rootstocks. Thus, the need for rootstock to overcome these limitations is essential for growing stone fruit, including almonds, in many regions of the Mediterranean basin (Felipe 1989). Proper rootstocks must be used in order to obtain

high quality and yield in different climatic and adverse soil conditions, to control the growth vigour of trees, to provide early tolerance for infestation and resistance against diseases and pests. There are also approaches to increase yield by applying frequent plantation methods which allow more trees to be kept in unit area in modern cultivation. For this reason, it is very important to use grafted cultivars on suitable rootstocks in commercial fruit growing. Factors such as technical errors during grafting, time of grafting, selection of materials to be used in grafting and some virus diseases affect grafting success. The main factor that determines the success of grafting is compatibility status between the rootstock and cultivar. Almond seedlings are traditionally used for almond rootstock. In addition, peaches, plums and apricot seeds are also used as rootstock. In addition to studies on seedling rootstocks, clonal rootstock studies in almond are also widely carried out. Peach x almond hybrids (GF 557, GF 677, Hansen 2168, Hansen 536 etc.) (Özcan 2000), plum clones (Marianna GF 8-1, Marianna 2624 etc.), plum x peach x almond hybrids (Garnem etc.) (Atlı et al 2011) have become widespread in recent years. Many studies have been conducted on scion-rootstock compatibility between some almond cultivars and almond rootstocks. It has been reported that, graft compatibility with almond is highly variable; hence, preliminary trials are required before a particular combination of almond is utilized on plum (Rubio-Cabetas et al 2017). Considering their performance against soil-borne problems such as chlorosis or nematode, it is suggested to increase seedling production with clone rootstocks of GF 677, Garnem, Cadaman and Rootpac series instead of seedling rootstocks for modern almond cultivation (Arquero et al 2002; Felipe 2009; Bielsa et al 2015; Rubio-Cabetas 2015). In addition there are several new clones also commercially propagated, such as 'Replantpac' (Rootpac-R), a myrobalan x almond hybrid with compatibility with almond (Pinochet 2010). Although without prior extensive field evaluation, dwarfing rootstocks such as Rootpac 20, Rootpac 40, Rootpac 70 and Rootpac 90 were released and considered for almond (Rubio-Cabetas et al 2017). It is very important that incompatibility status of almond cultivars to be grafted on a large number of rootstocks with various characteristics are known before the orchard is established. The incompatibilities that will arise after the orchard plantation will cause serious economic losses. For this reason, anatomical examination of the coexistence of different rootstock-type graft combinations is a rescuer approach.

Graft incompatibility situations between different new rootstocks and almond cultivars should be evaluated. Rootpac clonal rootstocks have superior properties and also some of them allow intensive almond plantation in recent years. Until now, some of the almond cultivars on these rootstocks have not been assessed anatomically in terms of graft compatibility.

In this study, anatomical investigation of graft compatibility performance of Laurenne almond cultivar on almond seedling and clonal rootstocks (Rootpac 40, Rootpac 70, Rootpac 90 and Rootpac R) was aimed. Formation in the graft union was anatomically examined in the samples taken 30 days and 12 months after budding.

2. Material and Methods

2.1. Plant materials

In the study, five different rootstocks (almond seedling, Rootpac R, Rootpac 90, Rootpac 70, Rootpac 40) and Lauranne cultivar were used as plant material. Almond seedlings are used as control rootstock. The research was carried out in the greenhouse conditions of Gaziantep Pistachio Research Institute. Clonal rootstocks were grown by tissue culture and obtained from a commercial company, and were stuck in tubes of 18x32 cm size filled with soil, burnt stallion and peat mortar in a ratio of 1: 1: 1. One year old rootstocks, that reached the grafting thickness, were done T-budding in June 2017 period. Laurenne cultivars as scions are prepared from annual exiles from the trees in the

collection parcel of Gaziantep Pistachio Research Institute.

Lauranne, a self fertile and highly productive cultivar which blooms late, is resistant to cold weather and tolerant to fungal diseases.

'Replantpac' (Rootpac® R) is a new plum-almond hybrid selected by Agromillora Iberia, S.L., Barcelona, Spain, for use mainly as a rootstock for Japanese plum (*Prunus salicina* Lindl.), peach, and nectarine [*P. persica* (L.) Batsch] cultivars, but it can also be used for almond [*P. dulcis* (Mill.) D. A. Webb, syn. *P. amygdalus* Batsch] and some apricot (*P. armeniaca* L.) cultivars (Pinochet 2009).

Rootpac R (*P. cerasifera x P. dulcis*): It is a rootstock that is compatible with cultivars of peach, nectarine, plum, almond and apricot, able to withstand hard soil conditions. It is highly productive and tolerant to asphyxia, highly tolerant to chlorosis and root-knot nematodes (Jiménez et al 2013).

Rootpac 90 (*P. persica x P. davidiana) x (P. dulcis x P. persica*): It is a new rootstock that is well adapted to intensive production conditions, and produces fruit with high yield and good quality. It is more productive than GF 677 and Garnem. It adapts very well to all climate conditions, both warm and colder climates. It is sensitive to asphyxia. It is highly tolerant to chlorosis. It is moderately resistant to root-knot nematodes (Pinochet 2009).

Rootpac 70 (*P. persica x P. davidiana) x (P. dulcis x P. persica*): It is a new rootstock suitable for intensive plantations, reducing the cost of production with good quality of fruit and early harvest. It adapts very well to all climate conditions, but particularly to warm conditions (low chilling areas). It is sensitive to asphyxia and salinity. It is tolerant to chlorosis. It is moderately resistant to root-knot nematode (Jiménez et al 2011).

Rootpac 40 *(P. dulcis x P. persica*) *x (P. dulcis x P. persica*): It is a new rootstock suitable for intensive plantations, with good fruit quality and early harvesting properties. It is extremely well adapted to the most warm production conditions and especially recommended for cultivars of peach, nectarine, almond and some Japanese plum. It is more tolerant to asphyxia than most peach and peach x almond hybrids and is moderately tolerant to chlorosis, salinity and root-knot nematodes (Yahmed et al 2016).

Almond seedling: It is a traditional rootstock widely used in almond cultivation. The compatibility with almond cultivars is high. The almond seedlings are suitable for calcareous, dry and gravelly soils and increase the growth vigor of the cultivar grafted on it (Yılmaz 2010).

The growth vigor of Rootpac R, Rootpac 90 and almond seedling rootstocks is high while that of Rootpac 70 and Rootpac 40 are moderate.

2.2. Methods

The rootstocks used in the study were grafted with the Laurenne cultivar by T budding method in June, 2017. 30 days and 12 months after budding, budded samples from each combination were taken from 5 cm below and above the graft union (Figure 1) and were stored in FAA solution until the sections were taken. Cross sections, 20-25 microns thick, were taken from the graft union by hand, stained using the safranin dyeing method and observed under microscope (Seferoğlu 1991).

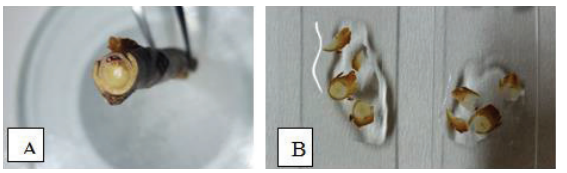

Figure 1- View of grafted union 5 cm above (A) and transversal cuts of *Prunus dulcis* (B)

3. Results and Discussion

3.1. 30 days after grafting

It was determined that, callus cells developed but cambial differentiation between rootstock and scion tissues has not begun in the sections budded onto seedling, Rootpac R and Rootpac 70 rootstocks

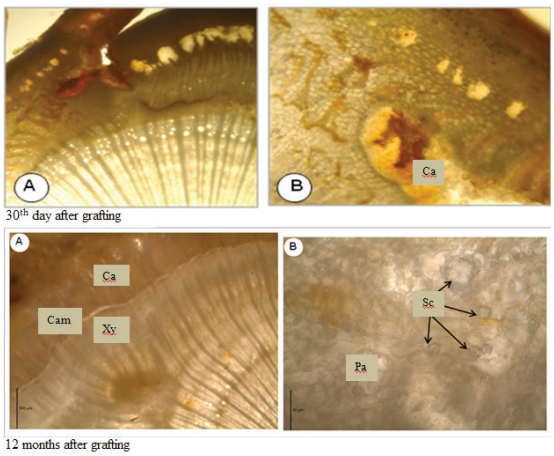

Figure 2- Cross sections taken 30 days and 12 months after the budding from budded parts of Laurenne cultivar on almond seedling; A, callus cells (Ca), cambium (Cam) and new xylem elements (Xy); B, parenchymatic cells (Pa), sclereids (Sc)

(Figure 2, Figure 3, Figure 4). Similarly, too many callus cells developed but cambial continuity between rootstock and scion tissues has not been established yet in the sections budded onto Rootpac 90 and Rootpac 40 rootstocks (Figure 5, Figure 6).

3.2. 12 months after grafting

In the cross sections taken 12 months after budding of Laurenne onto seedling rootstock, budding formation was found successful, cambial continuity sustained, new transmission tissues formed from the cambium, callus cells gained regular parenchymatic tissue properties and numerous sclereid cells were encountered in the parenchymal cells (Figure 2).

In the cross sections taken from the budded parts on Rootpac R rootstock, it was observed that the callus cells filled the callus completely between the budding elements, gained regular parenchymatic

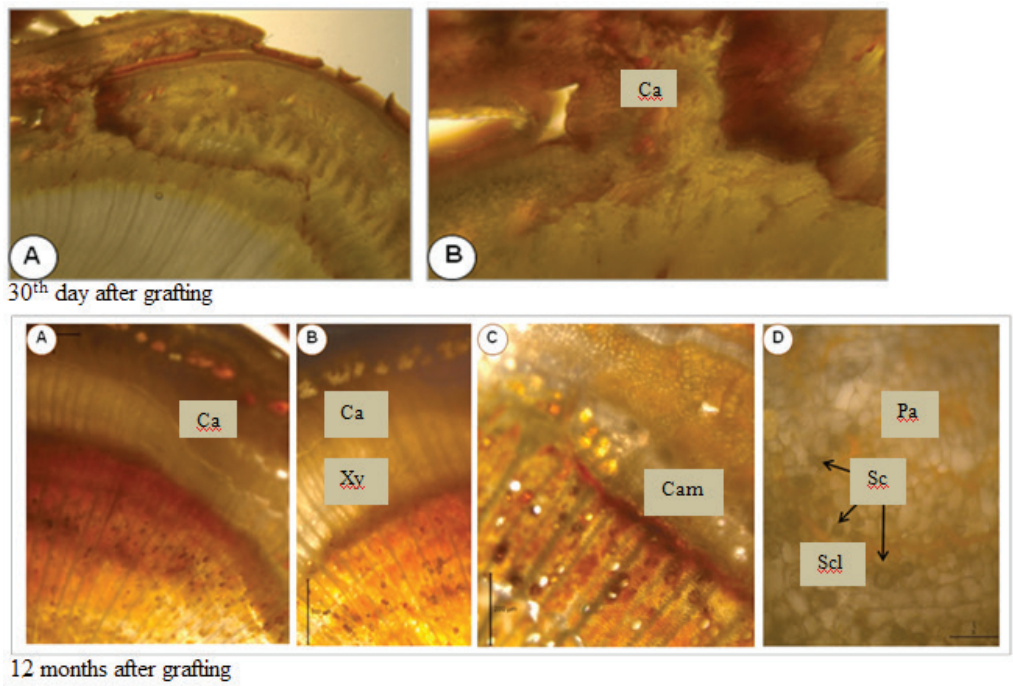

Figure 3- Cross sections taken 30 days and 12 months after the budding from budded parts of Laurenne cultivar on Rootpac R rootstock; A-B, callus cells (Ca), new xylem elements (Xy); C, cambium (Cam); D, parenchymatic cells (Pa), sclereids (Sc), sclerenchyma bundles (Scl.)

tissue properties and vascular differentiation increased. The differentiated cambium cells reached 6-8 rows (Figure 3).

It was observed that cambial continuity was established, vascular differentiation increased, the callus cells got regular parenchymatic tissue properties, and the parenchymatic cells were found to have a lot of sclereid cells in the cross sections taken from the budded parts on Rootpac 90 and Rootpac 70 rootstocks. In the samples budded onto Rootpac 70 rootstock, the differentiated cambium cells reached 6-8 rows (Figure 4, Figure 5).

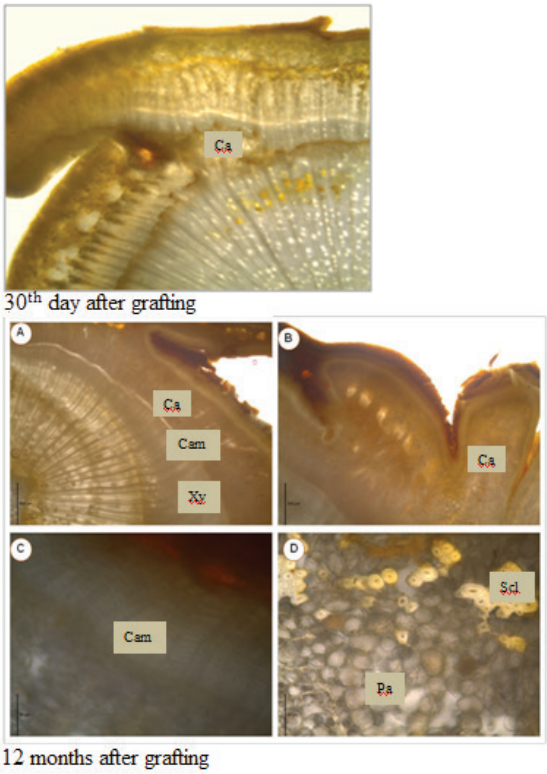

Figure 4- Cross sections taken 30 days and 12 months after the budding from budded parts of Laurenne cultivar on Rootpac 70 rootstock; A, callus cells (Ca), new xylem elements (Xy), cambium (Cam); B, callus cells (Ca); C, cambium (Cam); D, parenchymatic cells (Pa), sclerenchyma bundles (Scl.)

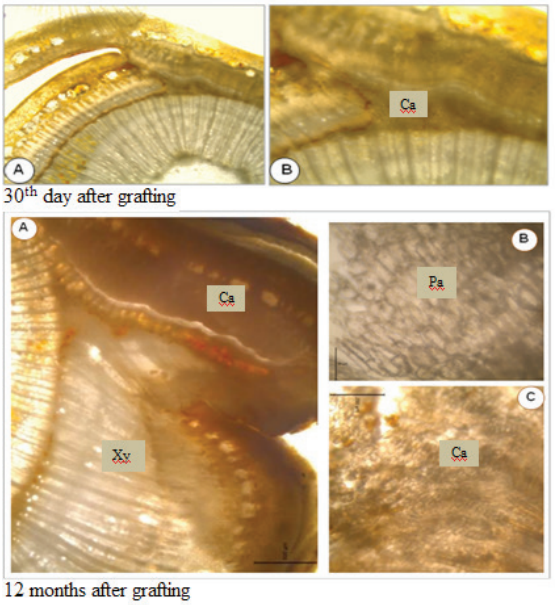

Figure 5- Cross sections taken 30 days and 12 months after the budding from budded parts of Laurenne cultivar on Rootpac 90 rootstock; A, new xylem elements (Xy), callus cells (Ca); B, parenchymatic cells (Pa); C, callus cells (Ca)

In the cross sections taken from the budded parts on Rootpac 40 rootstock, differentiation was similar to that of other rootstocks. Intensive callus formation was detected among the budded components, the differentiated cambium cells reached 5-8 rows, callus cells were found to gain regular parenchymatic tissue properties, and parenchymatic cells were found to contain a very large amount of sclereid cells (Figure 6).

Two plant parts, which are used as rootstock and scion, are required to be merged with each other over time and continue to live as a single plant in grafted combinations of fruit species. The connection between the rootstock and the scion takes place through the callus tissue, conflicting cambium tissues of rootstock and scion generate the callus formed by meristematic cells and these meristematic cells, which are composed of two different sources, merge along a line. The

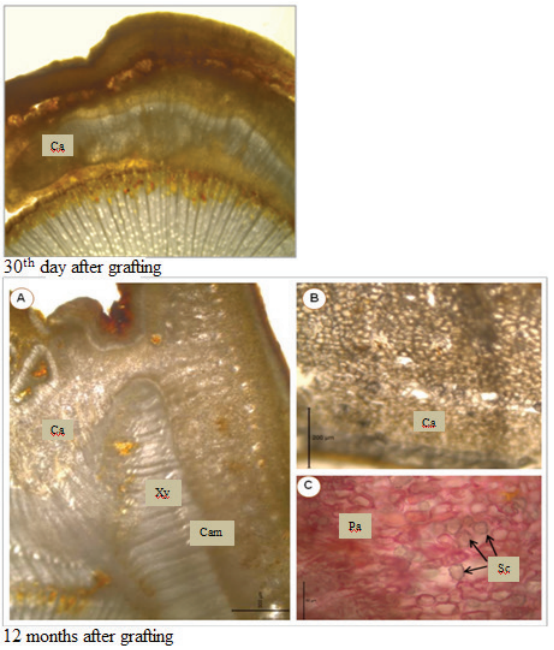

Figure 6- Cross sections taken 30 days and 12 months after the budding from budded parts of Laurenne cultivar on Rootpac 40 rootstock; A-B, callus cells (Ca), new xylem elements (Xy), cambium (Ca); C, parenchymatic cells (Pa), sclereids (Sc)

formation of the callus, which begins to occur two days after the grafting, takes about 2-3 weeks, and the new cambium tissue forms from callus that fills between the rootstock and the scion. It is also indicated that completion of the vascular system has been occurred within 6-8 weeks. As a result of the union, both the rootstock and the scion side of the wood and phloem tissues allow the passage of water and plant nutrients with assimilation products from the point of graft union. The formation of callus tissue from both graft members, establishment of callus bridge, cambial differentiation, cambial continuity and completion phases of vascular system development; type of grafting, the method of grafting, grafting time can demonstrate alterations based on species used as rootstocks and scions. While the graft formation occurs in these phases at successfully developed compatible combinations, development disorders occur in cambial continuity and the development of vascular system phases at incompatible combinations. On the basis of these knowledges, following the status of the necrotic layers between the rootstock and the scion in the graftings, the formation of the callus tissue and establishment of the callus bridge, cambial differentiation and continuity also the formation status of new vascular tissues, it was reported that there may have been a reliable idea whether the combinations were compatible or incompatible (Hartmann & Kester 1961; Torabi 1975; Moore 1984; Tekintaş 1991; Hartmann et al 1997; Koyuncu et al 2007; Darikova et al 2011; Bayram et al 2014).

Grafting incompatibility between some new almond rootstocks and almond cultivars was anatomicaly examined in this study. It was determined that callus formation between the grafting components was completed within 30 days after grafting and new transmission bundles were occured after 12 months. Our findings are consistent with the results determined in anatomically investigated combinations of rootstock-scion relationships in different species. It was stated that the vascular differentiation between the graft components in the compatible combinations was seen within the next 2-3 weeks (15-20 days) after grafting (Mosse 1962; Hartmann et al 1990).

Ünal & Özçağıran (1986) established that cambium, xylem and phloem tissues occured with different quantities in each some pear/quince graft combination one month after the grafting and four months later, xylem and phloem tissues occured in places where cambium was regular. In a study conducted in hazelnut, the first cambial differentiation in callus tissue was determined in the sections taken after 18 days. Cambial continuity was determined to be established after 26 days of grafting on chip budding and 42 days after splice grafting (Balta 1993).

In graft combinations of *Prunus* species, it was reported that callus tissues occured one week after grafting, cambium cells occured after 10 days and

vascular differentiation occured after 13 days (Errea & Felipe 1994; Errea et al 1994).

It was determined that formation of callus, cambium, new xylem and phloem was more rapid and regular in very good or good compatible peach/plum graft combinations while this formation was slow and deficient in incompatible or very poorly compatible combinations (Hartmann et al 1997), even the callus did not differ (Errea & Felipe 1994).

It was determined that callus cells in peach and nectarine cultivars grafted on almond rootstock occured within 14 days after grafting whereas vascular differentiation within 28 days. In the sections taken 4 months after the grafting, it was determined that the formation of cambium was completed in spite of locally interrupt, vascular differentiation continued but not completely in any combination (Tekintaş & Dolgun 1996).

Grafted samples which were examined 40 days after in walnut, the newly formed cambium tissue produced new vascular tissues (Kazankaya 1996). In some apricot cultivars grafted on Pixy rootstock, a weak callus formation occurred at the graft unions 15 days after grafting and cambial differentiation and new vascular tissues were identified in highly compatible cultivars (Kankaya et al 2001).

It was also detected that cambial continuity between the graft elements was achieved in all graft types within approximately 3 weeks after grafting at different buddings on *Pistacia vera* seedlings and this persistence was earlier in the T-budding than in the chip budding (Okay & Büyükkartal 2001). In the compatible combination of peach/plum, callus, cambium formation and vascular differentiation occured within four months after grafting, while a significant part of callus cells did not differentiate, cambium occured partly in some regions within a month after grafting, within 4 months after grafting, vascular differentiation didn't occur completely and necrotic layers were observed to increase in incompatible combinations (Demirsoy & Bilgener 2002; 2006).

Koyuncu et al (2007) stated that callus tissue between the rootstock and scion occured 2 weeks after grafting in the nectarine and peach cultivars that was highly compatible with the plum rootstock and the callus bridge was established also cambial continuity was achieved in following periods. Similar results were obtained in apricot cultivars grafted on some clonal rootstocks (Coşkun 2012).

An intense and regular callus formation occurred at early stage in all almond rootstocks used in our study similarly in all compatible combinations determined by other researches and no anatomical improvement was determined towards incompatibility in the investigated graft combinations.

4. Conclusions

It has been observed that callus cells were formed and started to develop in graft union taken after 30 days of budding, but cambial activity between rootstock and scion has not yet begun. This shows that in all rootstocks used, graft formation successfully continued at the graft union 30 days after budding. In all samples taken 12 months after budding, it was seen that graft formation in the budded parts has been completed successfully. Cambial continuity has been established, new transmission tissues have emerged, and callus cells have gained a regular parenchymatic tissue property. No significant difference was observed among rootstocks in terms of the period of callus cell formation after budding and establishment of cambial continuity. The Rootpac R, Rootpac 90 and almond seedling (high vigor) with Rootpac 70 and Rootpac 40 (medium vigor) showed almost the same process of differentiation. This indicates that Rootpac R, Rootpac 90, Rootpac 70 and Rootpac 40 clone rootstocks were successful in terms of graft compatibility with the Laurenne cultivar, as well as almond seedling. As a matter of fact, similar results were obtained in some studies in which the performance of different almond cultivars grafted on these rootstocks were evaluated (Pinochet 2010; Rubio-Cabetas 2015).

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Use of Principal Coordinate Analysis for Measuring GE Interactions in Rain-Fed Durum Wheat Genotypes

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ABSTRACT

Genotype × environment interactions complicate selection of superior genotypes for narrow and wide adaptation. Multi-environment yield trials of twenty durum wheat genotypes were conducted at five locations of Iran (Gachsaran, Gonbad, Moghan, Ilam and Khorram abad) over four years (2009-2013). Combined ANOVA of yield data of the twenty environments (year/location combined) revealed highly significant differences among genotypes and environments as well as significant genotype-environment interaction indicated differential performance of genotypes over test environments. The GE interaction was examined using multivariate analysis technique as principal coordinate analysis (PCOA). According to grand means and total mean yield, test environments were grouped into two main groups as high mean yield (H) and low mean yield (L). There were eleven H test environments and nine L test environments which analyzed in the sequential cycles. For each cycle, both scatter point diagram and minimum spanning tree plot were drawn. The identified most stable genotypes with dynamic stability concept and based on the minimum spanning tree plots and centroid distances were G12 (3342 kg ha^{-1}), G10 (3470.3 kg ha^{-1}), G5 (3203.0 kg ha^{-1}), and G1 (3263.5 kg ha^{-1}), and therefore could be recommended for unfavorable or poor conditions. Genotypes G10 (3470.3 kg ha^{-1}) and G9 (3404.2 kg ha^{-1}) were located several times in the vertex positions of high cycles according to the principal coordinates analysis (PCOA) and therefore could be recommended for favorable or rich conditions. Finally, the results of principal coordinates analysis in general confirmed the breeding value of the genotypes, obtained on the basis of the yield stability evaluation.

Keywords: Adaptation; Environment; Interaction; Genotype; Stability; *Triticum turgidum*

1. Introduction

During the past 50 years, agricultural research and technology transfer have helped increase the output of world crops two and a half-fold. Ruttan (1998), while summarizing the world's future food situation, referred to the "2-4-6-8" scenario, which means a doubling of population, a quadrupling of agricultural production, a sextupling of energy production, and an octupling of the size of the global economy by 2050. Durum wheat (*Triticum turgidum* var. *durum*) is one of the most important cereal crops in the world and grown on only 8 to 10% of all the wheat-cultivated area (FAO 2015). Durum wheat is better adapted to semiarid environments compered to bread wheat and is a crop adapted to marginal lands (Sabaghnia et al 2013a; Karimizadeh et al 2016). Iran imports considerable amount of durum wheat due to low quantity and quality of its own produced durum wheat (Sabaghnia et al 2013b; Karimizadeh et al 2016). Durum wheat production has been increasing globally since the 1950s and has currently reached about 33 million tons per year (Ma et al 2013). Crop performance, the observed phenotype, is a function of genotype-variety or cultivar, environment, and GE interaction. Genotype-environment interaction occurs when different cultivars or genotypes respond differently to diverse environments (Karimizadeh et al 2016). Expression of a phenotype is a function of the genotype, the environment, and the differential sensitivity of certain genotypes to different environments, also known as GE interaction (Leon et al 2016). The penultimate success of a plant breeding program depends on its ability to provide farmers with genotypes with guaranteed superior performance (phenotype) in terms of yield and/or quality across a range of environments. While there can be genotypes that do well across a wide range of conditions (widely adapted genotypes), there are also genotypes that perform well exclusively under a restricted set of environments or specifically adapted genotypes (Akcura et al 2009; Karimizadeh et al 2012a; Mohammadi et al 2012; Karimizadeh et al 2016).

Different yield stability statistics proposed to characterize GE interactions in multi-environment trials and several methods have been proposed to evaluate stability. These methods could be dividing by parametric, nonparametric and multivariate types. Several multivariate procedures have been proposed to explore GE interaction including principal component analysis (PCA), additive main effects and multiplicative interactions (AMMI), genotype plus GE interaction biplot (GGE) analysis and principal coordinate analysis (PCOA).

Principal Coordinates Analysis (multidimensional scaling) is a method to explore and to visualize similarities or dissimilarities of data (Westcott 1986). It starts with a similarity matrix or dissimilarity matrix (distance matrix) and assigns for each item a location in a low-dimensional space, e.g. as a 3D graphics (Gower 1966; Ibanmez et al 2001; Zuur et al 2007). PCOA is an eigen-analysis and computes a series of eigenvalues and eigenvectors that each eigenvalue has an eigenvector, and there are as several eigenvectors and eigenvalues. PCOA is a generalization of PCA and involves with measurement of similarity between variables. The main differences between PCOA and PCA are; (1) PCA explores for structure in the variables, PCOA explores for similarities between items, (2) PCA decreases variable dimensionality while PCOA analyses a distance matrix, and (3) the output of a PCOA is a set of coordinates on a number of derived axes such that similar cases are close together. It is not possible to associate these axes with any variables (Tabachnick & Fidell 2012).

Several investigations have studied the effects of environments on grain yield of different crops in arid and semi-arid regions and reported that the large magnitude of GE interaction are observed in these environmental conditions (Finlay & Wilkinson 1963; Becker & Leon 1988; Ilker et al 2011; Mladenov et al 2012; Mohebodini et al 2012; Sabaghnia et al 2012; Karimizadeh et al 2012b, Karimizadeh et al 2012c; Karimizadeh et al 2013; Sabaghnia et al 2013a).

The objectives in this study were to (i) evaluate the GE interaction for grain yield of durum wheat, (ii) evaluate the relationship of test environments for selecting superior genotypes within the mega-environment, and (iii) examine the results obtained with PCOA method.

2. Material and Methods

The data used in the yield analyses were recorded from 18 genotypes with two local check cultivars (Dehdasht and Seimareh) that grown for 4 cropping seasons (2009-2013) across five locations in Iran (Table 1). The locations were preventative of climatic and edaphic conditions in rain-fed durum wheat growing areas of Iran (Table 2). Moghan (Mn) in north of Iran has a sandy loam soil, Gonbad (Gd) in the north-east of Iran has a silty clay loam soil, that these areas were characterized by semi-arid conditions. Khorram abad (Kd) and Ilam (Im), in western Iran, Gachsaran (Gn), in southern of Iran, were relatively semi-arid and has silt loam, clay loam and silty clay loam soil respectivelly. The experimental design was a randomized complete block with four replicates. Seed density for each genotype was 300 seed per m^2 and planting was done experimental planters machine. Each plot size had six plant rows, 7.03 m length with row space of 17.5 cm at 80 kg ha^{-1} of phosphorus as triple super phosphate at planting time, first half 160 kg ha^{-1} of nitrogen as ammonium nitrate at tillering, and the other half at booting stage. No disease was shown during growth period, and weed control was made by chemical method. Clodinafop-propargyl ($C_{17}H_{13}C_1FNO_4$) and Tribenuron methyl ($C_{15}H_{17}N_5O_6S$) poisons by Topic and Granstar commercial names were used in field area. After physiological maturity, plots were harvested by WINTERSTEIGER AG trial thresher machine. Geographical properties in five locations presented in Table 2.

Analyses of variance were performed for each test environment. Initial statistical analyses including normality test using the Anderson-Darling normality test and homogeneity test of variances using Levene test were applied. After determination of homogeneity of residuals variance via Bartlett's homogeneity test, a combined ANOVA was performed. To partition out the year (Y), site (S), genotype (G) and their interactions effects, genotypes and sites were considered as fixed effects while years were considered as random effects. The PCOA was performed for stability analysis with computation of a measure of similarity between two genotypes, m and n, in a given test environment as equation 1 (Westcott 1986).

$$S_{i(m,n)} = [H_i - (m_i + n_i)/2] / (H_i - L_i) \quad (1)$$

Where; H_i is the highest mean yield of a genotype in test environment i; L_i is the lowest mean yield of a genotype in test environment i; m_i is the mean yield of genotype m in test environment i and n_i is the mean yield of genotype n in test environment i. Similarity index between two genotypes (m and n) was measured as the average of $S_{i(m,n)}$ across test environments when more than one test environment was used. The analysis was based on the sequential accumulation of the test environments according to their rank order, the environments being ranked in ascending order according to their overall means. Each cycle produced a two dimensional plot based on the first two PCOA scores and the minimum spanning tree plot was drawn for identification the most stable genotypes. All of the mentioned computations were done by GENSTAT 12.1 software (VSN International 2009).

3. Results and Discussion

The results of Anderson-Darling normality test and the Levene variances homogeneity test verified the assumptions of ANOVA and the combined analysis of variance was performed to determine the effects of environment, genotype, and GE interaction on grain yield of durum wheat genotypes. All of the studied effects including the main effects of genotype and environments as well as the GE interaction were highly significant (Table 3). Complexity of grain yield is a result of diverse processes that occur during plant development and the larger degrees of GE interaction cause to the more dissimilar the genetic systems which are controlling the physiological processes conferring adaptation to different test environments (Sabaghnia et al 2013b). The relative contributions of GE interaction effects for grain yield in this study were similar to those found in other crop adaptation investigations in rain-fed environments of semi-arid areas (Karimizadeh et al 2012a; Sabaghnia et al 2013a).

Table 1- Pedigree and origin of the 20 durum wheat genotypes studied in multi-environmental trials

Code	*Name/Pedigree*	*Origin*
G1	ACUATICO_1/RASCON_33//ACUATICO_1/3/AJAIA_12/F3LOCAL(SEL.ETHIO.135. 85)// PLATA_13CDSS96Y00570S-8Y-0M-0Y-1B-0Y-0B-0B	CIMMYT
G2	GAUNT_10/SNITANCDSS97Y00638S-4Y-0M-0Y-0B-0B-3Y-0BLR-1Y-0B	CIMMYT
G3	SOMO/CROC_4//LOTUS_1/3/KITTI/4/STOT//ALTAR 84/ALDCDSS99Y00636S-0M-0Y-34Y-0M-0Y-0B	CIMMYT
G4	CMH82A.1062/3/GGOVZ394//SBA81/PLC/4/AAZ_1/CREX/5/HUI//CIT71/CII/6/STOT//ALTAR 84/ ALDCDSS99Y00643S-0M-0Y-16Y-0M-0Y-0B	CIMMYT
G5	SRN_1/6/FGO/DOM//NACH/5/ALTAR 84/4/ GARZA/AFN//CRA/3/GGOVZ394/7/GEDIZ/ FGO//GTA/3/CNDO/8/GREEN_ 38/9/2*STOT//ALTAR 84/ALDCDSS00B00227T-0TOPY-0B-6Y-0M-0Y-1B	CIMMYT
G6	LLARETA INIA/YEBAS_8/3/MINIMUS_6 /PLATA_16//IMMERCDSS00Y01047T-0TOPB-5Y-0BLR-1Y-0B-0Y-1B-0Y	CIMMYT
G7	RASCON_21/3/MQUE/ALO//FOJACDSS94Y00099S-7M-0Y-0B-1Y-0B-0BLR-5Y-0B	CIMMYT
G8	GEDIZ/FGO//GTA/3/SRN_1/4/TOTUS/5/ENTE/MEXI_2//HUI/3/YAV_1/GEDIZ/6/SOMBRA_20/7/ STOT//ALTAR 84/ALDCD SS97Y00835 S-0TOPM-4Y-0M-0Y-0B-0B-3Y-0BLR-1Y-0B	CIMMYT
G9	STOT//ALTAR 84/ALD/3/THB/CEP7780// 2*MUSK_4CDSS99Y00366 S-3Y-0M-0Y-0BLR-1Y-0B-1M-0Y	CIMMYT
G10	ALTAR 84/STINT//SILVER_ 45/3/STOT// ALTAR 84/ALDCDSS99Y 00373S-7Y-0M-0Y-0BLR-6Y-0B-1B-0Y	CIMMYT
G11	STOT//ALTAR 84/ALD/3/GREEN_18/ FOCHA_1 //AIRON_1CDSS 99B00467S-0M-0Y-75Y-0M-0Y-2M-0Y	CIMMYT
G12	RASCON_21/3/MQUE/ALO//FOJA/4/GREEN_38/BUSHEN_4/5/CADO/BOOMER_33CDSS99B01055T-0TOPY-0M-0Y-10Y-0M-0Y-1M-0Y	CIMMYT
G13	STOT//ALTAR 84/ALD*2/3/AUK/GUIL// GREENCDSS00Y00786T-0TOPB-9Y-0BLR-5Y-0B-0Y-1M-0Y	CIMMYT
G14	SRN_1/6/FGO/DOM//NACH/5/ALTAR 84/4/ GARZA/AFN//CRA/3/GGOVZ394/7/GEDIZ/ FGO//GTA/3/CNDO/8/GREEN_38/9/2*STOT//ALTAR 84/ALDCDSS00B00227T-0TOPY-0B-28Y-0M-0Y-1M-0Y	CIMMYT
G15	AINZEN-1/SORD_3CDSS99B00317S-0M-0Y-104Y-0M-0Y-1M-0Y	CIMMYT
G16	PLATA_8/4/GARZA/AFN//CRA/3/GTA/5/RASCON/6/CADO/BOOMER_33/7/STOT//ALTAR 84/ ALDCDSS99B00843S-0TOPY-0M-0Y-5Y-0M-0Y-1B-0Y	CIMMYT
G17	ALTAR 84/STINT//SILVER_45/3/CBC 503 CHILE/4/AUK/GUIL// GREENCD SS99B01115T -0TOPY-0M-0Y-1Y-0M-0Y-1B-0Y	CIMMYT
G18	ALTAR 84/BINTEPE 85/3/ALTAR 84/STINT// SILVER_45/4/LHNKE/RASCON//CONA-DCD SS99B01265T-0TOPY-0M-0Y-12Y-0M-0Y-1M-0Y	CIMMYT
G19	Saimareh	Iran
G20	Dehdasht	Iran

Table 2- Agro-climatic properties of the location tested in Iran

Location	*Longitude latitude*	*Altitude (m)*	*Soil texture*	*Soil type*	*Rainfall (mm)*
Gachsaran	50° 50′ E 30° 20′ N	710	Silty Clay Loam	Regosols	455
Gonbad	55° 12′ E 37° 16′ N	45	Silty Clay Loam	Regosols	367
Khorram Abad	48° 12′ E 33° 29′ N	1125	Silt-Loam	Regosols	433
Ilam	46° 36′ E 33° 47′ N	975	Clay-Loam	Regosols	502
Moghan	47° 88′ E 39° 39′ N	100	Sandy-Loam	Cambisols	271

Table 3- Combined ANOVA of durum wheat performance trial yield data

Source	*df*	*SS*	*MS*
Genotype (G)	19	21661149	1140060**
Environment (E)	19	2346434521	123496554**
Genotype × Environment	361	123280605	341498**
Error	1140	115619600	101421
Total	1599	2694270223	1684972

**, significant at 1% probability level

The grain yield of durum wheat genotypes varied from 1065.3 kg ha^{-1} in genotype G16 grown at Ilam in the third year to 6598.3 kg ha^{-1} at Moghan in genotype G1 grown in the first year (The table is not shown). Average mean yields varied from 2994.7 kg ha^{-1} in G13 to 3470.3 kg ha^{-1} in G10 (Table 4). Minimum mean yields varied from 1065.3 kg ha^{-1} in genotype G16 to 1287.8 kg ha^{-1} in G12, while maximum mean yield varied from 4916.0 kg ha^{-1} in genotype G20 to 6598.3 kg ha^{-1} in genotype G1. Average yield was not correlated with minimum mean yield (r= 0.3924, P>0.05) and while significantly and positively correlated with maximum mean yield (r= 0.5926, P<0.01), and amplitude yield (r= 0.5616, P= 0.01). Minimum mean yield was not correlated with maximum mean yield (r= 0.3322, P>0.05), and amplitude yield (r= 0.2104, P>0.05). Maximum mean yield was very high positively correlated amplitude yield (r= 0.9920, P<0.001). Yield amplitudes were very large, from 3738.5 kg ha^{-1} for G20 to 5414.5 kg ha^{-1} in G1 (Table 4). The correlation results for grain yield found in this study were similar to those found in other investigations in rain-fed environments of semi-arid areas (Sabaghnia et al 2013a; Sabaghnia et al 2013b).

According to grand means and total mean yield (3192.2 kg ha^{-1}), test environments were grouped into two main groups as High mean yield (H) and Low mean yield (L). There were eleven H test environments and nine L test environments which analyzed in the sequential cycles. Grain yields are analyzed for the lowest test environment (cycle L1); the second cycle (L2), the third cycle (L3) the fourth cycle (L4) involves analyzing the four lowest environments, and so on. Minimum spanning tree plots for first six low cycles in Figure 1 and for other three low cycles in Figure 2 are shown. The differences in the lengths of the branches are grotesque relative to the differences between genotypes, because the minimum spanning tree is represented in two dimensions ignoring information in the next principal coordinates axis. Regarding

Table 4- Average, maximum, minimum and amplitude of grain yield in 20 durum wheat genotypes

Genotype	*Average*	*Minimum*	*Maximum*	*Amplitude*	*Genotype*	*Average*	*Minimum*	*Maximum*	*Amplitude*
G1	3263.5	1183.8	6598.3	5414.5	G11	3207.5	1109.0	5173.8	4064.8
G2	3097.6	1103.5	5312.8	4209.3	G12	3342.7	1287.8	6430.5	5142.8
G3	3073.7	1078.8	4959.8	3881.0	G13	2994.7	1147.0	5145.3	3998.3
G4	3230.8	1136.0	5803.0	4667.0	G14	3072.7	1189.5	4962.8	3773.3
G5	3203.0	1318.5	5449.5	4131.0	G15	3252.1	1115.5	5822.3	4706.8
G6	3065.6	1085.8	5987.3	4901.5	G16	3142.7	1065.3	5125.5	4060.3
G7	3153.0	1108.8	5383.8	4275.0	G17	3199.6	1130.5	5025.0	3894.5
G8	3239.2	1123.3	5239.5	4116.3	G18	3221.6	1141.0	5963.0	4822.0
G9	3404.2	1132.5	5805.3	4672.8	G19	3089.1	1141.3	4980.3	3839.0
G10	3470.3	1222.5	5857.4	4634.9	G20	3120.0	1177.5	4916.0	3738.5

this limitation, Flores et al (1996) suggested using a parameter as centroid distances which is benefits from all PCOA dimensions. Rather than including all nine scatter diagrams of L cycles, the stability structures of the genotypes are explained in the text and minimum spanning tree plots corresponding to all L cycles are shown.

In the minimum spanning tree plots for L cycles, the high-yielding genotypes which are furthest from the center (genotypes G12, G5 and G1) were detected as the high yielding genotypes in L1 cycle while genotypes G12, G5 and G4 were detected as the high yielding genotypes in L2 cycle (Figure 1). In the minimum spanning tree plots for other L

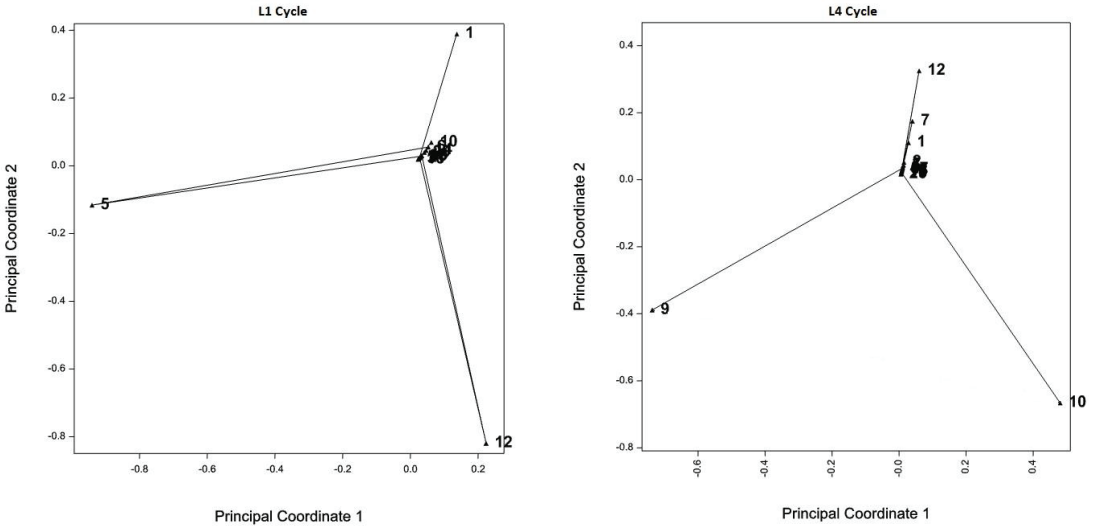

Figure 1- Example of minimum spanning tree of the first two PCOA axes for two low cycles

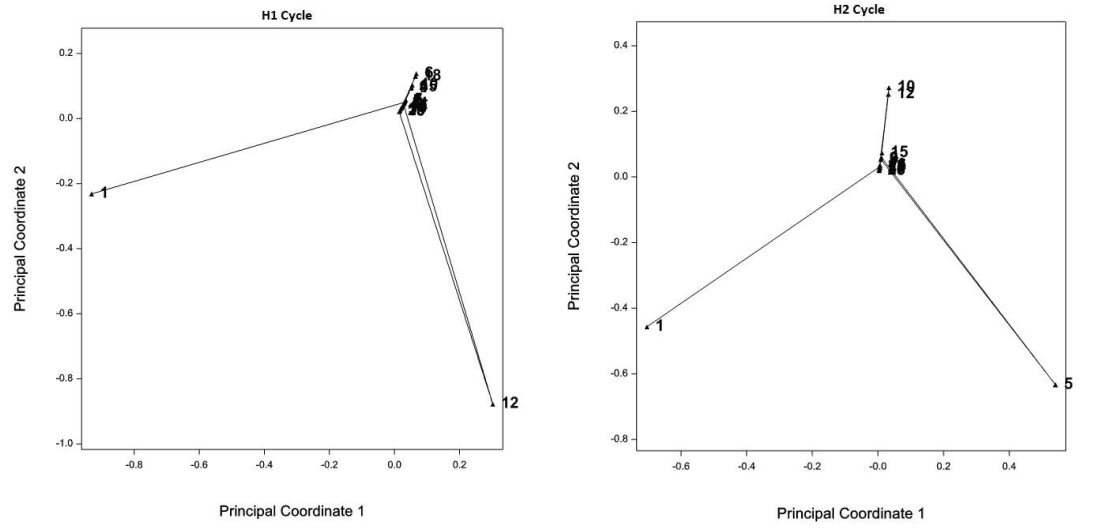

Figure 2- Example of minimum spanning tree of the first two PCOA for two high cycles

cycles, the high-yielding genotypes are those which are furthest from the center (genotypes G5, G1 and G12) were detected as the high yielding genotypes in L3 cycle while genotypes G10, G9 and G12 were detected as the high yielding genotypes in L4 cycle (Figure 1). For using all PCOA dimensions, Flores et al (1996) and Sabaghnia et al (2013a) used first two L cycles plots but in this research use of nine low cycle plots. Ranking of superior genotypes based on distance from center in low cycles was given in Table 5. According tothese ranks in all L cycles, genotypes G12, G10, G5 and G1 could be selected for poor environmental conditions and could be identified the most favorable genotypes with high mean yield and good stability.

In the minimum spanning tree plots for H cycles, the high-yielding genotypes which are furthest from the center (G1, G6 and G12) were detected as the high yielding genotypes in H1 cycle while genotypes G1, G10 and G5 were detected as the high yielding genotypes in H2 cycle (Figure 2). In the minimum spanning tree plots for other H cycles, the high-yielding genotypes G5, G10 and G12 were detected as the high yielding genotypes in H3 cycle, while genotypes G10, G7 and G17 were detected as the high yielding genotypes in H4 cycle (Figure 2). The high-yielding genotypes G7, G10 and G8 were detected as the high yielding genotypes in H5 cycle, while genotypes G10, G9 and G15 were detected as the high yielding genotypes in H6 cycle (Figure 2). For using all PCOA dimensions, Flores et al (1996), Mohebodini et al (2012) and Sabaghnia et al (2013b) used first two H cycles plots but in this research use of eleven high cycle plots.

Ranking of superior genotypes based on distance from center in H cycles was given in Table 6. Based on these values, genotype G10 on the list of the best genotypes was the best genotype in rich environmental conditions. Following to this favorable genotype (G10), genotypes G9 and G12 were located in the vertex positions for four times. Accordingly, Medina et al (1999) and Sabaghnia et al (2013a) noted that the results of the PCOA agree with those obtained using the other conventional

Table 5- Ranking of superior genotypes based on distance from center in low cycles

Genotype	*Yield*	*L1*	*L2*	*L3*	*L4*	*L5*	*L6*	*L7*	*L8*	*L9*	*No. of top position*
G1	3263.5	1	-	2	-	1	-	3	-	-	4
G2	3097.6	-	-	-	-	-	-	-	-	-	0
G3	3073.7	-	-	-	-	-	-	-	-	-	0
G4	3230.8	-	1	-	-	-	-	-	-	-	1
G5	3203.0	3	3	3	-	-	1	-	-	-	4
G6	3065.6	-	-	-	-	-	-	-	-	-	0
G7	3153.0	-	-	-	-	-	-	-	-	-	0
G8	3239.2	-	-	-	-	-	-	-	-	-	0
G9	3404.2	-	-	-	2	-	-	-	-	-	1
G10	3470.3	-	-	-	3	2	-	1	1	1	5
G11	3207.5	-	-	-	-	-	2	-	2	2	3
G12	3342.7	2	2	1	1	3	3	2	-	-	7
G13	2994.7	-	-	-	-	-	-	-	-	-	0
G14	3072.7	-	-	-	-	-	-	-	-	-	0
G15	3252.1	-	-	-	-	-	-	-	-	-	0
G16	3142.7	-	-	-	-	-	-	-	-	-	0
G17	3199.6	-	-	-	-	-	-	-	-	-	0
G18	3221.6	-	-	-	-	-	-	-	-	-	0
G19	3089.1	-	-	-	-	-	-	-	-	-	0
G20	3120.0	-	-	-	-	-	-	-	3	3	2
Mean	3192.2										

Table 6- Ranking of superior genotypes based on distance from center in high cycles

Genotype	*Yield*	*H1*	*H2*	*H3*	*H4*	*H5*	*H6*	*H7*	*H8*	*H9*	*H10*	*H11*	*No. of top position*
G1	3263.5	2	2	-	-	-	-	-	-	-	-	-	2
G2	3097.6	-	-	-	-	-	-	-	-	2	-	-	1
G3	3073.7	-	-	-	-	-	-	-	-	-	-	-	0
G4	3230.8	-	-	-	-	-	-	-	-	-	-	-	0
G5	3203.0	-	3	3	-	-	-	-	-	-	-	-	2
G6	3065.6	1	-	-	-	-	-	-	-	-	-	-	1
G7	3153.0	-	-	-	1	2	-	-	-	-	-	-	2
G8	3239.2	-	-	-	-	3	-	1	3	-	-	-	3
G9	3404.2	-	-	-	-	-	3	3	1	-	1	-	4
G10	3470.3	-	1	2	2	1	1	2	2	3	3	1	10
G11	3207.5	-	-	-	-	-	-	-	-	-	-	-	0
G12	3342.7	3	-	1	-	-	-	-	-	-	2	2	4
G13	2994.7	-	-	-	-	-	-	-	-	-	-	-	0
G14	3072.7	-	-	-	-	-	-	-	-	1	-	-	1
G15	3252.1	-	-	-	-	-	2	-	-	-	-	-	1
G16	3142.7	-	-	-	-	-	-	-	-	-	-	-	0
G17	3199.6	-	-	-	3	-	-	-	-	-	-	-	1
G18	3221.6	-	-	-	-	-	-	-	-	-	-	3	1
G19	3089.1	-	-	-	-	-	-	-	-	-	-	-	0
G20	3120.0	-	-	-	-	-	-	-	-	-	-	-	0
Mean	3192.2												

multivariate stability analysis such as AMMI (the additive main effects and multiplicative model) model or univariate stability analysis such as joint linear regression analysis.

4. Conclusions

There are different methods quantifying different components of the GE interaction. The principal coordinate analysis gives a simple measure of yield stability which allows a ranking of genotypes. This model is also an effective tool in understanding complex GE interactions in multi-environment trials of durum wheat.

The principal coordinate analysis as a stability method performed in the present study quantified yield stability of genotypes. The PCOA model provided useful information for reaching definitive conclusions. According to the present study, the best genotypes available for poor dryland environmental conditions are G12 and G10, while genotypes G10 and G9 are available for rich dryland environmental conditions.

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Genetic Effects Assessment through Line × Tester Combining Ability for Development of Promising Hybrids Based on Quantitative Traits in *Gossypium hirsutum* L.

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ABSTRACT

Line × Tester combining ability analysis involving five lines (cultivars i.e., CIM-446, CIM-473, CIM-506, CIM-554 and SLH-284) and three testers (cultivars viz., CIM-496, CIM-499 and CIM-707) was carried out during 2015 and 2016 to determine the inheritance for earliness, yield and lint traits in upland cotton. Genotypes revealed significant ($P \leq 0.01$) variations for all the traits. On average, F_1 hybrids showed the significant increase over parental means for yield traits. Mean squares due to general (GCA) and specific combining ability (SCA) were highly significant, which suggested that additive and non-additive gene actions were involved in controlling all the characters. However, the preponderance of non-additive type of gene action observed for majority of the traits. Lines (SLH-284, CIM-473) and pollinators (CIM-707, CIM-496) were leading general combiners for majority of the traits. F_1 hybrids (CIM-473 × CIM-496, SLH-284 × CIM-707 and CIM-446 × CIM-496) which involve best general combiners, showed the leading performance for yield and lint traits. Heritability was moderate to high with appreciable genetic gain for majority of the traits. Except for lint %, the correlation of seed cotton yield was positive with other traits. The significance of additive and non-additive components suggested integrated breeding strategies with delayed selection for development of cotton hybrids with improvement in earliness and seed cotton yield.

Keywords: Combining ability; General and specific combiners; Additive and nonadditive gene action; Earliness and yield traits; Upland cotton

1. Introduction

In Pakistan, the cotton research work has been started since independence and a large number of cultivars acquiring harmonious combination of characters were evolved. Nevertheless, our per unit seed cotton yield is still low as compared to other cotton growing countries. In 2017-18, the cotton crop was grown on 2.699 million hectares and seed cotton production was 11.935 million bales (170 kg) with average seed cotton yield of 752 kg ha^{-1} (PBS

2017-2018). Cotton production showed remarkable growth of 12.3% to 11.935 million bales over 2016-2017 production (10.671 million bales) (Economic Survey of Pakistan 2017-2018). Pakistan is facing a problem of low production as compared to other cotton growing countries. One of the main causes of low productivity is inferior cotton genotypes being grown in the country. To cope-up with the present level of production, it is imperative to develop new high yielding cultivars/hybrids with higher yield potential along with early maturity, desirable fiber quality and natural resistance to insect pests and diseases up to some extent.

Line × tester combining ability analysis is an appropriate breeding approach for choosing desirable parental genotypes and F_1 hybrids. General combining ability (GCA) is defined as average performance of a genotype in a series of cross combinations while specific combining ability (SCA) connotes those instances where certain hybrids are either better or poorer than would be expected on average performance in specific hybrid combinations (Usharani et al 2016). Thus, the SCA is important for hybrid crop development, whereas the GCA is useful for identification of potential parental genotypes for hybridization and then selection in segregating population. However, GCA is due to parental genes which are largely additive in nature, while SCA is due to new gene complexes with dominance or epistatic effects (Sprague & Tatum 1942). In addition, in combining ability the maternal effects were also explored (Griffing 1956). Later on the line × tester analysis was designed which is an extension to the said method in which several lines and testers are used to predict the GCA and SCA of genotypes and their F_1 cross combinations, respectively (Kempthorne 1957; Singh & Chaudhary 1985).

Combining ability of the inbred lines has been worked-out in cotton crop but most of the times the results remained discordant. Since the development of new cultivars through hybridization is a time consuming and continuous process, therefore information on the potentiality of new inbred lines through combining ability analysis becomes an important objective of the cotton breeders. Significance of the GCA and SCA variances, suggesting the importance of additive as well as dominant genes, nevertheless in their studies, ratio of GCA/SCA was greater than unity further indicating the preponderance of additive genes in the inheritance of seed cotton yield, bolls per plant, boll weight, seed index and lint % in cotton (Abro et al 2009; Basal et al 2009; Prakash et al 2018). However, appreciable degree of variance due to GCA was observed for these characters in upland cotton (Khan et al 2011; 2015). Contrary to above findings, the GCA and SCA variances were important yet the magnitude of SCA was higher than GCA implying the predominance of dominant genes controlling number of bolls, seed cotton yield, ginning outturn % and seed index (Panhwar et al 2008; Khan et al 2009a; Sivia et al 2017; Bilwal et al 2018; Roy et al 2018). Non-additive type of gene action for various earliness, morphological and yield related traits was observed in upland cotton populations (Khan et al 2017; Kumar et al 2017; Reddy et al 2017; Khokhar et al 2018). These controversial findings might be primarily due to mating designs, breeding material used, and the environment in which the breeding material was evaluated.

Mean squares due to GCA and SCA were also highly significant; however, the SCA variances were greater than GCA and more important for boll weight, boll number and seed cotton yield per plant, showing the predominance of non-additive gene action in cotton (Khan et al 2009b; Karademir et al 2016; Talpur et al 2016; Choudhary et al 2017; Lokesh et al 2018; Rajeev & Patil 2018). In some studies, high × low and low × high GCA parents performed well in SCA determination in upland cotton (Khan et al 2011). Mean squares due to GCA and SCA were highly significant, however, GCA mean squares were higher than SCA for majority traits which revealed that additive genes controlled the inheritance in upland cotton (Khan et al 2015). Many commercial cotton cultivars despite their high/low agronomic performance combine in a better way/poorly when used as a parental cultivars in cross combinations.

Heritability and the genetic variability studies of various genotypes in form of their expression for different morpho-yield traits are earnestly needed for selection of parental lines for breeding program. Knowledge about the genetic potential of different genotypes and inheritance of the morphological and yield traits is indispensable for the breeders to tackle with the problems of low yield (Khan et al 2009b; Thombre et al 2018). Substantial genetic variances and high heritability estimates implied that characters could be improved through selection from segregating populations. Heritability estimates sense were generally found to be high in magnitude in the *G. hirsutum* crosses comparative to *G. barbadense* L. for majority traits except for earliness and fiber % (Esmail 2007).

In view of the importance of knowing the combining ability of the parental genotypes, thereby determining the type of gene action involved in the expression of various plant characters in cotton, the line × tester analysis along with heritability and correlation studies was carried out to determine the efficient breeding strategies to improve the valuable characters. The objectives behind this research work were to determine a) genetic variability of parental lines, testers and their F_1 hybrids for earliness and yield traits, b) GCA of lines/testers and SCA of L × T interactions for various traits, c) best general and specific combiners, which could be used as a source material for further improvement in upland cotton.

2. Material and Methods

2.1. Breeding material and procedure

Present investigations were carried out during 2015 and 2016 at the University of Agriculture, Peshawar, Pakistan (Peshawar, 34°, 02′N, 71° and 37′E, Pakistan). Five upland cotton cultivars i.e. CIM-446, CIM-506, CIM-554, SLH-284 and CIM-473 were used as lines/female parents and three cultivars i.e., CIM-496, CIM-499 and CIM-707 as testers/pollinators (Table 1). These genotypes were hand sown in a crossing block during 2015, and were crossed through line × tester (5 × 3) methodology to make 15 F_1 crosses. During 2016, 23 genotypes (08 parents and 15 F_1 hybrids) were hand sown in a randomized complete block (RCB) design. Each Parent and F_1 hybrid was planted in four rows of five meter length, with three replications. The rows and plants spacing were 75 and 30 cm, respectively. Thinning was practiced after two weeks days when the plants gained the height of around 10 cm to ensure single plant per hill. Recommended cultural practices like fertilizer, hoeing, irrigation and pest control were applied uniformly to all the entries in order to minimize the environmental variations in the field. Picking was made during the month of November on individual plant basis and ginning was performed with eight saw-gins.

Table 1- Eight diverse genotypes of upland cotton used in 5 × 3 line × tester crosses

Cultivars	*Parentage*	*Breeding centre*	*Release (year)*	*Seed cotton yield (kg ha^{-1})*	*GOT (%)*	*Staple length (mm)*
Lines						
CIM-446	CP-15/2 × S-12	CCRI, Multan, Pakistan	1998	3,000	36.1	27.0
CIM-473	CIM-402 × LRA-5166	-do-	2002	3,000	39.7	29.5
CIM-506	CIM-360 × CP-15/2	-do-	2004	3,000	38.6	28.7
CIM-554	2579-04/97 × W-1103	-do-	2009	4,241	41.5	28.5
SLH-284	Not yet released	CRS, Sahiwal, Pakistan	-	3,707	39.0	28.5
Testers						
CIM-496	CIM-425 × 755-6/93	CCRI, Multan, Pakistan	2005	3,000	41.1	29.7
CIM-499	CIM-433 × 755-6/93	-do-	2003	3,000	40.0	29.6
CIM-707	CIM-243 × 738-6/93	-do-	2004	3,000	39.0	32.2

2.2. Traits measurement and data analysis

Data were recorded on ten randomly selected plant in central two rows on earliness (days to flowering), bolls per sympodia, bolls per plant, boll weight, seed cotton yield per plant and lint %. All the data were subjected to analysis of variance to test the null hypothesis of no differences among various F_1 hybrids and their parental cultivars (Steel et al 1997). The genotype means for each parameter were further separated and compared by using the least significant difference (LSD) test at 5% level of probability (Hayter 1986). Line × Tester analysis was carried out to ascertain the variances due to GCA and SCA for genotypes and gene action for various traits (Kempthorne 1957; Singh & Chaudhary 1985). For each trait the genetic, environmental and phenotypic variances, broad sense heritability and expected response to selection (Re) and its value as percentage were estimated according from the mean squares (Burton 1951). The correlation coefficient (r) of seed cotton yield with earliness and yield contributing traits was also worked out (Kwon & Torrie 1964).

3. Results and Discussion

Analysis of variance revealed significant ($P \leq 0.01$) variations among parental genotypes (lines and testers) and their F_1 hybrids (L × T interactions) for all the traits except lint % for which the mean differences were merely significant ($P \leq 0.05$) (Table 2). This provides evidence of sufficient genetic variability among lines, testers, and F_1 hybrids and allows further assessment through combining ability analysis. The total genetic variability was further partitioned into its components i.e., GCA and SCA. Past studies also revealed highly significant differences among line, testers and L × T interactions with greater genetic variability for earliness, yield and lint traits in upland cotton (Panhwar et al 2008; Samreen et al 2008; Ahuja et al 2009; Ashokkumar et al 2010; Karademir et al 2009; 2016; Sivia et al 2017; Khokhar et al 2018; Rajeev & Patil 2018).

3.1. Genetic variability among lines, testers and their F_1 hybrids

Days to flowering varied from 57.67 (CIM-473) to 71.67 days (SLH-284) among lines, testers ranged from 56.00 (CIM-499) to 59.67 days (CIM-496)

Table 2- Mean squares for various traits of line x tester analysis in upland cotton

Source of variation	*d.f*	*Days to flowering*	*Bolls sympodia^{-1}*	*Bolls plant^{-1}*	*Boll weight*	*Seed cotton yield plant^{-1}*	*GOT*
			Analysis of variance				
Replications	2	16.97 ns	0.39 ns	53.02*	0.16 ns	1052.24	4.07 ns
Genotypes	22	48.30**	1.40**	164.37**	0.20**	33303.31**	12.47*
CV%		4.49	16.10	13.15	9.45	30.60	7.00
			ANOVA for Line × Tester analysis				
Parents (P)	7	97.79	0.42	147.07	0.10	1732.10	8.46
P vs. C	1	308.41	2.92	192.25	1.25	9875.92	85.29
Crosses (C)	14	24.45**	1.79**	171.03**	0.18 ns	7828.99**	15.66**
Lines	4	37.08**	2.15 ns	156.86**	0.13 ns	5294.36**	14.23 ns
Testers	2	11.09 ns	0.97**	242.89**	0.05 ns	9727.13**	45.08**
Lines × Testers	8	21.48**	1.81**	160.15**	0.24**	8621.77**	9.02 ns
Error	44	6.77	0.16	14.61	0.11	1044.36	5.80
σ^2 GCA	-	0.217	-0.208	3.310	-0.013	-92.585	1.720
σ^2 SCA	-	4.903	0.55	48.513	0.043	2525.803	1.073
σ^2 GCA / σ^2 SCA	-	0.044	-0.038	0.068	-0.288	-0.037	1.602

*, **, significant at $P \leq 0.05$ and $P \leq 0.01$; ns, non-significant

while their F_1 hybrids varied from 53.33 to 65.33 days (Table 3). Overall, minimum days to flowering took by F_1 hybrids CIM-506 × CIM-707 (53.33 days) and CIM-554 × CIM-496 (54.00 days) and were found at par with nine other F_1 hybrids and one tester (CIM-499) ranging from 54.33 to 57.33 days. Maximum days to flowering were observed in line SLH-284 (71.67 days), followed by its F_1 hybrid SLH-284 × CIM-707 (65.33 days), two other lines i.e., CIM-446 (62.67 days) and CIM-506 (58.33 days) and one tester CIM-496 (59.67 days). Other genotypes showed medium days to flowering (57.67 to 58.33). Results further revealed that most of the F_1 hybrids showed early flowering and have more time for boll formation than lines and testers.

Table 3- Mean performance of parental cultivars and their F_1 hybrids for various traits in upland cotton

Parental cultivars/ F_1 hybrids	*Days to flowering*	*Bolls sympodia^{-1}*	*Bolls plant^{-1}*
	Lines		
CIM-446	62.67	2.55	36.26
CIM-473	57.67	1.68	22.86
CIM-506	58.33	2.36	32.23
CIM-554	58.00	1.54	30.52
SLH-284	71.67	2.48	29.19
	Testers		
CIM-496	59.67	2.38	25.25
CIM-499	57.00	2.19	13.31
CIM-707	56.67	2.58	24.67
	F_1 hybrids (Lines × Testers)		
CIM-446 × CIM-496	57.00	2.62	37.79
CIM-446 × CIM-499	54.33	1.86	23.54
CIM-446 × CIM-707	55.00	2.49	32.64
CIM-473 × CIM-496	55.33	3.50	45.33
CIM-473 × CIM-499	57.00	2.17	23.27
CIM-473 × CIM-707	56.67	2.91	37.97
CIM-506 × CIM-496	55.33	2.28	31.81
CIM-506 × CIM-499	58.33	1.76	20.95
CIM-506 × CIM-707	53.33	1.90	19.00
CIM-554 × CIM-496	54.00	3.38	30.51
CIM-554 × CIM-499	55.00	2.50	31.47
CIM-554 × CIM-707	57.67	2.07	30.75
SLH-284 × CIM-496	58.00	1.96	21.64
SLH-284 × CIM-499	57.33	3.32	29.52
SLH-284 × CIM-707	65.33	4.48	38.17
$LSD_{0.05}$	4.283	0.686	6.289

Bolls per sympodia ranged from 1.54 (CIM-554) to 2.55 (CIM-446) among lines, testers ranged from 2.19 (CIM-499) to 2.58 (CIM-707) while their F_1 hybrids obtained 1.76 to 4.48 bolls per sympodia (Table 3). Overall, maximum bolls per sympodia were recorded for F_1 hybrid SLH-284 × CIM-707 (4.48) followed by four other F_1 hybrids (CIM-473 × CIM-496, CIM-554 × CIM-496, SLH-284 × CIM-499, and CIM-473 × CIM-707) ranging from 2.91 to 3.50. Minimum bolls per sympodia were achieved by line CIM-554 (1.54) and it was similar in performance with line (CIM-473) and six F_1 hybrids ranging from 1.76 to 2.17. Other genotypes showed medium bolls per sympodia (2.19 to 2.28).

Bolls per plant ranged from 22.86 (CIM-473) to 36.26 (CIM-446) in lines, 13.31 (CIM-499) to 25.25 (CIM-496) in testers, and 19.00 (CIM-506 × CIM-707) to 45.33 (CIM-473 × CIM-496) in F_1 hybrids (Table 3). Among F_1 hybrids, the highest number of bolls per plant was recorded for F_1 hybrid CIM-473 × CIM-496 (45.33). With at par performance, the promising hybrid was followed by four other F_1 hybrids (SLH-284 × CIM-707, CIM-473 × CIM-707, CIM-446 × CIM-496, CIM-446 × CIM-707) and two lines (CIM-446, CIM-506) ranging from 32.23 to 38.17 bolls per plant. Minimum bolls per plant (13.31) were obtained by tester (CIM-499) and was found same with F_1 hybrid CIM-506 × CIM-707 (19.00). Other genotypes were found with medium number of bolls per plant.

Boll weight varied from 2.88 (CIM-554) to 3.53 g (CIM-446) in lines, 3.22 (CIM-707) to 3.33 g (CIM-499) in testers, while in F_1 hybrids the range was 3.21 to 4.12 g (Table 4). Maximum boll weight (4.12 g) was observed in F_1 hybrid CIM-473 × CIM-499 and it was found same with five other F_1 hybrids (CIM-554 × CIM-707, CIM-446 × CIM-499, CIM-506 × CIM-496, CIM-473 × CIM-496 and SLH-284 × CIM-707) ranging from 3.62 to 3.83 g. Minimum boll weight was shown by line CIM-554 (2.88 g) and it was found at par with three testers i.e., CIM-707, CIM-496 and CIM-499. The remaining genotypes exhibited medium boll weight.

Table 4- Mean performance of parental cultivars and their F_1 hybrids for various traits in upland cotton

Parental cultivars/ F_1 hybrids	*Boll weight (g)*	*Seed cotton yield plant^{-1} (g)*	*GOT (%)*
	Lines		
CIM-446	3.53	125.90	33.28
CIM-473	3.28	79.96	35.59
CIM-506	3.27	109.60	37.42
CIM-554	2.88	101.40	32.73
SLH-284	3.36	101.20	32.55
	Testers		
CIM-496	3.26	103.80	35.68
CIM-499	3.33	46.77	34.49
CIM-707	3.22	81.51	35.66
	F_1 hybrids (Lines × Testers)		
CIM-446 × CIM-496	3.47	136.39	37.12
CIM-446 × CIM-499	3.74	76.81	33.03
CIM-446 × CIM-707	3.30	119.50	28.91
CIM-473 × CIM-496	3.71	198.50	34.80
CIM-473 × CIM-499	4.12	84.54	33.44
CIM-473 × CIM-707	3.47	146.40	32.58
CIM-506 × CIM-496	3.73	126.90	34.08
CIM-506 × CIM-499	3.22	66.58	32.18
CIM-506 × CIM-707	3.51	70.92	33.59
CIM-554 × CIM-496	3.21	104.20	35.80
CIM-554 × CIM-499	3.50	118.20	37.32
CIM-554 × CIM-707	3.83	101.70	33.53
SLH-284 × CIM-496	3.36	73.70	37.33
SLH-284 × CIM-499	3.48	104.38	36.15
SLH-284 × CIM-707	3.62	150.00	33.28
$LSD_{0.05}$	0.535	53.18	3.948

For seed cotton yield per plant, the lines were in the range of 79.96 (CIM-473) to 125.90 g (CIM-446), testers ranged from 46.77 (CIM-499) to 103.80 g (CIM-496), while their 15 F_1 hybrids ranged from 66.58 to 198.50 g (Table 4). Maximum seed cotton yield per plant was exhibited by F_1 hybrid CIM-473 × CIM-496 (198.50 g). The said promising F_1 hybrid was found equal in performance with two other F_1 cross combinations SLH-284 × CIM-707 (150.00 g) and CIM-473 × CIM-707 (146.40 g) for seed cotton yield per plant. Lowest seed cotton yield per plant was recorded in tester CIM-499 (46.77 g) and was found at par with one each line (CIM-473) and tester (CIM-707) and five F_1 hybrids having involvement of almost above tester and line ranged from 66.58 to 84.54 g. However, other genotypes showed medium seed cotton yield per plant.

In case of lint %, lines ranged from 32.55 (SLH-284) to 37.42% (CIM-506), testers ranged from 34.49 (CIM-499) to 35.68% (CIM-496), while their F_1 cross combinations were in the range of 28.91 to 37.33% (Table 4). Maximum lint % was recorded for line CIM-506 (37.42%) and it was found at par with nine other F_1 hybrids (SLH-284 × CIM-496, CIM-554 × CIM-499, CIM-446 × CIM-496, SLH-284 × CIM-499, CIM-554 × CIM-496, CIM-473 × CIM-496, CIM-506 × CIM-496, CIM-506 × CIM-707 and CIM-554 × CIM-707) and two each lines and testers ranged from 35.53 to 37.42%. Minimum lint % was recorded for F_1 hybrid CIM-446 × CIM-707 (28.91%) and was found at par with two each lines (SLH-284 and CIM-554) and F_1 hybrids (CIM-506 × CIM-499, CIM-473 × CIM-707) ranged from 28.91 to 32.73%. Other genotypes revealed medium values for lint %.

Overall, F_1 hybrid CIM-473 × CIM-496 manifested maximum values for bolls and seed cotton yield per plant, and was also found 2nd scoring hybrid for boll weight and earliness. The F_1 hybrid SLH-284 × CIM-707 showed maximum boll weight, and average values for yield related traits. The F_1 hybrid CIM-446 × CIM-496 revealed maximum lint %, and average values for yield contributing traits.

Using broad based genotype as a tester, the GCA of the lines is tested in the top cross method. The line × tester analysis is an extension of this method in which several testers are used (Kempthorne 1957; Singh & Chaudhary 1985). The L × T design provides information about GCA and SCA of the lines and testers and their F_1 hybrids, respectively and helpful in estimating the gene effects involved in the inheritance of various traits. Combining ability in general play key role in identifying the precious genotypes having specific cross combinations that can be used for hybrid program and for further selection in segregating generations. The L × T combining ability also work as principal method for improved production of crops in the form of F_1 hybrids and for screening the germplasm to

determine the ability of the parental genotypes to be included in a breeding program based on their desirable GCA and SCA effects.

Yield is a highly complex character and is directly influenced by the various morphological and yield contributing traits. Therefore, knowledge about the genetic potential of different genotypes and inheritance of the various morpho-yield traits is indispensable for the breeders to tackle with the problems of low yield (Ahuja et al 2009; Khan et al 2017; Swetha et al 2018). In present results, the lines, testers and their interaction showed significant mean squares and greater genetic variations revealed by genotypes and their F_1 hybrids for majority traits. Past findings elucidated significant mean squares for combining ability and genotypic variability in upland cotton populations (Samreen et al 2008; Ahuja et al 2009; Reddy et al 2017). Overall, the F_1 hybrids i.e., CIM-473 × CIM-496, SLH-284 × CIM-707 and CIM-446 × CIM-496 revealed best performance for earliness, yield and lint traits. In past studies, significant variances were observed among F_1 hybrids and their parental genotypes for bolls per plant, boll weight, seed cotton yield and lint % in upland cotton (Khan et al 2009a, 2009b; Choudhary et al 2017). Similarly significant differences were noted among parental cultivars and their F_1 hybrids for morphological and yield traits in upland cotton (Talpur et al 2016; Sivia et al 2017). Greater genetic variability indicated variable performance of the F_1 populations and their parental lines for yield and lint traits and their vital role in managing seed cotton yield in upland cotton (Basal et al 2009; Ashokkumar et al 2010).

3.2. Combining ability analysis

Combining ability of the parental genotypes i.e., lines and testers (GCA) and their F_1 hybrids (SCA) was studied through line × tester analysis to determine the ability of parent cultivars to combine their favorable genes during the hybridization and their transfer to the F_1 progenies. Two types of combining abilities i.e. GCA and SCA were studied, the GCA is due to additive gene effects whereas SCA is due to dominant and epistatic gene effects. Female lines and male pollinators having maximum GCA effects were considered as the best general combiners for the said trait. However, the F_1 hybrids having maximum SCA effects were treated as specified best hybrid combination for the concerned trait. The proportional contribution of lines, testers and their L × T interactions to total genetic variance for different traits was also studied which revealed the share of each component.

According to mean squares of combining ability, lines showed highly significant differences for days to flowering, bolls and seed cotton yield per plant, while non-significant for bolls per sympodia, boll weight and lint % (Table 2). Testers also revealed highly significant differences for bolls per sympodia, bolls and seed cotton yield per plant and lint %. The line × tester interactions showed highly significant differences for earliness, bolls per sympodia, bolls per plant, boll weight and seed cotton yield per plant. These results authenticated sufficient genetic variability among lines, testers, and F_1 hybrids and allows further assessment through general combining ability.

For majority traits, the variances due to GCA were lower than SCA, suggesting the preponderance of non-additive gene action in controlling these variables (Table 2). Therefore, it appeared that inheritance of all the studied characters was controlled by non-additive gene effects. These results were supported by ratio of variance of GCA to SCA which was smaller than unity. Such type of gene action clearly indicated that selection of superior genotypes in terms of seed cotton yield and its contributing traits should be postponed to the later generations, where these traits can be improved by making selections among the recombinants within the segregating populations. However, for lint % the variance due to GCA was greater than SCA, and it was also confirmed by ratio of variance of GCA to SCA which was greater than unity.

The proportional contribution of L × T interactions was maximum to total sum of squares for days to flowering, bolls per sympodia, bolls per plant, boll weight and seed cotton yield per plant,

followed by lines (Table 5). However, for lint %, testers were the major contributors followed by their interaction with lines. These results showed that lines × testers interaction and lines brought much variation in the expression of the studied traits.

In proportional contribution of various components, L × T interaction contributed more to the total sum square of majority traits as compared to lines and testers, and provided room for further improvement through intensive selection. Significant differences of line x tester interaction was observed for all the traits under the study indicating the prevalence of non-additive variance (Reddy et al 2017; Sivia et al 2017; Prakash et al 2018). Earliness and yield related traits (days to flowering, bolls per sympodia, bolls per plant, boll weight and seed cotton yield per plant) were controlled by nonadditive genes by manifesting maximum SCA variances than GCA. Similarly, in various past studies the nonadditive type of gene action was observed for yield and yield contributing traits in upland cotton (Usharani et al 2016; Chattha et al 2018; Prakash et al 2018). Combining ability with nonadditive type of gene action for yield and fiber related traits were observed in upland cotton (Samreen et al 2008; Karademir et al 2009; Karademir & Gener 2010). However, some studies revealed additive type of gene action for yield related traits in F_1 and F_2 populations of upland cotton (Lukonge et al 2007; Khan et al 2009a; Kumar et al 2017). The GCA variances were higher than SCA and revealed preponderance of additive gene action with enough genetic variability for most of the yield and fiber traits in *Gossypium hirsutum* L. (Esmail et al 2007). Such contradictions might be due to varied genetic background of breeding material and environmental conditions under which the experiments were conducted.

3.2.1. General combining ability

For days to flowering, the lines GCA ranged from -1.2 to 3.58, while in testers the GCA ranged from -0.71 to 0.96 (Table 6). For earliness, negative GCA is desirable which encourage earliness and fewer days to flowering. Maximum negative GCA was revealed by lines CIM-446 (-1.20) and CIM-554 (-1.09). However, positive GCA was revealed by one each line SLH-284 (3.58) and tester CIM-707 (0.96), respectively. All other genotypes showed negative GCA effects. For bolls per sympodia, three out of five lines showed positive GCA values and ranged form 0.03 to 0.64, while in testers the positive GCA values of 0.14 and 0.16 were obtained by genotypes CIM-496 and CIM-707, respectively. Overall, maximum GCA effects of 0.64 and 0.16 reveled by line SLH-284 and tester CIM-707, respectively for bolls per sympodia. For bolls per plant, lines GCA ranged from -6.37 (CIM-506) to 5.24 (CIM-473), testers GCA ranged from -4.54 to 3.13. Overall, highest positive GCA effects of 5.24 and 3.13 were exhibited by line CIM-473 and tester CIM-496, respectively for bolls per plant. Except two lines and one tester, all other genotypes showed positive GCA effects for bolls per plant.

In case of boll weight, lines GCA ranged from -0.07 to 0.22 while testers varied from -0.05 to 0.06 (Table 6). Except line CIM-473 (0.22) and tester CIM-499 (0.06), all other lines and testers

Table 5- Proportional contribution of lines, testers and their L × T interaction (%) for various traits in upland cotton

Lines / Testers & L × T interaction	*d.f.*	*Days to flowering*	*Bolls sympodia^{-1}*	*Bolls plant^{-1}*	*Boll weight*	*Seed cotton yield plant^{-1}*	*GOT*
Lines	4	43.33	34.41	26.20	21.21	19.32	25.96
Testers	2	6.48	7.74	20.29	3.83	17.75	41.12
Lines × Testers	8	50.19	57.85	53.51	74.96	62.93	32.92

Table 6- General combining ability effects of lines and testers for various traits in upland cotton

Parental cultivars	*Days to flowering*	*Bolls sympodia^{-1}*	*Bolls plant^{-1}*	*Boll weight*	*Seed cotton yield plant^{-1}*	*GOT*
			Lines			
CIM-446	-1.2	-0.29	1.03	-0.05	-7.96	-1.19
CIM-473	-0.31	0.25	5.24	0.22	24.3	-0.60
CIM-506	-0.98	-0.63	-6.37	-0.06	-30.74	-0.93
CIM-554	-1.09	0.03	0.62	-0.04	-10.83	1.34
SLH-284	3.58	0.64	-0.51	-0.07	25.24	1.38
			Testers			
CIM-496	-0.71	0.14	3.13	-0.05	9.07	1.62
CIM-499	-0.24	-0.29	-4.54	0.06	-28.76	0.21
CIM-707	0.96	0.16	1.41	-0.01	19.69	-1.83

showed negative GCA effects for boll weight. For seed cotton yield, GCA ranged from -30.74 to 25.24 and -28.76 to 19.69 among lines and testers, respectively. However, two each lines SLH-284 (25.24) and CIM-473 (24.30) and testers CIM-707 (19.69) and CIM-496 (9.07) showed maximum positive GCA effects and excelled other parental cultivars for yield. In lint %, two each lines SLH-284 (1.38) and CIM-554 (1.34) and testers CIM-496 (1.62) and CIM-499 (0.21) manifested highest positive GCA effects, while other lines and testers revealed negative GCA effects.

Overall, line SLH-284 revealed maximum positive GCA effects for bolls per sympodia, seed cotton yield and lint %, and was found as best general combiner (Table 6). The maternal parent CIM-473 was found as 2nd leading general combiner by having maximum positive GCA effects for bolls per plant, boll weight and desirable negative GCA values for earliness. In case of testers, cultivar CIM-496 was also appeared to be the best general pollinator for bolls per plant, lint % and desirable negative GCA value for days to flowering. The tester CIM-707 was 2nd best general pollinator and revealed maximum positive GCA effects for bolls per sympodia and seed cotton yield per plant. Therefore, earliness and yield related traits could be further improved by identifying best general combiners and to use them hybridization program.

According to GCA effects, the lines SLH-284 and CIM-473 were found as best general combiners and exhibited highest positive GCA effects for maximum traits. In mean performance, cultivar SLH-284 also expressed maximum mean values in its F_1 derivatives by crossing with CIM-707 and CIM-496 for bolls per sympodia and lint %, respectively. The line CIM-473 also showed prominent contribution through its promising F_1 hybrids by crossing with CIM-496 for bolls and seed cotton yield per plant, and CIM-499 for boll weight. Results revealed that lines SLH-284 and CIM-473 could be safely used as best general combiners in breeding program for development of superior specific hybrids. F_1 hybrids involve general combiners in combination with other cultivars showed highest mean performance for yield related traits in upland cotton (Khan et al 2009a; 2009b).

In case of testers, the pollinators CIM-496 and CIM-707 were appeared to be the best general combiners for having desirable GCA for majority traits. The tester CIM-496 manifested remarkable mean performance by crossing with lines CIM-473 and SLH-284 for bolls and seed cotton yield per plant and lint %. In average mean performance, the tester CIM-707 showed best recital as maternal parent by crossing with tester CIM-506 and revealed early maturity. In present studies, it was also concluded that high × high, high × low and low × high general combiners performed better in

manifestation of specific F_1 hybrids for yield and lint traits. Previous studies also revealed that for getting good cross combination, at least one of the parents should have good GCA effect i.e., high × high, high × low and low × high GCA parents in cotton (Chinchane et al 2018). In past studies, in some cases high × low, low × high and high × high GCA parents showed best performance for majority traits in upland cotton (Panhwar et al 2008; Khan et al 2011, 2015; Karademir et al 2016). Therefore, the said traits can be further improved and strengthened by using the general combiners. In upland cotton, the parental genotypes with best GCA, used as a pollen parent produced better cross combinations, however, higher GCA of parents does not necessarily confer higher SCA, and the GCA and SCA were independent (Basal et al 2009; Khan et al 2009a; Sivia et al 2017).

3.2.2. Specific combining ability

For days to flowering, nine out of fifteen F_1 hybrids revealed desirable negative SCA effects (ranging from -0.29 to -3.29) to boost early maturity (Table 7). However, six F_1 hybrids showed positive SCA effects (0.38 to 4.16). Desirable maximum negative SCA effects were possessed by F_1 hybrids i.e., CIM-506 × CIM-707 (-3.29), SLH-284 × CIM-499 (-2.64) and SLH- 284 × CIM-496 (-1.51) and were found as best specific cross combinations for early maturity. At GCA level, the maternal line CIM-506 and testers CIM-499 and CIM-496 have manifested desirable negative GCA effects for days to flowering and played best response in presentation of desirable negative SCA effects in their F_1 hybrids through involvement as high × low and low × high general combiners.

For bolls per sympodia, nine F_1 hybrids revealed positive SCA effects ranging from 0.07 to 0.60, while negative SCA effects (-0.11 to -1.43) revealed by six F_1 hybrids (Table 7). However, F_1 hybrids CIM-554 × CIM-496 (0.60), CIM-473 × CIM-496 (0.50) and SLH-284 × CIM-499 (0.63) enunciated maximum positive SCA effects and were established as best specific combiners for bolls per sympodia. The involvement of best general combiners SLH-284 and CIM-473 revealed the F_1 hybrids with promising SCA values. Therefore, low × high, high × high and high × low general combiners revealed promising SCA for bolls per sympodia.

Table 7- Specific combining ability effects of lines x testers for various traits in upland cotton

F_1 hybrids	*Days to Flowering*	*Bolls sympodia^{-1}*	*Bolls plant^{-1}*	*Boll weight*	*Seed cotton yield plant^{-1}*	*GOT*
CIM-446 × CIM-496	2.27	0.16	3.34	0.02	16.42	2.48
CIM-446 × CIM-499	-0.87	-0.17	-3.24	0.18	-5.33	-0.20
CIM-446 × CIM-707	-1.40	0.01	-0.10	-0.20	-11.09	-2.28
CIM-473 × CIM-496	-0.29	0.50	6.68	0.00	46.29	-0.42
CIM-473 × CIM-499	0.91	-0.40	-7.71	0.29	-29.86	-0.38
CIM-473 × CIM-707	-0.62	-0.11	1.03	-0.29	-16.43	0.80
CIM-506 × CIM-496	0.38	0.17	4.76	0.30	29.67	-0.82
CIM-506 × CIM-499	2.91	0.07	1.57	-0.33	7.22	-1.32
CIM-506 × CIM-707	-3.29	-0.23	-6.34	0.03	-36.89	2.14
CIM-554 × CIM-496	-0.84	0.60	-3.53	-0.25	-12.90	-1.37
CIM-554 × CIM-499	-0.31	0.14	5.10	-0.07	38.93	1.56
CIM-554 × CIM-707	1.16	-0.74	-1.57	0.32	-26.03	-0.19
SLH-284 × CIM-496	-1.51	-1.43	-11.26	-0.07	-79.47	0.12
SLH-284 × CIM-499	-2.64	0.36	4.28	-0.06	-10.96	0.35
SLH-284 × CIM-707	4.16	1.07	6.98	0.14	90.43	-0.47

It is obvious from Table 7 that eight F_1 hybrids exhibited positive SCA effects (1.03 to 6.98) for bolls per plant, while remaining seven F_1 hybrids exhibited negative SCA effects (-0.10 to -11.26). Maximum positive SCA effects were possessed by F_1 cross combinations i.e. SLH-284 × CIM-707 (6.98), CIM-473 × CIM-496 (6.68) and CIM-554 × CIM-499 (5.10) and were found as best specific combiners for bolls per plant. The line CIM-473 and testers CIM-496 and CIM-707 were already announced as best general combiners and the same stability performance have shown by these lines and pollinators as low × high, high × high and high × low general combiners.

For boll weight, eight F_1 hybrids exhibited positive SCA effects ranged from 0.02 to 0.32, while seven F_1 hybrids showed negative SCA values (-0.06 to -0.33) (Table 7). Highest SCA effects owned by F_1 hybrids CIM-554 × CIM-707 (0.32), CIM-506 × CIM-496 (0.30) and CIM-473 × CIM-499 (0.29). For the former two hybrids the parental genotypes i.e., CIM-554, CIM-707, CIM-506 and CIM-496 have shown negative GCA effects, whereas in later hybrid the genotypes CIM-473 and CIM-499 have highest GCA effects. Therefore, low × low and high × high GCA parents were involved in producing F_1 hybrids with desirable SCA effects for boll weight.

For seed cotton yield per plant, positive SCA effects were manifested by six F_1 hybrids ranged from 7.22 to 90.43, while nine F_1 hybrids revealed negative SCA effects (-5.33 to -79.47) (Table 7). However, the utmost desirable SCA effects were shown by F_1 hybrids i.e., SLH-284 × CIM-707 (90.67), CIM-473 × CIM-496 (46.29), CIM-554 × CIM-499 (38.93) and CIM-506 × CIM-496 (29.67) for seed cotton yield per plant. Results further revealed that high × high, high × low, low × high and low × low GCA parental genotypes were involved in manifestation of F_1 hybrids with desirable SCA effects for seed cotton yield.

For lint %, six F_1 hybrids revealed positive SCA with range of 0.12 to 2.48, while negative SCA values (-0.19 to -2.28) were noted in nine F_1 hybrids (Table 7). However, F_1 hybrids CIM-446 × CIM-496 (2.48), CIM-506 × CIM-707 (2.14) and CIM-554 × CIM-499 (1.56) were found as best specific combiners by having maximum SCA effects and excelled all other F_1 hybrids for lint %. In above F_1 hybrids, the lines (CIM-446, CIM-506 and CIM-554), and testers (CIM-496, CIM-707 and CIM-499) used were having low GCA effects except line CIM-554 and tester CIM-496 which has maximum positive GCA effects.

Overall, the F_1 hybrid SLH-284 × CIM-707 revealed desirable SCA effects for bolls per sympodia, bolls per plant and seed cotton yield per plant (Table 7). The 2nd best scoring F_1 hybrids were CIM-473 × CIM-496 (bolls and seed cotton yield per plant), CIM-506 × CIM-707 (earliness and lint %), CIM-554 × CIM-496 (bolls per sympodia), CIM-554 × CIM-707 (boll weight) and CIM-446 × CIM-496 (lint %) which showed desirable SCA effects for earliness and yield traits.

In present studies, F_1 hybrid SLH-284 × CIM-707 was the leading cross combination for having desirable SCA effects for majority of the traits and involve high × high GCA parents. The high × high general combiners were involved in the expression of majority morphological and yield related traits in *Gossypium hirsutum* L. (Karademir et al 2009; Khan et al 2009b). Present studies also proved that parental cultivar with best GCA and its utilization as one of the parents produced superior F_1 hybrids. However, in some previous studies, it was concluded that both parents with higher GCA were found to produce high yielding F_1 hybrids in upland cotton (Lukonge et al 2007; Abro et al 2009; Basal et al 2009). The promising F_1 hybrids always involve at least one parent with high/average GCA for a particular trait in cotton. Some lines and testers by having desirable GCA effects performed better and provided maximum genetic variability in F_1 hybrids. However, in contradiction, parental genotypes performance in their specific F_1 hybrids exhibited that it is always not necessary that general combiners will provide best F_1 hybrids, however, parents with low GCA may have the potential to produce promising hybrids (Karademir & Gener 2010; Talpur et al 2016; Reddy et al 2017).

The 2nd best scoring F_1 hybrids were CIM-473 × CIM-496 (bolls and seed cotton yield per plant) and CIM-506 × CIM-707 (earliness and lint %) which showed highest SCA effects. In the remaining populations, F_1 hybrids CIM-554 × CIM-496 for bolls per sympodia, CIM-554 × CIM-707 for boll weight and CIM-446 × CIM-496 for lint %, showed desirable SCA effects. Therefore, these F_1 hybrids could be further explored in segregating generations for improvement in their genetic potential for said traits. In these F_1 hybrids, lines (SLH-284 and CIM-473) and testers (CIM-707 and CIM-496) were the best general combiners. General combiners involvement itself (high × high) and also as high × low and low × high GCA parents manifested desirable SCA for majority yield traits. However, the SCA effects authenticated that best specific combinations were having high × low and low × high GCA parents and performed well in SCA determination, with outstanding mean performance and heterosis (Abro et al 2009; Basal et al 2009; Karademir et al 2009). Significant SCA differences were determined among F_1 hybrids for morpho-yield and fiber quality traits (Lukonge et al 2007; Ahuja et al 2009).

3.3. Heritability and correlation

Heritability is the proportion of phenotypic variation in a population that is attributable to genetic variation among individuals. For each trait, genetic, environmental and phenotypic variation, heritability broad sense (bs) and expected response to selection were estimated from mean squares (Table 8). Overall, heritability ranged from 0.45 to 0.91 for various traits. For days to flowering, the genetic variance (13.84) was two times greater than environmental variance (6.77). Heritability (bs) for said trait was also high (0.86) with genetic advance of 4.83 days and its value as percent of population mean was 8.34%. High broad sense heritability (0.89) and moderate expected response to selection (0.82#, 33.60%) were recorded for bolls per sympodia. For bolls per plant, the genetic variance (49.92) was almost three times greater than environmental variance (14.60) with high broad sense heritability (0.91) and selection response values of 9.44# and 32.48%. Heritability (bs) for boll weight was moderate (0.45) with genetic gain values of 0.16 g and 4.71%. In seed cotton yield, the genetic variance (2259.95) was two times greater than environmental variance (1044.36) with high broad sense heritability (0.68) and selection response values (31.77 g, 30.09%). For lint %, heritability (bs) was 0.54 and expected response to selection was 1.54 its value as percent of population mean was 4.48%. Correlation of seed cotton yield was highly significant positive with bolls per sympodia (r= 603) and bolls per plant (r= 0.720), nonsignificant positive with days to flowering (r= 0.202) and boll weight (r= 0.186) (Table 9). However, correlation of lint % (r= -0.060) was negative and nonsignificant with seed cotton yield. Overall, high heritability with appreciable genetic advance and positive association of yield with yield components and earliness revealed that yield traits could be improved through selection in early segregating populations.

Table 8- Genotypic (Vg), environmental (Ve) and phenotypic (Vp) coefficient of variance, heritability (bs), genetic advance (G.A) and its percentages

Parameters	*Vg*	*Ve*	*Vp*	*Heritability (bs)*	*G.A.*	*G.A. (%)*
Days to flowering	13.84	6.77	16.10	0.86	4.83 days	8.34
Bolls sympodia^{-1}	0.39	0.15	0.44	0.89	0.82 #	33.60
Bolls plant^{-1}	49.92	14.61	54.79	0.91	9.44 #	32.48
Boll weight	0.03	0.11	0.06	0.45	0.16 g	4.71
Seed cotton yield plant^{-1}	753.01	1044.36	1101.13	0.68	31.77 g	30.09
Lint %	2.24	5.76	4.16	0.54	1.54%	4.48

Table 9- Correlation of various traits with seed cotton yield in upland cotton

Parameters	*Correlation (r) with seed cotton yield plant^{-1}*	*Standard error*
Days to flowering	0.202	0.010
Bolls sympodia^{-1}	0.603**	0.001
Bolls plant^{-1}	0.720**	0.013
Boll weight	0.186	0.001
Lint %	-0.060	0.006

Heritability and genetic potential studies of various cotton cultivars in form of their expression for various yield traits are earnestly needed for selection of parental lines for successful breeding program (Hague et al 2008). Present findings revealed high heritability (bs) with appreciable genetic advance for almost all the traits. Substantial genetic variances and high heritability estimates implied that characters could be improved through selection in segregating populations in early generations. Higher heritability and genetic gain is an indication of additiveness with partial dominance type of gene action suggesting the feasibility of selection in the early generations (Khan et al 2009a). However, bolls per plant and boll weight exhibited moderate to high heritability and low genetic gain with over dominance type of gene action thereby revealing that selection might be useful if delayed (Khan et al 2009b). In present studies, high genetic variance and heritability revealed that there are better chances of improvement by getting early maturity in some genotypes. Majority traits revealed positive association with seed cotton yield except lint %. Basal et al (2009) mentioned that fiber quality traits were negatively associated with the most basic within-boll lint yield components. Both at genotypic and phenotypic levels, seed cotton yield exhibited significant positive association with bolls, seed cotton weight per boll, plant height, monopodia/ plant and fiber strength revealed that any selection among correlated traits might lead to improvement in seed cotton yield, while with other fiber traits the correlation was negative (Ahuja et al 2009). Correlation of seed cotton yield was significantly positive with yield related traits, however, the association was negative with lint % (Khan et al 2011; 2015).

4. Conclusions

Seed cotton yield and its contributing traits were controlled non-additively, and hence, the selection might be effective if delayed. Lines (SLH-284, CIM-473) and pollinators (CIM-707, CIM-496) were leading general combiners, and their involvement in F_1 hybrids (CIM-473 × CIM-496, SLH-284 × CIM-707 and CIM-446 × CIM-496) showed best performance for earliness, yield and lint traits. Therefore, these promising hybrids may be preferred for hybrid crop development.

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Production of Double Haploid Plants Using *In Vivo* Haploid Techniques in Corn

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ABSTRACT

This research was conducted at the breeding station of the Turkish breeding company Agromar A.Ş in the city of Bursa in Turkey during the 2013 and 2014 growing seasons. Used from within the same heterotic group crossings, 7 donor materials were obtained during the 2012 winter season in the greenhouse. The inducer line RWK-76xRWS, provided by University of Hohenheim, Germany, was used for generating haploid seeds. The donor and inducer crossing was performed during the 2013 summer season. The haploid selection and chromosome doubling were performed during the 2014 summer season. Seven donors were used for haploid induction which name are DNR1, DNR2, DNR3, DNR4, DNR5, DNR6, DNR7 respectively, from each donor different amount of ear crosses were performed (DNR1:16 ears, DNR2:10 ears, DNR3:10 ears, DNR4:12 ears, DNR5:11 ears, DNR6:13 ears, DNR7:11 ears). According to the present study, the average induction rate found ranged from 7.1 to 12.8%, and the average seedling survival rate in the greenhouse after colchicine application ranged from 57.9 to 77.6%. After transplanting to the field, 78.3-92.6% of these plants survived. As a result of this research, the chromosome doubling rate ranged from 22.5 to 48.3% depending on the donor material. These result indicates that maternal haploid selection visually is easy. Haploid induction rate (HIR) changes from donor to donor, its mean genotype and environment is effective for HIR. Average chromosome doubling rate is lower than other researchers' results, it is also effected by genotype and chromosome doubling methods.

Keywords: *Zea mays*; Haploid line; Double haploid line; *In vivo* induction

1. Introduction

The new phenomenon in maize breeding is called the double haploid technique, and the offspring of these plants are called double haploid lines (DHL). This technique is prevalent throughout the world and is being applied by maize breeders. Today, many private Turkish breeding firms and institutes use this method. The progress of science and technology and the economy have accelerated and advanced to allow creation of new plant varieties in the 20th century. The most critical stage of maize breeding is the improvement of inbred lines, which have the highest general and specific combining ability.

This kind of work takes a long time and requires a high budget and labor force. Inbred lines used for hybrid development require at least 7-10 years.

The key to increased genetic gains and accelerated development of improved varieties is reducing the time needed for inbred development. This can be most effectively achieved by application of double haploid (DH) technology (Prigge 2012). Currently, double haploid lines (DHL) produced by *in vivo* induction of maternal haploids are prevalent in maize (*Zea mays* L.) breeding (Gordillo & Geiger 2008; Rotarenco et al 2012; Battiselli et al 2013; Couto et al 2013). Haploids in maize can be obtained either through *in vitro* or *in vivo* techniques (Geiger 2009; Dang 2010; Dang et al 2011; Prasanna et al 2012). For maize, the widely used induction technique is *in vivo* induction of maternal haploids (Mureşeanu et al 2013). The limitations of in vitro haploid induction techniques in maize coupled with their requirement to have a good laboratory and skilled staff makes them less efficient methods (Chidzanga at al 2017). Two modes of *in vivo* haploid induction can be distinguished in maize, leading to maternal and paternal haploids (Röber et al 2005). In induction crosses aimed at paternal haploids, the inducer is used as the female and the donor plant as the male parent. Thus, the cytoplasm of paternal haploids originates from the inducer, but the chromosomes exclusively come from the donor plant. For production of maternal haploids, on the other hand, the inducer is used as the pollinator, leading to haploids carrying both the cytoplasm and chromosomes from the donor (Geiger 2009).

The *in vivo* production of maternal DHL involves the following four steps: (i) inducing haploidy by pollinating the source germplasm with pollen of the haploid inducer; (ii) identifying those seeds with haploid embryos based on a visual scorable morphological marker; (iii) duplicating chromosomes of putative haploids by treating the seedlings with a mitotic inhibitor; and (iv) self-pollinating DH plants to multiply their seed (Prigge 2012; Prasanna et al 2012; Tseng 2012). To induce maternal haploids, the donor plant is pollinated by a specific maize stock (line, single cross or population), which is called the inducer (Geiger & Gordillo 2009). For *in vivo* induction of maternal haploids, the pollen of maize inducer genotypes is used to pollinate the source germplasm from which DHL are to be developed (Prigge et al 2012). The haploid inducers are specialized genetic stocks that, when crossed to a diploid (normal) maize plant, result in progeny kernels in the ear segregating for diploid (2n) kernels and a certain fraction of haploid (n) kernels due to anomalous fertilization (Prasanna et al 2012). The goal of this research is to determine how the double haploid technique is applied and what sort of results are obtained.

2. Material and Methods

This research was conducted at the Agromar A.Ş breeding station department of the Plant Breeding and Genetics, Karacabey, district of Bursa, a province in Turkey (40°13'N, 28°22'E), during the growing seasons of 2013 and 2014. Plant materials were developed using from within the same heterotic group crossing in the 2012 winter season in greenhouse conditions. DHL were induced from F1 donor plants. Bernardo et al (2010) observed that DHL should be induced from F2 plants rather than from F1 plants. Seven donor genotypes were used for this study. The donor names are as follows: DNR1, DNR2, DNR3, DNR4, DNR5, DNR6, and DNR7. The DHL were obtained in two steps, as follows.

2.1. Generation of haploid plants (donor and ınducer crossing)

The donor and inducer crossings were performed during the 2013 summer season. In this study, the inducer line RWK-76xRWS provided by University of Hohenheim, Germany was used for generating haploid seeds. Averaged across a wide range of donors and environments, it has an induction rate of approximately 8%. A sister line, RWK-76, developed from the reciprocal cross (WS14xKEMS), even reached an average induction rate of 9-10%. This same rate was observed for the cross RWSxRWK-76. Although having related parents, this cross is much more vigorous and is better at shedding pollen than both of its parents; this cross is therefore easier to handle, particularly in adverse environments (Geiger 2009). The use of

F1 plants as inducers may be more advantageous because of their higher vigor, which generally is associated with more abundant pollen shedding (Röber et al 2005). Each plant was planted in 2 rows at a row spacing of 70x20 cm and a row length of 3 m. Each plot contained 32 plants. Because of the early maturity of the inducer line, the inducers were sowed two times: 4 and 7 days after planting of the donors. The haploid induction rates (HIR) of some inducer lines are given in Table 1 below (Roeber 2014).

Table 1- Haploid induction rates of some inducer line according to usage

Using type	*Line name*	*Haploid induction rate (%)*
Maternal	Stock 6	2.3-3.2
Maternal	WS14	2.0-5.0
Maternal	KEMS	6.3
Maternal	RWS	10.0
Maternal	UH400	8.0-9.0
Maternal	BRZO6	12.3
Maternal	B-432	13.9
Maternal	ZMS	0.6-3.4
Maternal	AC'R	5.5
Maternal	ACR	8.3
Maternal	KMS	0.8-2.9
Maternal	PK6	6.0
Maternal	AT-1	2.0-3.0
Maternal	AX6012	10.0
Paternal	W23ig	2.6-8.0

2.2. Identification of putative haploids and artificial chromosome doubling

The most efficient haploid identification marker is the 'red crown' or '*navoja*' kernel trait encoded by the dominant mutant allele R1-*nj* of the 'red color' gene R1. In the presence of dominant pigmentation genes A1 or A2 and C2, R1-*nj* causes deep pigmentation of the *aleurone* (endosperm tissue) in the crown (top) region of the kernel (Geiger 2009). The R1-*Navajo* (R1-*nj*) color marker facilitates easy and quick identification of haploid kernels at the seed stage during the *in vivo* haploid induction process in maize (Chaikam et al 2015). The R1-*nj* marker gene is widely used for screening haploids of dry seed. However, the expression of this gene has strong female influence: sometimes the screening of haploids can be very confusing or even impossible, especially in those cases where there are inhibitor genes (C1-I) in females (common for flint maize) (Rotarenco et al 2010). There are some newly methods provided by some researchers for haploid identification. Chaikam et al (2016) developed haploid inducer lines with triple anthocyanin color markers, including the expression of anthocyanin coloration in the seedling roots and leaf sheaths, in addition to the R1-*nj* gene. The other method is suggested by Melchinger et al (2014) that UH600 high oleic inducer line using for haploid identification. Jones at al (2012) recommend that near infrared spectroscopy for the haploid identification.

In the present study, haploid or diploid kernels were distinguished by means of the expression of the dominant anthocyanin marker gene R1-*nj* (Figure 1). Which mechanism is controlling maternal haploid induction? The answer is that there are two major ways that the maternal haploid inducer might produce kernels with haploid embryos. First, an abnormal fertilization might occur in which one sperm fertilizes the two polar nuclei and other sperm fails to fertilize the egg of an embryo sac, producing a kernel that has a triploid endosperm and haploid embryo. Second, the normal double fertilization event would occur, and then the chromosomes from the female parent are eliminated from the embryo after fertilization (Weber 2014). There are three types of seed obtained. The first one is diploid seed; kernels with purple coloration in the endosperm (*aleurone*) and the embryo (*scutellum*) are accepted as diploid. The second one is putative haploid kernels; purple endosperm but no coloration of the embryo is accepted as putative haploid. The last one is unpigmented kernels; without purple coloration of the embryo or endosperm is accepted as an outcross because of pollen contamination. Putative haploid seed is identified visually according to their *aleurone* and embryo color. Pigmented ears shelled individually and by HIR (haploid induction rate) are found according to each donor genotype. The haploid induction rate of the maize inducer denotes

the proportion of seeds with a haploid embryo detected in the total number of seeds harvested from source germplasm pollinated with inducer pollen (Prigge et al 2012).

Figure 1- Haploid and diploid kernels

In this research, the haploid induction rate (HIR), haploid germination rate (HGR), surviving seedling rate (SSR), surviving plant rate (SPR), and chromosome doubling rate (CDR) were calculated using the following formulas:

The genome of the haploid plant material should be doubled to obtain 100% homozygous plant materials after the putative haploids are identified. To achieve this, colchicine treatment is the most widely used procedure for chromosome doubling (Prasanna et al 2012; Mureşeanu et al 2013). Chromosome doubling of the selected haploid seedlings was performed according to methods of Deimling et al (1997). Haploid seeds were germinated in a growth chamber for 4 or 5 days. When coleoptiles reached 2-3 cm in length, the tops were cut and removed from the roots and immersed in a solution of 0.06% colchicine and 0.05% dimethyl sulfoxide (DMSO) for 12 hours at 18 °C. One day after the solution application, coleoptiles were washed for 20 minutes and transplanted into pots in the greenhouse. When the first cycle of DHL reached the 4- or 5-leaf-stage in greenhouse conditions, all of the seedlings were transferred to the field and transplanted for seed increase.

HIR= (Haploid kernels / Total kernels)*100 (1)

HGR= ((Haploid kernels - ungerminated kernels) / Total haploid kernels)*100 (2)

SSR= (Transplanted seedlings / Colchicine-treated seedlings)*100 (3)

SPR= (Surviving plant number / Transplanted seedling number)*100 (4)

CDR= (Fertile plant number / Selected plant number)*100 (5)

3. Results and Discussion

3.1. Haploid ınduction rate (HIR)

Seven donor genotypes were induced by the inducer line RWK-76xRWS in the summer season of 2013. The ears were shelled individually, and haploid kernels were selected according to visual selection. The mean haploid induction rate of each ear is given in Table 2.

There is a significant difference between donors at 5% level. The putative HIR changed from donor to donor such that DNR1 changed from 3.1 to 14.7%, DNR2 changed from 1.2 to 12.8%, DNR3 changed from 1.1 to 13.6%, DNR4 changed from 5.3 to 11.7%, DNR5 changed from 3.5 to 32.7%, DNR6 changed from 2.1 to 20.1%, and DNR7 changed from 2 to 19.8%. The highest average HIR belonged to DNR7 (12.8%) and lowest to DNR2 (7.1%), on average. Chalky (1994) reported that, on average, 27.4 kernels per ear of haploid plants were obtained in the first year of study and 26.3 in the second year from the inducer line ZMS. Cengiz (2016) reported that the highest HIR was established as 20.42% in the RWK-76 inducer line and that the lowest HIR was calculated as 17.75% in WS14. Cerit at al (2016) reported that the RWK-76 has highest HIR and the lowest one is Stock-6. Dang et al (2011) reported that the rates of putative haploids among progenies induced with the inducer lines RWK76 and RWK-76xRWS (15.7 and 15.0%, on average, respectively) were significantly lower ($P<0.05$) than those observed with the inducer line

Table 2- Putative haploid induction rate (%)

Ear no	*DNR1*	*DNR2*	*DNR3*	*DNR4*	*DNR5*	*DNR6*	*DNR7*
1	7.4	6.7	8.1	6.4	3.6	4.8	12.7
2	11.4	5.7	9.5	6.4	7.1	4.2	2.0
3	9.5	1.2	5.7	8.5	3.8	5.3	7.6
4	7.2	4.7	1.1	5.3	3.5	2.1	5.8
5	14.7	7.5	9.5	10.2	12.7	12.6	19.8
6	8.9	11.4	8.5	11.7	20.2	11.2	16.6
7	3.1	9.2	9.7	6.3	12.8	5.7	16.4
8	9.4	12.8	6.9	7.7	10.7	20.1	17.6
9	8.3	5.1	5.5	9.9	32.7	9.6	11.7
10	8.2	6.6	13.6	6.8	9.9	15.6	13.8
11	6.4			5.6	18.8	14.2	17.3
12	8.8			9.3		10.0	
13	8.3					7.1	
14	8.3						
15	14.1						
16	9.9						
Average	9.0 bc	7.1 c	7.8 c	7.8 c	12.3 ab	9.4 abc	12.8 a

significant at 5% probability levels

RWS (23.8%) for the same maternal plant material. Those induction rates are higher than those of the present study. Prasanna et al (2012) reported that the tropical inducer UHo induction rate is 8-10% and that of TAILs is 8-12%, which are similar to the results of the present research. The HIR of other inducer are given in the materials and methods section in Table 1.

3.2. Haploid germination rate (HGR)

Haploid kernels were treated with colchicine in the summer season of 2014. For this process, putative haploid kernels should be germinated to have seedlings ready before treating with colchicine. Haploid seeds were germinated in a growth chamber at 25 °C. The germination rates of each donor changed from 63.6 to 95.2%, with an average of 81.8% (Table 3.). The lowest HGR belonged to DNR6 (63.6%) and the highest to DNR7 (95.2%). At CIMMYT, a germination percentage of 85-90% is commonly achieved among the putative haploid kernels (Prasanna et al 2012). Our results are similar to those of this study.

Table 3- Haploid germination rate (%)

Donors	*Haploid kernels*	*Ungerminated kernels*	*HGR*
DNR1	406	31	92.4
DNR2	128	21	83.6
DNR3	209	57	72.7
DNR4	227	13	94.3
DNR5	211	62	70.6
DNR6	239	87	63.6
DNR7	294	14	95.2
Total	1714	Average	81.8

3.3. Colchicine treatment and surviving seedling rate (SSR)

Colchicine treatments were applied according to the methods of Deimling et al (1997). Other methods have been presented by Gayen et al (1994), who used three rates of colchicine in a DMSO solution. At CIMMYT, a solution of 0.04% colchicine and 0.5% DMSO is used for chromosomal doubling (Prasanna et al 2012). The application procedure is given in the materials and methods. The average SSR was 66.3% (Table 4). Cengiz (2016) reported

that 89% of plants lived of the 2178 seedlings that were planted the field. The highest donor SSR 77.6% belonged to DNR4; the lowest, 57.9%, belonged to DNR3.

Table 4- Surviving seedling rate (%)

Donors	*Colchicine-treated seedlings*	*Transplanted seedlings*	*SSR*
DNR1	375	243	64.8
DNR2	107	73	68.2
DNR3	152	88	57.9
DNR4	214	166	77.6
DNR5	149	87	58.4
DNR6	152	98	64.5
DNR7	280	204	72.9
		Average	66.3

3.4. Transplanted seedlings and surviving plant rate (SPR)

Surviving seedlings were transplanted to the field. Each seedling was transplanted to the field at a spacing of 70x20 cm. The surviving plant rates are given in Table 5. The average SPR was 86.8%. The highest SPR was 92.6%, which belonged to DNR1, and the lowest one was 78.3%, which belonged to DNR4.

Table 5- Surviving plant rate (%)

Donors	*Transplanted seedlings number*	*Surviving plants number*	*SPR*
DNR1	243	225	92.6
DNR2	73	66	90.4
DNR3	88	77	87.5
DNR4	166	130	78.3
DNR5	87	70	80.5
DNR6	98	89	90.8
DNR7	204	179	87.7
		Average	86.8

3.5. Chromosome doubling rate (CDR)

There were some misclassified plants that were identified in the field. They were determined by their agronomic attributes, such as purple stem, advanced vigor, etc. All of the misclassified plants were discarded before selfing. The first stage of DH lines is called D0, and second generation of DH lines is called D1 indicates the CDR percentages (Table 6). The average CDR found was 34.3%. The CDR changed for each donor, ranging from 22.5 to 48.3%. Doubling rates have reached 50%, 0-40%, and 55.31%, as reported by Vanous (2011), Tseng (2012) and Prasanna et al (2012), respectively. Cengiz (2016) reported that fertile plants made up 57% of live plants. These rates are higher than those of the present study. The chromosome doubling may vary depending on the genotype and application. The reason why CDR is different for many authors that the genotype and application methods for chromosome doubling is different. Beside haploid selection effects this rate.

Table 6- Chromosome doubling rate (%)

Donors	*Selected plants number*	*Fertile plant number*	*CDR*
DNR1	204	46	22.5
DNR2	52	15	28.8
DNR3	60	29	48.3
DNR4	115	49	42.6
DNR5	58	17	29.3
DNR6	80	25	31.3
DNR7	148	55	37.2
		Average	34.3

4. Conclusions

In the present study, the HIR, HGR, SSR, SPR, and CDR were determined. The values of these characteristics changed according to each donor genotype, and these terms can be used for this kind of research as common terms. The highest haploid induction rate obtained was 7.1-12.8% with the maternal inducer RWK-76xRWS. The average HGR found was 81.8%, and this rate is sufficient for utilizing haploid kernels. The SSR obtained in pots was lower than the SPR in the field because of the adverse effects of colchicine and acclimatization. As a result, the observed CDR was, on average, 34.3%. In conclusion, 1714 putative haploids were

identified based on the phenotypic marker system of the R1-*nj* allele. Among the 1714 putative haploids, only 87 D0 lines advanced to the D1 stage, which means only 5.1% of haploids can be advanced to the D1 stage. The most critical stage for DHL development is haploid identification. There are newly and efficient methods developed by the researcher for the identification of haploid induction like using high oleic inducer, near infrared spectroscopy and triple anthocyanin color markers inducer. These newly methods will helps to corn breeder for the selection of haploids. Most of the publication trace back maternal haploid system but the other important methods is paternal haploid system. This system specially work well in cms corn line development and backcross breeding. We suggest to corn breeders to utilize paternal haploid system.

To sum up DHL is invaluable and newly methods for corn breeders to have 100% homozygous line only in two generation. Accelerating the generation is less expensive than other corn breeding methods and less laboratory needed. It is recommended to corn breeders that they should use at least ten F1 donor ears crossing with inducer because of advanced D1 generation is only about 5 percent.

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Effect of Sowing Date and Humic Acid Foliar Application on Yield and Yield Components of Canola Cultivars

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ABSTRACT

In order to investigate the effects of different sowing dates and humic acid foliar application on some agronomic traits of six canola cultivars a two-year experiment was carried out in 2015 and 2016 growing seasons. The experiments were laid out in a randomized complete block design arranged in factorial split plot with three replicates. The factorial combination of three sowing dates (7th, 17th and 27th October) and two humic acid levels (0 and 0.2%) were allocated to the main plots and six canola cultivars (HW118, WPN6, HL3721, L14, Tassilo and Natali) were randomized in sub-plots. The results indicated that the main effects of experimental factors were significant on all studied traits, except for harvest index. Interaction between sowing date and cultivar was also significant on all traits except for branch number and harvest index. In general, early seed sowing caused the highest yield and yield component as well as oil percentage and yield. Similarly, humic acid foliar application could increase agronomic traits in canola cultivars. In sum, early seed sowing and humic acid foliar application are highly recommended in canola production.

Keywords: Canola; Humic acid; Oil content; Sowing date; Yield

1. Introduction

Canola (*Brassica napus* L.) is one of the most important oilseed crops grown extensively in Iran. The crop has been taken into account to reduce vegetable oil import dependence. Among oilseed crops, canola has become the second largest oil crop behind soybean in the world (USDA 2013) and is widely grown for its high quality oil for human consumption. Canola seeds contain about 40 to 42% oil and its meal is also great protein supplement in cattle rations and averages approximately 35% crude protein. Agronomic practices such as tillage, planting density, nitrogen fertilizer rate, and cultivar selection have received attention to improve survival and yield of canola (Holman et al 2011). Among these crop management practices, optimum sowing date plays key role in determining final crop yields and should be considered for the germination, seedling establishment and vegetative and reproductive stages not to be affected by unfavourable conditions (Usman et al 2016). Generally, canola should be sown before soil temperature falls under -4 °C as

canola is highly cold tolerant at eight leaf stage or rosette stage. In winter cropping, early sowing in autumn increases water and nutrients uptakes and vegetative growth resulting in the risk of cold tolerance loss. On the other hand, late sowing reduces total growth period and increases the risk of freezing. It has been reported that late sowing decreases biomass production, yield and yield components through increasing the risk that the crop is affected by late season drought stress (Wang et al 2012). Late sowing reduces canola vegetative growth period resulting in reduced assimilates production. In addition, late sowing increases the risk heat and drought stress during reproductive stages resulting in poor pollination, flower abortion and low seed set (Farre et al 2002). Optimum sowing date results in better seedling establishment and improved cold tolerance, which prevent cold injury and yield loss (Begna & Angadi 2016). Reduction in oil content due to late sowing has been reported in canola by (Robertson et al 2004; Turhan et al 2011). Oil content is a trait with high heritability in canola. Oil content depends on genotype, region, soil fertility and seed age. Among environmental factors temperature is the most important factor affecting oil synthesis so that oil percentage dramatically decreases with increasing temperature (Wang et al 2012). Siliques as an active photosynthetic organ play a key role in determining seed yield. In addition, increase in silique length means more photosynthetic area and more photosynthates transfer into the seeds (Gammelvind et al 1996). Furthermore, it has been documented that seed number in siliques increases with increasing plant dry weight (Gan et al 2008). In this regard, it has been stated that canola genotypes differ to each other in terms of seed number in siliques, a trait that plays a crucial role in final seed yield (Iqbal et al 2008). However, seed number in silique is more controlled by genetic than environmental factors.

In addition to organic and mineral fertilizers, application of humic acid is getting popular in improving the crops growth and yield. Humic compounds or humus are products of decomposing plants that have complex structures and large molecular weights (Lee et al 2004). It can be extracted from any material containing well-decomposed organic matter soil, coal, composts, etc. (Sani 2014). Humic acid as the most important component of soil humus (Sparks 2003) can be applied in liquid or powder form in soil or one plant leaves (Ulukan 2008) to reduce the negative effect of environmental stresses. Humic acid has numerous benefits and all farmers across the world have come to this conclusion that humic acid is considered as an inseparable and integral part of fertilization program and soil fertility (El-Ghamry et al 2009). It has been reported that humic acid application significantly increases soil organic matter which in turn improves plant growth and development (Erik et al 2000; Hafez 2003; Abd El-Aal et al 2005). In a study, humic acid application could significantly affect initial growth stages of wheat (Mirzamasoumzadeh et al 2012). Humic acid application has been advised to diminish drought stress effects, especially after flowering (Bassoa et al 2013). It has been reported that humic acid has limited promoting effect on growth, yield and quality of wheat (Delfine et al 2005). Application of 2% humic acid could increase grain and straw yield in wheat (Brunetti et al 2007).

As a result, there have been several studies on the effect of sowing date on the agronomic traits of numerous crops; however, studies on the effect of sowing date and humic acid on canola growth and production using different cultivars are still limited. Therefore, the objective of this research was to determine the effects of sowing dates and humic acid on some agronomic of six canola cultivars.

Therefore, the current study was aimed to evaluate the effects of sowing dates and humic acid foliar application on canola cultivars seed yield and yield components.

2. Material and Methods

In this study, the effects of different sowing date and humic acid foliar application were evaluated on plant height, branch number, seed yield and yield components, biological yield, harvest index,

oil percentage and oil yield of six canola cultivars using a two-year experiment carried out in Seed and Plant Improvement Institute, Karaj, Iran, in 2015 and 2016 growing seasons. Meteorological data during growing season are given in Table 1. The experiments were laid out in a randomized complete block design arranged in factorial split plot with three replicates. The factorial combination of three sowing dates (7th, 17th and 27th October) and two humic acid levels (0 and 0.2%) were allocated to the main plots and six canola cultivars (HW118, WPN6, HL3721, L14, Tassilo and Natali) were randomized in sub-plots. Soil samples were collected at the depth of 0-30 and 30-60 before seed sowing, the soil physicochemical properties are presented in Table 2. According to the soil analysis results 150 kg ha^{-1} ammonium phosphate and 150 kg ha^{-1} potassium sulphate were applied into the soil before seed sowing. In addition, 350 kg ha^{-1} urea was applied at three separate times (100 kg ha^{-1} at sowing time, 150 kg ha^{-1} at stem elongation and 100 kg ha^{-1} at flowering stage). Each plot consisted of 6 ridges, 60 cm apart and distance between seeds on each row was 5 cm (70,000 plant ha^{-1}). Seeds were sown using a drill sower on both side of a ridge (30 cm apart) and at 25 mm depth. Irrigation was perfumed immediately after seed sowing. Weeds were manually controlled from 4 to 8 leaf stage. Humic acid was sprayed on plants at 4 leaf stage and stem elongation stage using backpack sprayer. Control plants were sprayed with distilled water. In order to determine plant height, branch number, silique number per plant, seed number per silique, 1000-seed weight, four central rows were manually harvested at maturity stage. Silique number in plants was determined using 10 harvested plants and average values were recorded. To determine seed number in silique, 30 siliques were randomly detached from the plants and seed number was determined. The seeds were counted and weighted using laboratory scale. Finally seed

Table 1- Meteorological data during growing season

Growing season months										
Year	*Parameter*	*October*	*November*	*December*	*January*	*February*	*March*	*April*	*May*	*June*	*July*
2015	Rainfall (mm)	13.4	13.7	31.6	6	47.8	21.3	45.4	2.2	6.6	0
	Temperature (°C)	18.31	18.2	6.3	5.2	7.3	6.7	13.8	20	26.4	30.9
2016	Rainfall (mm)	3.5	77.4	28.6	15.6	8.7	17.8	75.5	13	0	0
	Temperature (°C)	19.4	10.5	4.6	5.1	4.9	11.8	11.7	19.9	24.2	28.9

Table 2- Soil physicochemical properties

Parameter	*2015*		*2016*	
	Depth (0-30 cm)	*Depth (30-60 cm)*	*Depth (0-30 cm)*	*Depth (30-60 cm)*
Electrical conductivity (dS m^{-1})	1.45	1.24	1.33	1.15
pH	7.9	7.2	7.8	7.4
Total neutralizing value (%)	8.56	6.68	8.25	8.46
Moisture content (%)	36	38	35	37
Organic carbon (%)	0.91	0.99	0.83	0.96
Total N (%)	0.09	0.07	0.08	0.06
Available P (mg kh^{-1})	14.7	15.8	14.2	15.3
Available K (mg kh^{-1})	197	155	165	148
Clay (%)	28	25	29	27
Silt (%)	47	49	45	46
Sand (%)	25	26	26	27
Soil texture	Clay loam	Clay loam	Clay loam	Clay loam

yield was calculated using yield components date. Oil percentage was measured using NMR (Mq20) and oil yield was calculated through multiplying oil percentage by seed yield. In order to determine biological yield, harvested plants were sun dried, weighed and converted into kg per hectare. Harvest index was calculated as the ratio of seed yield to biological yield (Kutcher et al 2010). The data were analysed using SAS 9.0 software program. Bartlett's test showed the homogeneity of variance in all traits in both years. Therefore, combined anlaysis of variance was carried out. The Duncan's multiple range test (DMRT) was used to compare means within the combined analysis of variance.

3. Results

The analysis of variance indicated that the main effects of year, sowing date, humic acid foliar application and cultivar were significant on all studied traits, except for harvest index (Table 3). In addition, the results revealed that interaction between sowing date and cultivars was significant for all traits except for branch number and harvest index (Table 3). No significant interaction between sowing date and humic acid or between cultivar and humic acid were detected; therefore, only main effects are discussed. According to Table 4, sowing date significantly affected canola plant height. The highest plants were observed when seed sowing was performed on 7th October (Table 4). The shortest plants were related to late sowing date (27th October) (Table 4). Similar results were found as to branch number (Table 4). The branch number decreased when late seed sowing was practiced (Table 4). The minimum and maximum silique number was found when seed sowing was done on 27th and 7th October, respectively (Table 4). Seed number in silique followed a similar trend and decreased when seed sowing delayed (Table 4). 1000-seed weight decreased when seed sowing was done later than 7th October (Table 4). In other words, the maximum 1000-seed weight was found when seed sowing was done on 7th October. In case of biological yield, delay in seed sowing caused a significant reduction in biological yield so that the minimum and maximum biological yield was observed when canola seeds were sown on 7th and 27th October, respectively (Table 4). Since seed yield is a function of interaction among yield components that are affected by sowing date, then delay in seed sowing could reduce final seed yield too (Table 4). Oil percentage and oil yield decreased as seed sowing was delayed (Table 4). As shown in Table 4, humic acid application could significantly increase plant height, branch number, yield components, biological yield, final seed yield as well as oil percentage and yield. Harvest index was the only trait that was not affected by humic acid foliar application (Table 4). The results indicated that there are significant differences among canola cultivars in terms of height, branch number yield and yield components as well as oil percentage and yield but not in terms of harvest index (Table 4). However, some cultivars were the same in terms of above mentioned traits. For example, no significant difference was found between WPN6 and Natali in terms of plant height or there was no significant difference among HW118, WPN6 and Natali cultivars in terms of final seed yield (Table 4). As mentioned before, interaction between cultivar and sowing date was found to be significant on all studied traits except for branch number and harvest index (Table 3). Comparison of means indicated that the maximum plant height was related to WPN6 and Natali cultivars when planted on 7th October (Table 5). The highest plant height was observed in HW118, WPN6 and Natali cultivars at the 17th October (Table 5). Similar results were found when seed sowing delayed and seeds were sown on 27th October (Table 5). In case of silique number per plant, when WPN6 and Natali cultivars were sown on 7th October, the maximum silique umber per plant was obtained (Table 5). On 17th October, the maximum silique number was related to HW118 cultivar (Table 5). The maximum silique number per plant was related to HW118 and WPN6 cultivars when sown on 27th October (Table 5). The maximum seed number in silique was related to WPN6 and Natali cultivars sown on 7th October (Table 5), however, when seed sowing was performed 17th or 27th October, the maximum seed number was observed in HW118, WPN6 and Natali

cultivars (Table 5). Almost in all sowing dates, the maximum 1000-seed weight was related to HW118, WPN6 and Natali cultivars (Table 5). The maximum biological yield was related to WPN6 and Natali cultivars when sown on 7th October (Table 5). Seed sowing on 17th October caused the maximum biological yield in HW118, WPN6 and Natali cultivars (Table 5). Late sowing date (27th October) decreased biological yield in all the cultivars (Table 5). Almost in all sowing dates, the maximum seed

Table 3- Analysis of variance on yield and yield components of canola cultivars as affected by sowing date and humic acid application

S.O.V	*Df*	*Plant height*	*Branch number*	*Silique number*	*Seed number in silique*	*1000-seed weight*	*Biological yield*	*Seed yield*	*Harvest index*	*Oil percentage*	*Oil yield*
Y	1	**	**	**	**	**	**	**	ns	**	**
E	4	23.66	0.69	356.49	1.81	0.38	2076708	1497597.80	33.97	0.26	291703.43
S	2	**	**	**	**	**	**	**	ns	**	**
Y×S	2	ns	ns	*	*	**	ns	ns	ns	ns	ns
H	1	**	**	**	**	**	**	**	ns	**	**
Y×H	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
S×H	2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Y×S×H	2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
E	20	38.14	0.82	112.84	1.32	0.28	912493	255431.20	12.30	0.26	50708.73
C	5	**	**	**	**	**	**	**	ns	**	**
Y×C	5	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
S×C	10	**	ns	**	**	**	**	**	ns	**	**
Y×S×C	10	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
H×C	5	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Y×H×C	5	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
S×H×C	10	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Y×S×H×C	10	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
E	120	51.80	1.39	110.09	1.30	0.17	1122770	235235.30	12.12	0.13	40811.81
C.V (%)		4.94	18.18	6.77	6.87	9.51	6.27	11.98	14.55	0.89	11.92

S.O.V, source of variation; df, degree of freedom; Y, year; E, error; P, sowing date; H, humic acid; C, cultivar; C.V, coefficient of variation; *, ** and ns indicate significance at P<0.05, 0.01 and not significant, respectively

Table 4- Main effects of sowing date, humic acid and cultivar on yield and yield components

Factors	*Levels*	*Plant height (cm)*	*Branch number*	*Silique number*	*Seed number in silique*	*1000-seed weight (g)*	*Biological yield (kg ha^{-1})*	*Seed yield (kg ha^{-1})*	*Harvest index (%)*	*Oil percentage*	*Oil yield (kg ha^{-1})*
Sowing date	October 7th	173.92a	8.63a	215.41a	19.80a	5.47a	21835a	5243a	24.06a	42.87a	2250a
	October 17th	144.43b	6.40b	151.09b	16.62b	4.41b	16689b	4069b	24.41a	41.57b	1694b
	October 27th	118.19c	4.42c	98.24c	13.40c	3.30c	12148c	2826c	23.29a	40.25c	1140c
Humic acid	-	142.12b	6.21b	147.21b	16.21b	4.26b	16229b	3894b	23.93a	41.38b	1624b
	+	148.90a	6.75a	162.62a	17.00a	4.53a	17553a	4199a	23.91a	41.75a	1765a
Cultivars	HW118	148.21b	6.76a	159.75b	17.02a	4.53a	17261b	4198a	24.36a	41.75b	1759a
	WPN6	152.42a	7.03a	170.1a	17.46a	4.68a	18353a	4355a	23.79a	41.94a	1840a
	HL3721	140.65c	6.08b	144.37c	16.01b	4.216b	15924c	3832b	24.00a	41.318c	1595b
	L14	141.08c	6.09b	145.6c	16.03b	4.217b	16069c	3834b	23.74a	41.311c	1598b
	Tassilo	138.94c	5.93b	140.72c	15.78b	4.12b	15608c	3736b	23.76a	41.19c	1554b
	Natali	151.76a	7.00a	168.95a	17.34a	4.62a	18130a	4321a	23.87a	41.88ab	1823a

In each column same letters show that there are no significantly difference according to DMRT

Table 5- Interaction between sowing date and cultivar on some agronomic traits of canola

	Plant height (cm)			*Silique number per plant*			*Seed number in silique*			*1000-seed weight (g)*		
	7th	*17th*	*27th*	*7th*	*17th*	*27th*	*7th*	*17th*	*27th*	*7th*	*17th*	*27th*
HW118	165.05c	154.07a	125.51a	194.99d	170.31a	113.95a	18.89b	17.73a	14.45a	5.14b	4.72a	3.72a
WPN6	184.51a	149.33a	123.42a	240.57a	160.24b	109.50ab	21.12a	17.11a	14.15a	5.87a	4.55ab	361a
HL3721	167.81bc	139.68b	114.46b	231.87cd	141.35c	90.84c	19.11b	16.04b	12.87b	5.27b	4.25bc	3.12b
L14	173.03b	137.45b	112.78b	206.37b	136.40cd	86.55cd	19.63b	15.83b	12.62b	5.34b	4.21c	3.00b
Tassilo	170.70bc	134.57b	111.54b	200.92bc	132.90d	82.90d	19.38b	15.56b	12.40b	5.34b	4.12c	2.91b
Natali	182.40a	151.47a	121.40a	235.76a	165.35b	105.72b	20.70a	17.45a	13.89a	5.80a	4.61a	3.46a
	Biological yield (kg ha-1)			*Seed yield (kg ha-1)*			*Oil percentage*			*Oil yield (kg ha-1)*		
	7th	*17th*	*27th*	*7th*	*17th*	*27th*	*7th*	*17th*	*27th*	*7th*	*17th*	*27th*
HW118	19943.20d	18189.30a	13649.80a	4892.90b	4452.90a	3249.20a	42.48c	42.00a	40.75a	2080.40b	1871.80a	1324.70a
WPN6	24321.30a	17534.80a	13201.60ab	5672.10a	4284.10a	3110.10a	43.41a	41.77a	40.63ab	2464.00a	1790.70a	1264.70a
HL3721	20487.50cd	15897.50b	11387.90c	5003.70b	3870.50b	2622.80b	42.58bc	41.35b	40.01c	2132.08b	1601.70b	1051.08b
L14	21536.90b	15556.40bc	11115.00c	5186.70b	3786.70b	2527.30b	42.78b	41.26b	39.88c	2219.30b	1565.08b	1008.90b
Tassilo	20982.20bc	15120.80c	10720.30c	5117.10b	3674.90b	2415.50b	42.68bc	41.14b	39.76c	2184.90b	1513.80b	962.50b
Natali	23740.90a	17834.30a	12813.50b	5584.20a	4344.30a	3033.30a	43.28a	41.90a	40.46b	2417.50a	1821.10a	1228.80a

In each column same letters show that there are no significantly difference according to DMRT

yield was obtained from HW118, WPN6 and Natali cultivars (Table 5). When canola seeds were sown on 7th October, WPN6 and Natali cultivars showed the maximum oil percentage (Table 5). When seed sowing was performed on 17th October, HW118, WPN6 and Natali cultivars showed the maximum oil percentage and in late sowing date (27th October) HW118 and WPN6 cultivars indicated maximum oil percentage (Table 5). Almost in all sowing dates, the maximum oil yield, was obtained from HW118, WPN6 and Natali cultivars (Table 5).

4. Discussion

The results indicated that sowing date has a significant effect on yield and yield components, biological yield, oil percentage and oil yield in canola cultivars. These results agree with previous reports on canola yield (Farre et al 2002; Kutcher et al 2010). A similar experiment carried out in the United State (Holman et al 2011), documented the advantage of optimum sowing date in canola, and showed that optimum sowing date can vary across a relatively short geographical distance, largely driven by substantial differences in annual precipitation and elevation. In another study it has also been reported that sowing date significantly affects winter survival, suggesting early sowing can assure sufficient canola plant growth to survive the winter (Holman et al 2011; Darby et al 2013), but sowing too early also can have negative effects on plant. The reduction in canola seed yield due to delay in seed sowing has been reported by several authors (Robertson et al 2004; Faraji et al 2009). The increase in canola seed yield due to early sowing date might be attributed to more light, water and nutrients absorption by plants thus, increasing photosynthetic capacity. These results are in agreement with those of Chauhan et al (1993). Sowing date is a critical factor that controls growing season length and hence, final seed yield. Early sowing postpones flowering which is an important factor leading to the highest yields (Jenkins & Leitch 1986). Jansinka et al (1989) indicated that seed and oil yields decreased with delay in sowing date. The differences between canola cultivars in terms of seed yield might be attributed to their differences in growth traits such as branches number that mirrored differences in yield components and hence, increased seed yield. Sharief & Keshta (2000) have found similar results. According to the results, the effect of humic acid foliar application was significant on plant height,

branch number, seed yield and yield components as well as biological yield, oil percentage and yield. It has been reported that humic acid application reduces the requirement of other fertilizers (Sani 2014). For instance, previous findings have shown that total chemical contents percentage in leaves of cucumber plants due to humic acid application (El-Nemr et al 2012).

The increase in plant height on account of humic acid application may be due to enhanced shoot growth. The results are in line with (Salwa & Eisa 2011) who stated that maximum plant height was recorded when 15 kg ha^{-1} humic acid was applied. Increase in branch number due to humic acid can also be attributed to increased plant growth as reported by Sani (2014). Regarding increase in silique number on account of humic acid application, similar results have been reported with regard to the increased number of pod in soya bush (Farnia 2006). Furthermore, in a study carried out by Hagh-Parast et al (2012), humic acid application caused noticeable increase in number of pod in chickpea. Since humic acid causes remarkable increase in photosynthesis activity (Saadati & Baghi 2014), therefore, more flowers will be formed in canola plant which is effective on formation of fertile silique and seed production. It has been documented that humic acid application leads to increased photosynthesis rate and consequently, assimilates. In the same direction, assimilates retransfer rate would increase and seed weight will be increased (Farnia & Nasrollahi 2010). Similar results were reported by Wang et al (2015) that humic acid application increases 1000-seed weight and biological yield. Rao et al (2000) also reported such results in case of increased dry matter yields of mustard due to humic acid application. In this study, humic acid application caused an increase in oil percentage and oil yield in comparison with control plants. These results are in agreement with the report of Rajpar et al (2011) which showed that application of humic acid had significant effect on oil percentage and yield. Similarly, it has been reported that foliar application of humic acid improved seed yield and oil content in mustard (Chris et al 2005). Generally, humates enhance nutrient uptake and increase the yield and quality of various oilseed crops (MacCarthy et al 2001; Salt et al 2001).

5. Conclusions

The canola cultivars responded to different sowing dates. Growth, seed yield and oil yield decreased with delayed sowing date. By contrast, early seed sowing could improve canola yield and oil production. In this experiment, application of humic acid could significantly increase yield and yield components of canola as well as oil percentage and oil yield. Therefore, choosing a suitable sowing date (as early as possible) is essential depending upon growing conditions and cultivar. According to this experiment, it can be suggested that the most appropriate sowing time for a desire seed yield in the experimental region is early October and humic acid foliar application as a way to increase canola yield and production is recommended.

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Inheritance of Indehiscent Capsule Character, Heritability and Genetic Advance Analyses in the Segregation Generations of Dehiscent x Indehiscent Capsules in Sesame

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ABSTRACT

The investigation was made to estimate heritability and genetic advance and thus understand the genetic behavior of indehiscent capsule character in sesame with the field experiments during three consecutive years. Muganli-57 (♀) parent with dehiscent capsule was crossed with ACS 344 (♂), an introduction material, which had indehiscent capsule character. The results in the F_1 generation indicated that dehiscent capsule character was dominant over indehiscent capsule. In F_2, 3:1 segregation ratio was monitored and further confirmed in F_3 showing indehiscent capsule character was controlled by a single recessive gene. Heritability was estimated by parent-offspring regression and the data were collected in F_1, F_2 and F_3 generations derived from the cross between dehiscent and indehiscent capsule types. Heritability estimates in narrow sense for number of branches, number of capsules per plant, 1000 seed weight and seed yield were low in indehiscent capsule types of F_2. Genetic advance had also low values except for number of branches (30.79%). Whilst low values obtained for indehiscent capsule, dehiscent capsule types in F_2 indicated high heritability for the characters studied (70.52-92.84%). Stem height to the first capsule and plant height were of high heritabilities in both capsule types of F_2 and F_3. Although indehiscent capsule types had low means and heritabilities due to pleiotropic effects, mutant cross had heterotic effect on dehiscent types for all the characters. This positive shift could be explained by additive gene effect and this information presented in this investigation was therefore highly beneficial for the genetic improvement of sesame and reaching to high yielding types. This research also provides an example that bad parent may have a good progeny.

Keywords: Capsule shattering; Genetic parameters; Mutation; Pleiotropy

1. Introduction

Sesame (*Sesamum indicum* L.) is one of the world's important oilseed crops and has been used for food and animal feeding in the form of seed, seed oil and meal (Hahm et al 2009). Sesame oil includes antioxidant lignans such as sesamin and sesamolin which have lots of health benefits. Especially sesamin has been linked with prevention of hyperlipidemia, hypertension, and carcinogenesis (Harikumar et al 2010). In addition, sesame has agricultural advantages: it grows well tropic, sub-tropic and Mediterranean climates, can grow on only soil moisture without rainfall or irrigation (Ashri 2007) and can be used as a second crop which is

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important for sustainable farming. Despite such desirable characteristics, sesame production suffers from certain major causes of low seed yields include capsule shattering, indeterminate growth habit, late maturity, low adaptation ability.

Normally, the seed-bearing capsule of sesame shatters at harvest. This wildish character is the most problematic issue in sesame farming because it causes seed losses at harvest which can be very high, up to 50% (Weiss 1971). This unimproved character also makes the crop unsuitable for mechanized harvesting (Langham & Wiemers 2002) and consequently restricts its commercial potential by limiting the growing areas to countries that have no access to cheap labor (Uzun et al 2003). Therefore non-shattering capsule genotypes are needed for mechanized harvesting of sesame.

Although an indehiscent mutation that held all of sesame seed was first discovered by Langham (1946), the majority of the world's sesame (probably over 99%) is shattering, and most of the harvest is manual (Langham 2001). The most important reason was the pleiotropic effects of the mutation including cupped leaves, short seed pods, twisted stems and low seed yield (Wongyai et al 2001). These unwanted side effects restrict non-shattering sesame usage and crossing with cultivars which have different genetic backgrounds should be beneficial to remove the undesirable side-effects (Uzun et al 2004). Because, selected parents from divergent origins have potential to present beneficial traits (Ashri 1998). Even though non-shattering sesame has been used as a parent in breeding programs, there is no report about heritability and genetic advance on yield and yield components in hybridization studies. To understand genetic behaviors of characters genetic parameters would be helpful (Majidi et al 2009). This type data is important for identification of agronomic potential of genotypes. Such data is usefully subjugated in articulating efficient selection program for synthesis and development of cultivars with desirable characters having genetic base (Khan et al 2010). From this perspective, the objectives of the current study were to evaluate the genetic parameters (heritability, inheritance and genetic advance) in indehiscent capsule type sesame, its cross with dehiscent capsule type sesame and exhibit their breeding potential.

2. Material and Methods

2.1. Experimental area

The field experiments were conducted at the West Mediterranean Agricultural Research Institute's fields of Antalya (36°52'N. 30°50'E. 15 m elevation). Antalya has coastline to Mediterranean Sea and it has 1060 mm annual average precipitation and 18 °C annual average temperature (TSMS 2010).

2.2. Plant materials

Indehiscent capsule sesame (PI 599446) was kindly provided by USDA, ARS, Georgia, USA and deposited in our collection with the code of ACS 344. Muganli-57 with dehiscent capsule (♀) was crossed with ACS 344 (♂) in 2008. F_1s were selfed in the growing season of 2009 and F_2 and F_3 populations were developed in the years of 2010 and 2011.

2.3. Genetic study

Parents, F_1s, F_2s and F_3s were grown in 70 cm row and plant to plant within a row of 10 cm. Fertilizer was applied at rates of 60 kg N, 60 kg P_2O_5 and 60 kg K_2O per hectare prior to sowing F_1, F_2 and F_3 generations. 56 seeds from hybridization of Muganli-57 and ACS 344 were used to produce F_1 plants. In F_2 generation, dehiscent capsule and indehiscent capsule plants were grown with the numbers of 803 and 254, respectively. Single dehiscent and indehiscent capsule plants in F_2 and F_3 were selected and compared with previous generations for heritability. The characters of stem length to the first capsule (SLFC), plant height (PH), number of branches (NB), number of capsules per plant (NCP), number of seeds per capsules (NSC), 1000 seed weight (TSW) and seed yield (SY) were used for the assessments (IPGRI & NBPGR 2004).

2.4. Statistical analysis for inheritance

Statistical analysis for inheritance was conducted according to Steel & Torrie (1980). Parent offspring

method was used for heritability estimates in narrow sense according to Smith & Kinman (1965) with the formula;

$h_{rg}2= b/2r_{op}$

Where; b is regression coefficient and r_{op} is relationship of parent-offspring.

20% selection intensity was applied for the genetic advance calculations. Genetic advance was conducted using the following formula;

$GA = K (S_{ph}) (H)$

Where; K is a constant which at a selection intensity of 20% is about 1.40; S_{ph} is the phenotypic standard deviation ($\sqrt{V_{ph}}$); H is the heritability ratio.

3. Results

3.1. Heritability and genetic advance

Heritability estimates in narrow sense of dehiscent x indehiscent capsule cross were calculated for seven quantitative characters (Table 1). Heritability and genetic advance of dehiscent and indehiscent capsule types were estimated separately and thus the plants in F_2 were classified as dehiscent and indehiscent capsule types. The highest heritability was obtained for SLFC (78.3%) in indehiscent capsule types in F_2. PH indicated latter the highest heritability (74.4%). Following to these characters, NB, NCP, NSC and TSW showed low heritability ranged from 54.5% to 44.0% (Table 1). On the other hand the lowest heritability was observed for SY (22.2%). Heritability estimates for dehiscent capsule types was higher than those of indehiscent capsule in F_2 generation for all characters except for SLFC (Table 1). Narrow sense heritability (h^2) was the highest (92.8%) for NCP, followed by NSC (90.1%) for dehiscent capsule types in F_2. Similar high heritability trends were observed for NB (87.9%) and PH (86.0%). However three characters, SLFC, TSW and SY, had heritability under 80%, particularly SY showed the lowest heritability (70.5%) for dehiscent capsule types in F_2.

Estimates of genetic advance (GA) ranged from 2.6% to 30.8% for indehiscent capsules in F_2. Generally, SLFC, PH, NCP and SY depicted GA values lower than 10%. In contrast, TSW showed relatively high GA estimation (>15%). GA valuation for dehiscent capsule types was much higher than those of indehiscent capsule in F_2 (Table 1). The characters of NB, TSW, SY, and NCP showed appreciable GA estimates with values of 49.7%, 28.4%, 26.0% and 12.4%, respectively. The remaining three characters,

Table 1- Heritability (h^2) of by parent-offspring regression and standard errors of dehiscent capsule x indehiscent capsule cross and its genetic advance (GA)

	F_1 to F_2		*F_2 to F_3 (ICT to ICT and DCT to DCT)*					
	h^2 (%)	*GA (%)*	*h^2 (%)*	*GA (%)*	*h^2 (%)*	*GA (%)*	*h^2 (%)*	*GA (%)*
Traits	*Indehiscent capsule types (ICT)*	*Indehiscent capsule types*	*Dehiscent capsule types (DCT)*	*Dehiscent capsule types*	*Indehiscent capsule types*	*Indehiscent capsule types*	*Dehiscent capsule types*	*Dehiscent capsule types*
SLFC	78.3±0.3	8.9	78.2±0.3	8.9	75.3±0.1	10.9	82.4±0.5	8.7
PH	74.4±0.7	3.7	86.0±0.4	4.3	89.3±0.9	34.6	87.8±0.3	17.6
NB	54.5±0.3	30.8	87.9±0.1	49.7	83.3±0.2	1.3	83.3±0.2	1.2
NCP	46.4±0.2	6.2	92.8±0.5	12.4	92.8±0.1	25.6	90.4±0.1	13.0
NSC	50.0±0.3	2.6	90.1±0.5	4.7	91.7±0.1	5.3	96.1±0.1	5.2
TSW	44.0±0.1	16.3	76.8±0.2	28.4	62.5±0.1	0.1	71.2±1.2	0.2
SY	22.2±0.1	8.2	70.5±0.8	26.0	63.1±0.6	0.2	69.7±0.6	2.2

ICT is stand for indehiscent capsule types; DCT is stand for dehiscent capsule types. SLFC is stand for stem length to the first capsule; PH is stand for plant height; NB is stand for number of branches; NCP is stand for number of capsules per plant; NSC is stand for number of seeds per capsules; TSW is stand for 1000 seed weight; SY is stand for seed yield

SLFC, NSC and PH, depicted genetic advance lower than 10%. Heritability and GA of dehiscent and indehiscent types of sesame were also calculated in F_3 generation to observe genetic stability of the characters (Table 1). In indehiscent capsule types, the highest heritability was estimated for NCP (92.8%) which was followed by NSC (91.7%), PH (89.3%) and NB (83.3%) as higher heritability. Lower heritability was observed in the characters of SY (63.1%) and TSW (62.5%). Among the characters, GA expected from selection of the progenies was the highest for PH (34.6%) and the lowest for TSW (0.1%). Higher heritability estimates was calculated in dehiscent capsule types for the characters of SLFC, PH and NSC characters in F_3 compared to F_2. The maximum heritability estimate was for NSC (96.1%) and the minimum was for SY (69.7%) in dehiscent capsule types. Across these characters, NCP and PH showed relatively high heritability values (90.4% and 87.8%, respectively).

Mean values of dehiscent capsule plants in F_2 were higher than parental lines for all characters (Table 2). However indehiscent capsule plants in F_2 had lower values than the parent, ACS 344 for NB, NCP, NSC and TSW. In dehiscent F_3 progenies, the characters of NSC, NCP and SY had the highest mean values compared to F_2s and F_3s indehiscent types, F_1s and both parents. The characters of PH, NCP and NB showed higher means for indehiscent progenies in F_3 than F_2 (Table 2).

3.2. Inheritance

Following to Muganli-57 (♀) x ACS 344 (♂) cross, all the F_1 filials had dehiscent capsules. In F_2, segregation for dehiscent to indehiscent capsule was 803 to 254 (Table 3). Chi-square value showed a good fit for a monogenic inheritance with the F_2 phenotypic ratio of 3:1. In F_3, dehiscent and indehiscent capsule type plants obtained from F_2 progenies were assessed separately. Totally, 165 plants originating from 5 indehiscent F_2 plants indicated indehiscent capsule type and there is no dehiscent capsule type segregation in F_3 (Table 4). Similarly dehiscent capsule type F_2 plants were advanced to F_3 separately and of 192 F_3 plants (offspring 1, 2, 4, 5, and 8) showed dehiscent capsule

Table 2- Means and standard errors of the parental lines, F_1, F_2 and F_3 generations for seed yield and yield components

Characters	*Parental line*		*Muganli-57 x ACS 344*				
	Muganli-57	*ACS 344*	F_1	$F_{2\text{-}id}$	$F_{2\text{-}ID}$	$F_{3\text{-}id}$	$F_{3\text{-}ID}$
SLFC (cm)	52.0±4.9	52.0±4.9	42.2±3.1	57.0±5.3	58.0±3.3	41.0±4.6	57.0±3.4
PH (cm)	127.0±8.9	86.0±2.4	111.6±3.7	157.0±7.8	142.9±5.0	162.8±6.3	146.0±6.6
NB	3.8±0.2	3.2±0.2	3.8±0.4	3.7±0.5	4.6±0.3	7.2±0.5	6.0±0.5
NCP	56.0±1.0	48.0±3.0	69.6±4.7	49.5±6.9	82.6± 4.7	83.2±8.8	97.2±4.6
NSC	77.6±0.7	66.8±1.5	74.8±1.5	68.8±1.3	78.2±1.5	70.4±1.8	81.6±1.7
TSW (g)	3.04±0.1	2.06±0.1	3.2±0.1	2.7±0.1	3.4± 0.1	2.6±0.1	3.4±0.1
SY (g)	5.91±1.9	1.24± 0.1	6.74±1.4	1.2±0.2	12.3± 3.4	2.2±0.2	14.4±2.0

Muganli-57 is dehiscent capsule genotype, ACS 344 is indehiscent capsule genotype. $F_{2\text{-}id}$ is indehiscent capsule genotypes in F_2, $F_{2\text{-}ID}$ is dehiscent capsule genotypes in F_2. $F_{3\text{-}id}$ is indehiscent capsule genotypes in F_3, $F_{3\text{-}ID}$ is dehiscent genotypes in F_3. SLFC is stand or stem length to the first capsule; PH is stand for plant height; NB is stand for number of branches; NCP is stand for number of capsules

Table 3- Segregation ratio of dehiscent and indehiscent characters of sesame in F_2

Cross	*Experimental*		*Theoretical*		χ^2	*P*	*Ratio*
	Dehiscent capsule	*Indehiscent capsule*	*Dehiscent capsule*	*Indehiscent capsule*			
Muganli-57 x ACS 344	803	254	792.75	264.25	0.53	0.45-0.50	3:1

type (Table 4). The remain F_3 plants sourced from offsprings 3, 6, 7 and 9 consisted of 160 dehiscent and 36 indehiscent capsule type plants, totally. This result indicated that indehiscent and dehiscent plants fitted the expected 3:1 ratio in the offsprings of 3, 6, 7 and 9 (Table 4).

Table 4- Segregation ratios of dehiscent and indehiscent capsule characters of sesame in F_3

Cross	*Number of plants*			
Muganli-57 x ACS 344	*Dehiscent capsule plants*	*Indehiscent capsule plants*	*Ratio*	*Symbol*
F_2 progeny with dehiscent capsule				
Offspring 1	56	0	1:0	*Id/Id*
Offspring 2	59	0	1:0	*Id/Id*
Offspring 3	65	14	3:1 (P= 0.14)	*Id/id*
Offspring 4	35	0	1:0	*Id/Id*
Offspring 5	13	0	1:0	*Id/Id*
Offspring 6	24	6	3:1 (P= 0.52)	*Id/id*
Offspring 7	23	8	3:1 (P= 0.92)	*Id/id*
Offspring 8	29	0	1:0	*Id/Id*
Offspring 9	48	8	3:1 (P= 0.06)	*Id/id*
F_2 progeny with indehiscent capsule				
Offspring 1	0	28	0:1	*id/id*
Offspring 2	0	25	0:1	*id/id*
Offspring 3	0	29	0:1	*id/id*
Offspring 4	0	20	0:1	*id/id*
Offspring 5	0	63	0:1	*id/id*

4. Discussion

Indehiscent capsule plants indicated low heritability for seed yield and important yield components. This result might be sourced of the mutation event because a large deletion of a chromosome segment could be occurred in development of indehiscent capsule type plants. By the deletion event, if the plants are indehiscent in F_2, then the chromosome with a large deletion are paired with a normal one. Thus, the plant is indehiscent capsule type but the deleterious effect as a result of the natural mutation is still continuous in the F_2 plants though different background crosses were made.

TSW of indehiscent capsule types in F_1 to F_2 and F_2 to F_3 had low heritability compared to dehiscent capsule types. This low heritability indicated that it would not be a close correspondence between genotype and phenotype for the mutant types due to the relative high contribution of the environment to the phenotype. Small capsules and weak seeds might also be responsible for the lower values which might effect to seed yield because seed size (Kumar 2010) and capsule length (Chowdhury et al 2010; Yol et al 2017) have strong correlations with seed yield in sesame. Similarly Hika et al (2015) obtained low heritability for TSW in dehiscent capsule type sesame plants. Only PH had higher mean in indehiscent capsules in F_2 compared to F_1 and parents. This positive improvement could be explained by additive gene effect because mutant crosses might have heterotic effect. Similar effects were observed by Murty (2001) and Maluszynski et al (2001) for sesame and different plants, respectively. This prediction is reasonable because similar improvement was also observed in PH of dehiscent capsule types in F_2 and it was consistent

with high heritability for this character as reported by Govidarasu et al (1990). In contrast to low heritability values of indehiscent capsule types, dehiscent capsules showed high heritability in F_1 to F_2 and F_2 to F_3. NB had high genetic advance and heritability with higher mean values compared to F_1. Mutant crosses therefore might have heterotic effect on character of NB which was identified as one of the most contributing character to seed yield (Yol et al 2010). Greater heritability and high genetic advance in SY of dehiscent capsules showed that this parameter was under the additive genetic effects (Mahmood et al 2004). Selection of high yield genotypes obtained from mutant x cultivar crosses therefore has agricultural benefit because additive effects have positive contribution to SY (Hoballah 2001). This positive effect was also observed for dehiscent capsule types in F_2 compared to parents and F_1. Similarly, Uzun et al (2004) reported that all hybrids showed heterosis for seed yield in mutant x cultivar crosses. This approach was also suggested to develop new genotypes having more than one of the desired characters using with crosses 'local varieties x mutants', 'mutants x mutants', and 'mutants x introduced lines' by Van Zanten (2001).

The cross made in current study indicated that dehiscent capsule in sesame was under the control of a single gene and dehiscent character was dominant to indehiscent capsule. Dehiscent capsule had monogenic inheritance and indehiscent capsule was controlled by a recessive allele. Firstly, Langham (1946) discovered an indehiscent mutation and this character was symbolized as *id* (indehiscent gene). *id/id* genotype capsules were so rigid and its capsules open when it took a hammer (Langham & Wiemers 2002). Other indehiscent capsule character was also recessive and it's called "*seamless*", symbolized *gs/gs* (Langham 2001), appear to have only one carpel although it has 4 rows of seeds (Ashri 1998). Even though the single gene effect was identified, the indehiscent capsule types show deleterious characteristics such as semi-sterility, twisted stems, and low yield as a result of unwanted side-effects. In addition threshing the hard capsule without damaging the seed is too difficult (Weiss 1971). Due to undesirable characters, the *gs* and *id* allele can only be used in a timely manner to minimize the effects of seed damage from threshing (Van Zanten 2001). However it does not seem a commercial. Rather than direct using of indehiscent capsule, cross breeding will be useful for development of superior varieties of sesame (Kang 2001). Because, this present study showed that "cultivar x mutant" crosses had positive effect on development of desirable genotypes.

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Seedling Response of Iranian Barley Landraces to *Pyrenophora teres* f. *teres* and *Pyrenophora teres* f. *maculata*

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ABSTRACT

Net blotch caused by *Pyrenophora teres* is an important pathogen of barley plants worldwide. There are two biotypes of the fungus. *Pyrenophora teres* f. *teres* (*Ptt*) causes the net form of the disease and *Pyrenophora teres* f. *maculata* (*Ptm*) causes the spot form of the disease. Barley landraces are good sources of disease resistance. In this study, seedling response of 25 barley landraces obtained from different regions of northwest Iran to 3 single spore isolates of *Ptt* and 3 single spore isolates of *Ptm* were determined under greenhouse conditions. Differences in virulence among the isolates were evident. Some landraces showed different responses to different isolates. Landraces # 9 and # 16 showed moderately resistant reactions to one isolate of *Ptt* and showed moderately resistant-moderately susceptible reactions to 2 isolates of *Ptt*. Landraces # 7, # 11, # 15, # 17, # 21, # 22, # 23 and # 25 showed moderately resistant-moderately susceptible reactions to all 3 isolates of *Ptt*. Landrace # 23 showed resistant-moderately resistant reaction to one isolate of *Ptm* and showed moderately resistant reactions to 2 isolates of *Ptm*. Landrace # 16 showed moderately resistant reactions to all isolates of *Ptm*. Landraces # 11, # 15, # 21 and # 25 showed moderately resistant reaction to one isolate of *Ptm* and showed moderately resistant-moderately susceptible reactions to 2 isolates of *Ptm*. Landraces that exhibited reactions between resistant and moderately resistant-moderately susceptible range could be used as a direct seeding material to the field or could be used as breeding materials.

Keywords: *Drechslera teres*; Barley; Landrace; Net form of net blotch; Spot form of net blotch

1. Introduction

Barley (*Hordeum vulgare* L.) is the most cultivated cereal crop after wheat, rice and maize in the world (FAO 2015). It is the most planted cereal after wheat in Turkey (TUIK 2016; Geçit 2016).

Archaeological findings showed that barley was domesticated in various places of the Fertile Crescent (Zohary & Hopf 1993). Parts of Turkey and Iran are located in the Fertile Crescent region. *Hordeum spontaneum*, progenitor of cultivated barley, is also common in this region (Harlan & Zohary 1966; Nevo 1992). Eight main regions including China, India, Near East, Central Asia, Ethiopia, Mediterranean, Central and South America and Southern Mexico are considered as plant gene centers in the world (Vavilov 1951). Turkey and Iran are very important phytogeographical regions due to presence of Mediterranean as well

as Central Asiatic taxa (Von Bothmer 1996). Barley landraces are still planted in these areas.

Barley plant is resistant to adverse conditions and has high adaptation capability. It can grow in various soil and climatic conditions (Mathre 1982; Geçit 2016). Wild barleys and barley landraces are new sources of genetic variation useful for different stress tolerances. They are regarded as a 'gold mine' because of their potential power to develop new genotypes against various biotic and abiotic stress factors (Yitbarek et al 1998; Ceccarelli & Grando 2000; Ellis et al 2000). Barley landraces show optimum adaptability to changing environmental conditions (Allard & Bradshaw 1964). Landraces have rich antioxidant and mineral contents and these properties can be used to develop varieties with better quality traits (Newton et al 2010). Iranian barley landraces can be considered as important gene sources for modern cultivar improvement (Khodayari et al 2012).

Net blotch is an important barley foliar disease and causes significant decreases in yield and quality of barley. Two biotypes of fungus cause different symptoms. *Pyrenophora teres* f. *maculata* (*Ptm*) incites spot type of net blotch and *P. teres*. f. *teres* (*Ptt*) incites net type of net blotch (Shipton et al 1973; Mathre 1982; Karakaya & Akyol 2006; Liu et al 2011). The prevalence of net blotch disease is related to the susceptibility of cultivated varieties. Yield losses can be 100% in severely affected fields where highly susceptible cultivars are planted. However, general losses range between 10-40% (Mathre 1982). The use of fungicides, cultural practices and planting resistant barley genotypes against the disease are recommended (McLean et al 2012). The most profitable and ecologically friendly method to control of net blotch is through using resistant barley cultivars.

In this study, we evaluated the seedling responses of 25 Iranian barley landraces obtained from different parts of Iran to 3 single spore isolates of *Ptt* and 3 single spore isolates of *Ptm* collected from different provinces of Turkey. An abstract of this study has been published previously (Çelik Oğuz et al 2017a).

2. Materials and Methods

2.1. Plant materials

Barley landraces were collected from Oshnaviye, Piranshahr, Bukan and Naghadeh regions of northwest Iran. From these, healthy looking individial seeds were selected and planted into 24 cm in diameter plastic pots filled with field soil. These pots were placed outside and watered as needed. Both light colored and dark colored seeds were selected. Seeds of these landraces were harvested after maturity and were used in this experiment.

2.2. Making single spore isolates

The infected barley leaves with net and/or spot form of net blotch were obtained from Sivas, Şanlıurfa, Kilis, Ankara, Konya and Diyarbakır provinces of Turkey. Leaf samples were cut into small pieces and surface sterilized with 1% sodium hypochloride. These pieces were incubated for 3 days on moist filter paper in sterile Petri dishes. Single spores were taken under a stereomicroscope and then transferred to the Petri dishes containing Potato Dextrose Agar (PDA).

2.3. Inoculation and incubation

No sporulation was observed in PDA, therefore, hyphal parts were used as inoculum. Previous studies showed that inoculation with hyphae was successful (Douiyssi et al 1998; Karakaya & Akyol 2006; Çelik Oğuz et al 2017b). Inoculum was prepared using 10 days old *Ptt* and *Ptm* cultures grown on PDA by brushing the culture and then filtering through cheesecloth. Mycelium particles (15-20 x 10^4 per mL) were adjusted using Thoma slides and 1 mL Tween 20 was added per 100 mL inoculum (Aktaş 1995; Douiyssi et al 1998; Karakaya & Akyol 2006; Çelik Oğuz et al 2017b). Inoculum was then sprayed onto barley leaves using a hand sprayer at the growth stages 12-13 (Zadoks et al 1974). The plants were kept in lid boxes for 76 hours under greenhouse conditions. After this period, ventilation

of the boxes was opened and they were kept for 48 hours. The temperature of the greenhouse was 17±2 °C night and 22±2 °C day with a 14h/10h light/dark regime. Three replications were employed.

2.4. Disease assessment

Disease evaluations were made 7 days after inoculation using Tekauz (1985) scales which are based on lesion morphology of net blotch biotypes.

3. Results and Discussion

Twenty-five Iranian barley landraces showed different responses to 3 *Ptt* and 3 *Ptm* isolates (Table 1). Pathogenic variation was observed between *P. teres* isolates and Iranian barley landraces. Response of landraces to *Ptt* and *Ptm* isolates ranged between moderately resistant and moderately susceptible-susceptible. The most virulent isolates of *Ptt* and *Ptm* were *Ptt* 1 Sivas and *Ptm* 1 Ankara isolates, respectively.

Table 1- Seedling reactions of 25 Iranian barley landraces to 3 *Pyrenophora teres* f. *teres* and 3 *Pyrenophora teres* f. *maculata* isolates. For disease evaluation scales developed for net form of net bloth and spot form of net blotch by Tekauz (1985) were used

Landrace no	*Location*	*Row type*	*Kernel color*	*Pyrenophora teres f. teres*				*Pyrenophora teres f. maculata*			
				Ptt 1 Sivas	*Ptt 2 Şanlıurfa*	*Ptt 3 Kilis*	*Mean*	*Ptm 1 Ankara*	*Ptm 2 Konya*	*Ptm 3 Diyarbakır*	*Mean*
1	Naghadeh	2	Light	7	7	5	6.3	7	7	7	7
2	Naghadeh	2	Light	8	7	3	6	5	7	7	6.3
3	Bukan	2	Light	6	8	5	6.3	7	5	5	5.6
4	Naghadeh	2	Dark	8	6	5	6.3	8	7	5	6.6
5	Oshnaviye	2	Light	8	4	3	5	8	5	7	6.6
6	Naghadeh	2	Dark	7	4	6	5.6	7	7	7	7
7	Bukan	2	Light	5	5	5	5	5	7	5	5.6
8	Oshnaviye	2	Light	7	6	3	5.3	5	7	3	5
9	Bukan	2	Light	4	4	3	3.6	7	2	5	4.6
10	Piranshahr	2	Light	6	6	7	6.3	7	3	5	5
11	Piranshahr	6	Light	5	4	5	4.6	5	3	5	4.3
12	Naghadeh	2	Light	8	4	6	6	7	7	5	6.3
13	Naghadeh	2	Dark	8	6	6	6.6	7	7	5	6.3
14	Naghadeh	2	Dark	7	4	6	5.6	7	7	7	7
15	Naghadeh	2	Light	5	5	5	5	5	3	5	4.3
16	Bukan	2	Light	6	6	3	5	3	3	3	3
17	Piranshahr	6	Light	6	6	4	5.3	7	5	3	5
18	Oshnaviye	2	Light	6	4	7	5.6	5	5	7	5.6
19	Piranshahr	2	Light	7	5	7	6.3	7	3	7	5.6
20	Naghadeh	2	Dark	7	4	5	5.3	7	7	5	6.3
21	Naghadeh	2	Light	5	5	4	4.6	5	3	5	4.3
22	Piranshahr	2	Light	5	4	4	4.3	7	7	3	5.6
23	Naghadeh	2	Light	4	6	4	4.6	3	3	2	2.6
24	Naghadeh	2	Dark	5	7	4	5.3	7	5	3	5
25	Bukan	2	Light	4	4	4	4	5	5	3	4.3
Mean				6.16	5.24	4.76		6.12	5.2	4.96	

Fourteen, 21 and 17 landraces exhibited moderately resistant-moderately susceptible reactions to *Ptt* 1 Sivas, *Ptt* 2 Şanlıurfa and *Ptt* 3 Kilis isolates, respectively. Five landraces (# 2, # 5, # 8, # 9 and # 16) showed moderately resistant reactions to *Ptt* 3 Kilis isolate. Landraces # 7, # 11, # 15, # 17, # 21, # 22, # 23 and # 25 showed moderately resistant-moderately susceptible reactions to all 3 isolates of *Ptt*. Landraces # 9 and # 16 were moderately resistant-moderately susceptible to two isolates of *Ptt* and were moderately resistant to one isolate of *Ptt*. Eight, 6 and 11 landraces showed moderately resistant-moderately susceptible reactions to *Ptm* 1 Ankara, *Ptm* 2 Konya and *Ptm* 3 Diyarbakır isolates, respectively. Two landraces (# 16 and # 23) were moderately resistant to *Ptm* 1 Ankara isolate. In addition, 7 landraces were moderately resistant and one landrace (# 9) was resistant-moderately resistant to *Ptm* 2 Konya isolate. Six landraces were moderately resistant and one landrace (# 23) was resistant-moderately resistant to *Ptm* 3 Diyarbakır isolate. Landrace # 23 showed resistant-moderately resistant reaction to one isolate of *Ptm* and showed moderately resistant reactions to 2 isolates of *Ptm*. Landrace # 16 exhibited moderately resistant reactions to all isolates of *Ptm*. Landraces # 11, # 15, # 21 and # 25 were moderately resistant to one isolate of *Ptm* and exhibited moderately resistant-moderately susceptible reactions to 2 isolates of *Ptm*. Landrace # 9 was resistant-moderately resistant to one isolate of *Ptm* and landraces # 8, # 10, # 11, # 15, # 17, # 19, # 21, # 22 and # 24 were moderately resistant to one isolate of *Ptm*.

Fertile Crescent is the most likely geographical area where the wild barley is domesticated and wild barley populations located in the Fertile Crescent have contributed genetic material to the cultured barley (Zohary & Hoph 1993; Badr et al 2000; Morrell & Clegg 2007). This creates a large variaton in the genetic base of barley. McLean et al (2009) reported the presence of resistant genotypes among barley genotypes in the Middle East.

Barley has been grown in Fertile Crescent region a long period of time and a rich genetic diversity exist in this area (Ceccarelli & Grando 2000; Khodayari et al 2012). Ebrahimi et al (2013) investigated the genetic diversity of 115 barley landraces and wild barleys from 5 *Hordeum* species and significant variation was observed between the landraces. Khodayari et al (2012), using microsatellite markers, investigated the genetic diversity among the Iranian barley landraces and Khazaei et al (2012) characterized the agronomic traits of winter barley landraces and 4 advanced varieties collected from Iran. Both studies reported high levels of polymorphism and genetic diversity among the Iranian barley genotypes.

There are limited studies on the resistance of Iranian barley landraces to *P. teres*. Ghazvini & Tekauz (2007) tested 160 barley accessions from Iran for their reactions to *Fusarium graminearum*, *Bipolaris sorokiniana* and *Dreschlera teres* f. *teres* (teleomorph: *Pyrenophora teres* f. *teres*) in order to find new resistance sources. Three accessions were found to be resistant to *Dreschlera teres* f. *teres*. No resistance to *Fusarium graminearum* and *Bipolaris sorokiniana* isolates was found. It is concluded that disease resistant landraces were important in achieving sustainability and they were valuable sources in germplasm collections. In the current study, we identified Iranian landraces that exhibited different levels of resistance to both forms of net blotch. In current study, 5 Iranian barley landraces showed moderately resistant reactions to *Ptt* 3 Kilis isolate. Landrace number 16 exhibited moderately resistant reactions to all *Ptm* isolates and landrace number 23 showed moderately resistant reactions to 2 isolates of *Ptm* and showed resistant-moderately resistant reaction to one isolate of *Ptm*.

Large number of disease resistant barley genotypes were found in the gene centers of barley (Afanasenko et al 2000). Numerous studies reported the resistance of barley landraces to *P. teres* from different parts of the world. Lakew et al (1995) and Yitbarek et al (1998) assessed the reactions of Ethiopian barley landraces to *P. teres* and observed significant variation in landraces. Legge et al (1996) tested the resistance status of 176 Turkish barley lines to *P. teres*. More lines showed resistance to spot form of net blotch than net form of net blotch. In our

study, similarly, Iranian landraces exhibited different levels of resistance to *P. teres* isolates and more Iranian landraces showed resistance to spot form of net blotch. In a study conducted by Semeane (1995) in Ethiopia only 4 of 900 barley landraces were found to be resistant to net blotch. Silvar et al (2010) tested 159 barley landraces and 16 barley cultivars from Spanish Barley Core Collection to 3 *P. teres* f. *teres* isolates. Landraces showed low resistance and only one landrace showed resistant reaction to all isolates used. Endresen et al (2011) evaluated trait-specific subset selection methods for net form of net blotch. Neupane et al (2015) evaluated 2062 barley accessions obtained from World Barley Core Collection to four *P. teres* f. *teres* isolates obtained from Australia, United States, Denmark and New Zealand. Fifteen accessions were found to be resistant to all isolates. In our study, a high number of Iranian barley landraces showed reactions in the range of resistant-moderately resistant to moderately resistant- moderately susceptible to both forms of the pathogen. Chakrabarti (1968) and Khan & Boyd (1969) tested barley varieties from World Barley Core Collection for their reactions to net blotch. In their studies, thirty of 6246 barley varieties and 6 of 8756 barley varieties were found to be very resistant, respectively. Turkey, which is located in the Fertile Crescent region, is one of the important gene centers of barley and has important barley genetic resources. Wild barley and cultivated barley landraces obtained from Turkey and Jordan were evaluated for their resistance status to *Cochliobolus sativus*, *P. teres* f. *maculata* and *P. teres* f. *teres* collected from Canada. Wild barley genotypes were found to be more resistant to *C. sativus* and *P. teres* f. *teres*. Equal amounts of wild barleys and cultivated landraces of barley were found to be resistant to *P. teres* f. *maculata* (Jana & Bailey 1995). Çelik Oğuz et al (2017b) tested 198 Turkish barley landraces to 6 virulent isolates of net form of net blotch and spot form of net blotch. 13 barley landraces showed resistant reactions to all *P. teres* f. *maculata* isolates and 7 barley landraces showed resistant reactions to all *P. teres* f. *teres* isolates. In addition, numerous landraces exhibited resistant reactions to at least one isolate. Similarly, in our current study, more Iranian barley landraces showed resistant group reactions to *Ptm* isolates than *Ptt* isolates. Several Iranian landraces were found to be resistant-moderately resistant or moderately resistant to both forms of the pathogen.

New gene resources resistant to diseases, pests and changing climatic conditions are needed for sustainable agriculture. Landraces have desirable agronomical traits and are sources of wide variation (Ceccarelli & Grando 2000; Ergün et al 2017). Useful agronomical traits could be transferred to advanced varieties successfully (Newton et al 2010). These genetic resources should be collected from natural habitats and should be protected (Frankel & Hawkes 1975).

4. Conclusions

Barley landraces are valuable sources of disease resistance. In this study, 25 barley landraces collected from different regions of northwestern Iran were tested to both forms of *P. teres* with the aim of finding sources of resistance. Fifteen of 25 landraces showed moderately resistant or resistant-moderately resistant reactions to 1 or more isolates. A wide variation was observed among the Iranian barley landraces to pathogen isolates.

Virulence changes can occur in various ways in fungi and resistant genotypes may show susceptible reactions to emerging virulent pathogens (Burdon & Silk 1997; Liu et al 2011). In order to control new pathotypes, resistance studies should be continuous and the establishment of a broad genetic base is necessary for durable and sustainable resistance. Iranian barley landraces determined in this study could be used as gene sources in future breeding studies in order to obtain net blotch resistant barley genotypes.

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Comparison of Physicochemical Attributes of Einkorn Wheat (*Triticum monococcum*) and Durum Wheat (*Triticum durum*) and Evaluation of Morphological Properties Using Scanning Electron Microscopy and Image Analysis

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ABSTRACT

Einkorn (*Triticum monococcum* L. subsp. *monococcum*) is a diploid hulled wheat strictly related to durum and bread wheat types. Many farms in northern Turkey, especially the province of Kastamonu, continue to cultivate the oldest type of wheat still in existence, *Triticum monococcum*, also known as Siyez. Although there is great potential for breeding, it's production and consumption has been locally limited and cultivation quantity has been very low. However nowadays, today's consumer demands to traditional or regional food products and grains have been increased einkorn cultivation in Kastamonu, and cultivation area has been reached to approximately from 5 thousand decares to 35 thousand decares. Einkorn is cultivated two times a year in Kastamonu. The variety which is named as "Çatal Siyez" (Kaplica) composed 60-70% of the production of einkorn, is sowed in April and used as animal feed after harvest. Another type which is "Tek Siyez" variety sowed in October is processed to produce einkorn flour and bulgur.

In this study, some physicochemical attributes of Einkorn (*Triticum monococcum*) and Durum Wheat (*Triticum durum*) were compared and their morphological properties were evaluated by using scanning electron microscopy and image analysis. The scanning electron microscopy micrographs shown that Einkorn had opaque but durum wheat had a vitreous appearance. In addition, the starch granules could be easily separated visually in Einkorn texture. In durum wheat, the protein bonds were more compact and the diameter of starch granules located in protein matrix lower than ones of Einkorn. Moreover, it was determined that visually the caryopsis layers of Einkorn were thinner than ones of the durum wheat. The kernel weight (g 1000 pieces^{-1}) of Einkorn was found lower (27.94 g) than durum wheat (54.6 g). Also, water absorption was 51.8% of Einkorn and 65.3% of durum wheat. The ash, protein, fat content and antioxidant activity of Einkorn sample were determined higher than durum wheat. Mineral content of the samples was analyzed, and Einkorn contained higher amount of trace and major elements especially in terms of Zn, Fe and Al.

The results show that many foods will able to fortified by einkorn or einkorn products such as semolina, flour, germ or bulgur as a solution of micronutrient malnutrition problem, and to meet todays consumers demand which are clean-label and additive-free food. Moreover, einkorn is a promising candidate for the development of new or special foods such as bakery products, baby food or products with high content of dietary fiber, protein, minerals, carotenoids and tocols.

Keywords: Siyez; Einkorn; *Triticum monococcum*; Durum wheat; Image analysis

1. Introduction

The archeological findings show that the wheat first occurred in parts of Turkey, Egypt, Ethiopia, Lebanon, Syria and Israel (Lev-Yadun et al 2000). Domesticated Einkorn wheat (*Triticum monococcum* L. subsp. *monococcum*) in Turkey dates back to 9000 B.C (Piperno et al 2004). Einkorn which is a diploid hulled wheat, is the first wheat species cultivated in Karacadağ, Şanlıurfa (Heun et al 1997; Hidalgo & Brandolini 2014) and strictly related to durum and bread wheats. It was most probably spread to Europe during the Agricultural Revolution. Today, traditional einkorn crops are found in mountain areas of the Mediterranean region (Turkey, southern Italy and France, Balkan countries, Morocco and Spain).The changing climate and socio-economic changes have seriously endangered the survival of many traditional foods and native varieties. Most underutilized species and ancestral cereals like einkorn are in danger of disappearing due to agronomic, genetic, economic, and cultural factors. The ancient food grains and cereals have been sustained in specific geographic areas by only small-scale producers.Many farms in the forested northern Turkish province of Kastamonu, continue to cultivate the oldest type of wheat still in existence, *Triticum monococcum*, known as Siyez in Turkey. In recent years the trend towards sustainable agriculture and increased attention to the nutritional attributes of food, has led to the rediscovery of several forgotten cereals and legumes, including einkorn.

Einkorn is highly competitive thanks to having one spikelet and tight hull nature and it can be grown on poor soil with limited fertility, in arid conditions and cold climates with lower input and technology. The studies show that Siyez wheat has high fat, protein and carotenoid content and it contains more lutein compared to bread wheat. As it is traditionally consumed in the form of whole wheat, it has low glycemic index. It is also known for being rich compared to the other wheat varieties in terms of protein, phenolics, tocopheroles and carotenoids. Ancestor wheats are also efficient for treating diseases like cholesterol, colitis and allergy and their starch is resistant to high enzymes (Strehlow et al 1991; Abdel-Aal et al 2002; Hidalgo & Brandolini 2011). Seed size of Einkorn has an important role for evaluation of the quality and technologic nature of wheat. Because, wheats have bigger and heavier kernels, high endosperm, lower quantities of outer pericarp and aleurone layer. Siyez kernels contain 3.0-3.2% germ while bread wheat has 2.9-3.0%; siyez contains 22.8-23.0% bran while bread wheat has 15.0-17.0%, Siyez's endosperm ratio is 73.0-75.0% while it is 80.0-82.0% for bread wheat. High bran fraction of Siyez wheat stems from its quite small kernel structure, so when Siyez wheat is processed for flour, it will be not appropriate to compare it with bread or durum wheat. Its technologic yield is lower (Hidalgo & Brandolini 2014).

The aim of this study was to draw attention to the issue of dissemination of the ancestral food grains such as einkorn, to increase of demand for its' sustainability. In this research, some physiochemical and morphological properties of einkorn and durum wheat have been compared. In this context, alternative uses of einkorn have been examined and new products have been proposed except usage as bulgur. For this aim, various physicochemical properties such as protein, gluten, fat, moisture, water absorption, antioxidant activity and microtextural properties of einkorn samples cultivated from Kastamonu and durum wheat from Konya were examined.

2. Material and Methods

The samples which were named "Tek siyez", were obtained from seeds which were sown in October 2016 and harvested in July 2017 at Devrekani and İhsangazi districts where are the most einkorn cultivation areas of Turkey. Two Einkorn (*Triticum monococcum*) samples from İhsangazi, and two samples from Devrekani district were supplied by the main producers in this study. Every sample was obtained randomly from four locations by the farmers. All samples (from 2 district x 2 sample x 4 location) were mixed homogeneously. The durum wheat (*Triticum durum*) sample (Selçuklu-97)

was supplied from Bahri Dagdas International Agricultural Researh Institute, Konya, Turkey. The chemicals used for analysis were purchased from Merck (Germany) and DPPH from Sigma (Germany).

Physicochemical properties: The composition of the wheat samples were determined according to AACC (2000) approved methods (Moisture No: 44-15A, Ash No: 08-03, Protein No: 46-10, Wet gluten No: 38-12, Fat No: 30-25) and AOAC (1995) method (Total dietary fiber No: 985.29). The color profile of the samples were determined using a colorimeter (Konica-Minolta, CR400, Japan) as *L*, a** and *b**. The measurement was performed on three different points. Farinograph properties (water absorption, degree of softening) were carried out according to AACC (2000) No:54-21 method.

Antioxidant activity: The extraction was carried out according to Banu et al (2010). Antioxidant activity was determined by the method of Karamac et al (2002) as inhibition % using the α,α diphenyl-β-picrylhydrazyl (DPPH) radical scavenging assay.

Mineral content: The mineral content was measured by using the microwave (Milestone MLS 1200, Italy) nitric acid digestion procedure and it was followed by induction-coupled plasma optical emission spectrometry (Spectro Blue ICP-OES) according to slightly modified method of Kilci & Gocmen (2014).

Scanning Electron Microscopy (SEM): The samples were adhered on sample holder with double sided tape, the samples coated with about 135 Angstrom Au/Pd (device coating speed 3 A° s^{-1}) with SC 7620 mini Sputter Coater then they were examined by SEM (Aponte et al 2014).

Image Analysis (IA): Image Pro Plus 6.0 (Media Cybernetics Inc., USA) software was used for the evaluation of the SEM views. The diameter of starch granules were measured through the software with appropriate calibration.

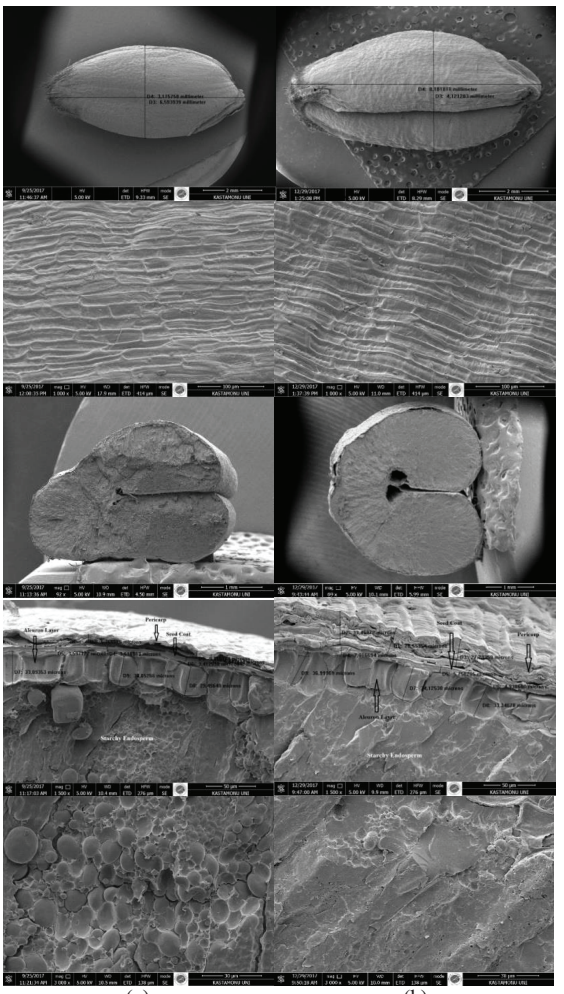
Statistical Analysis: One sample *t*-test (SPSS 17.0.1) were used for the comparison ($P<0.05$) of the results. All experiments on the wheat samples were carried out in triplicate on duplicate samples.

3. Results

Morphological properties: The scanning electron microscopy (SEM) micrographs shown that Einkorn had opaque but durum wheat had a vitreous appearance. In addition, SEM images were evaluated by Image ProPlus software, the diameters of starch granules were measured after appropriate calibration. Both wheat samples contained starch granules of spherical and lenticular shapes that were distributed throughout the protein matrix. The starch granules which were small as well as large could be easily separated visually in Einkorn texture. But, the protein bonds were more compact in the image of durum wheat and the diameter of starch granules located in the protein matrix lower than ones of Einkorn (Figure 1).

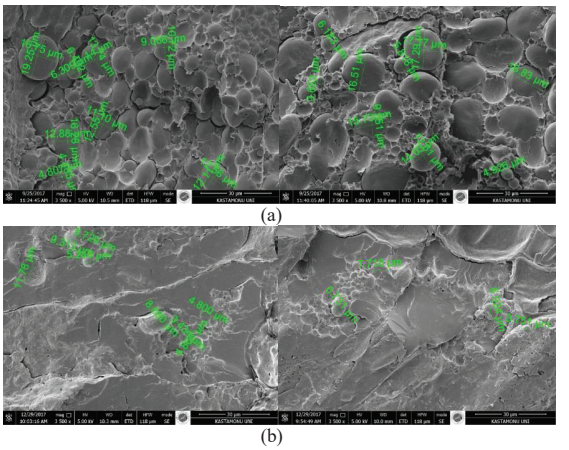
Wheat starch granules generally exhibit a bimodal distribution in the structure and can be classified as A-type: 20-35 µm and B-type: 2-10 µm (Delcour & Hoseney 2010). In Figure 2, the B type of granules was captured in SEM images of both wheat types. But the A-type starch granules were seen as separate phase and captured clearly in einkorn images, they were not captured in durum wheat images due to vitreous texture. Moreover, the diameter of A-type granules was lower than the classification of literature (Delcour & Hoseney 2010). But the diameters of granules of einkorn for both types granules were higher than ones of the durum wheat.

The SEM micrograph shows that Einkorn kernels had a higher proportion of endosperm and smaller amounts of the external pericarp and aleurone layers (Figure 1). The seed size of einkorn had a marked difference from durum wheat. The sizes were smaller than durum wheat (Figure 1 and Table 1). Although the bran fraction of einkorn is thinner than durum wheat (Figure 1 and Table 2), the percentage of this layer is higher than other haploid wheat types. This phenomonia is related to it's smaller and lightweight seed structure (Borghi



(a) (b)

Figure 1- Scanning Electron Microscopy (SEM) micrographs of wheat samples (a, einkorn; b, durum wheat)



(a)

(b)

Figure 2- Scanning Electron Microscopy (SEM) micrographs (3500X); Diameter of starch granules of wheat samples (a, einkorn; b, durum wheat)

et al 1996; Løje et al 2003), whose average weight was in the range 27.94±2.10 g. It was 54.6±8.45 g for durum wheat per 1000 kernels.

Physicochemical properties: Actually the wheat kernels are a good source of dietary fibers which some of them are been resistant to digestion and absorption in the small intestine, but some of them are been totally or partially fermented in the large intestine. In this study, it was found that einkorn had lower dietary fiber than the durum wheat. In conducted various studies, it was indicated that the dietary fiber content of *Triticum monococcum* varied to 93-128 g kg^{-1} (Loje et al 2003; Grausgruber et al 2004; Gebruers et al 2008). But it was higher in free-threshing wheats, as reported by Gebruers et al(2008) (durum wheat, 134 g kg^{-1} DM; bread

Table 1- Diameter of starch granules and thickness of layer of wheat samples

Property	*Granule/Layer*	*Einkorn (µm)/1000*	*Durum wheat (µm)/1000*	*P value**
Diameter of granules	Class A	16.77±2.31	9.18±2.01	0.005
	Class B	5.07±1.41	4.20±0.71	0.091
Thickness of layer	Pericarp	5.77±0.34	29.74±3.29	0.000
	Seed coat	3.54±0.12	5.91±1.05	0.010
	Aleuron	30.88±1.94	34.83±1.93	0.072

*, P<0.05 means statistically different

Table 2- Physicochemical properties of wheat samples

Property		*Einkorn*	*Durum wheat*	*P value**
Kernel weight(g/1000 pieces)		27.94±2.10	54.60±8.45	0.000
Softening degree (BU)		186.00±1.50	28.00±0.90	0.000
Water absorbtion (%)		51.80±0.20	65.30±0.00	0.000
Protein (%)		12.74±0.80	11.85±1.05	0.194
Wet gluten (%)		6.75±0.20	9.82±0.50	0.000
Gluten index (%)		42.00±1.10	85.00±0.30	0.000
Ash (%)		2.34±0.13	1.96±0.079	0.037
Fat (%)		2.64±0.21	1.85±0.090	0.023
Moisture (%)		10.31±0.05	11.18±0.07	0.001
Total dietary fiber (%)		10.76±1.36	13.10±0.02	0.228
Total carotenoids (µg/g dw)		2.79±0.21	3.58±0.10	0.023
Antioxidant activity (Inhibition%)		18.60±0.20	9.23±0.55	0.000
Color	*L**	50.16±1.13	60.19±0.93	0.000
	*a**	8.14±0.12	3.32±0.09	0.000
	*b**	18.85±0.16	22.44±0.79	0.001

*, P<0.05 means statistically different

wheat, 147-152 g kg^{-1} DM) and Andersson et al (2013) (bread wheat, 134 g kg^{-1} DM). Einkorn was poor in dietary fiber compared to durum wheat, but rich in proteins, lipids and mostly all elements (including Zn and Fe).

It was indicated that by Hidalgo & Brandolini (2014), the good concentration of several antioxidant compounds (carotenoids, conjugated polyphenols, tocols, and phytosterols) and low lipoxygenase and β-amylase activities (which limit antioxidant degradation during food processing) contribute to the excellent nutritional properties of einkorn.

In the study, it was found that einkorn sample was softer than durum wheat and its water absorption quite lower than durum wheat. These parameters are directly related to baking and milling technology, and they should be taken into account when the einkorn processes to cereal products such as flour, semolina, bulgur or various doughs.

Mineral content: Statistically significant differences were detected among einkorn and durum wheat in terms of both major and trace elements (Table 3). Especially the contents of Fe, Zn and Al were great significantly higher (P<0.05) in einkorn. Einkorn is considered a good source of micronutrients (Ozkan et al 2007). The genotype, climate and soil all play significant roles in mineral

Table 3- Mineral contents of wheat samples

Minerals	*Einkorn*	*Durum wheat*	*P value**
Na, mg kg^{-1}	59.70±0.01	26.30±0.01	0.000
K, mg kg^{-1}	4963.60±0.05	4769.52±0.09	0.000
Ca, mg kg^{-1}	549.45±0.03	479.21±0.05	0.000
Fe, mg kg^{-1}	73.10±0.00	27.82±0.16	0.000
Zn, mg kg^{-1}	67.90±0.00	14.74±0.12	0.000
P, mg kg^{-1}	3924.30±0.17	3348.90±0.22	0.000
Mg, mg kg^{-1}	1294.70±0.06	1118.60±0.09	0.000
Al, µg kg^{-1}	5343.50±0.44	<100	0.000
Co, µg kg^{-1}	< 31.70	< 31.70	-
Cu, µg kg^{-1}	6120.00±0.30	5890.32±0.12	0.000
Cd, µg kg^{-1}	< 6.30	< 6.30	-
Ba, µg kg^{-1}	1746.20±0.08	698.50±0.02	0.000

*, P<0.05 means statistically different

content levels in cereals. In addition, most mineral elements are more abundant in the bran fraction. Therefore the higher trace and major mineral contents of einkorn may be partially attributed to the smaller size of its seeds, which increases the (bran+germ)/endosperm ratio and leads to a non-genotypic or non-climatic concentration effect.

Cereals and especially their refined flours are poor in concentration and bioavailability of some of the trace and major minerals. So they may fail to satisfy human daily requirements, and various regions where have cereal based daily diet in the world suffer from micronutrient deficiencies.

4. Conclusions

In the study, it has been found that einkorn has a high-nutritional value, especially considered for its high protein and antioxidant content. The grain differs from conventional varieties due to its low level of gluten, high protein content. Also, it has high fat and mineral content level. Despite its extraordinary characteristics, the cultivation of Eincorn, has been declining constantly. Current trends toward slow-impact and sustainable agriculture as well as an increase in the utilization of ''biological'' and ''functional'' products suggest that this cereal may still play a role in human consumption. The results and several studies concluded that einkorn is a promising candidate for the development of new or special foods such as bakery products, baby food or products with high content of dietary fiber, carotenoids and tocols. Durum wheat is rich in lutein due to selection for yellow color. Einkorn wheat has also high contents of the carotenoid. Furthermore, because of its high protein content, Siyez wheat gives larger particulate flour like fine semolina. For this reason, Einkorn can be a good supplement raw-material to durum wheat and it should be tried for several types of pasta. Already traditional Siyez erişte (traditional einkorn pasta), Siyez ezmesi (flake) and Siyez bread have just been produced in Kastamonu for many years. The functional properties of these products should be examined and used for the developing of the new products.

On the other hand, the content of major and trace elements such as Fe and Zn were higher statistically significant in einkorn than durum wheat. The micronutrient malnutrition effects over two billion people in the developing countries. Iron (Fe) deficiency alone affects most of the women globally and >47% of all preschool-aged children, often leading to impaired physical growth, learning capacity and mental development. Zinc (Zn) and iron (Fe) deficiency are thought to affect billions of people, hampering growth and development, and destroying immune systems. In many micronutrient-deficient shown countries, wheat and cereals are the dominant staple food of the diet. For that reason, many foods can be fortified through einkorn or einkorn products such as semolina, flour, germ or bulgur for the regions affected by malnutrition. The natural fortification is the power and a new approach to improve the nutritional quality of foods consumed in the daily diet for today's consumers who demand clean-label and additive-free food.

To ensure the sustainability of Siyez wheat, the main steps should to make the direct sale of the product possible domestic and abroad, increase harvesting area and production rate, to investigate functional and nutritional qualities and to find alternative usage areas. Moreover, agricultural production incentives should be provided by the government and domestic or abroad advertisement should be made about Siyez wheat and its products.

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Effect of Infrared, Ultraviolet-C Radiations and Vacuum Drying on Certain Chemical and Microbial Characteristics of Stuffed Pasta (Manti)

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ABSTRACT

Stuffed pasta (manti) is one of the famous and nutritious traditional foods produced and consumed in Turkey. Traditional foods are special products having some cultural aspects of specific regions of countries. In addition to home-made traditional production, industrial production of manti has increased in recent years. Industrial production of manti using various techniques is likely to result in some risks or modifications in terms of its sensory and microbiological features. Therefore, the purpose of this study was to examine quality properties of manti samples produced by sole or combined applications of infrared (IR) and ultraviolet-C (UV-C) radiations and vacuum drying as compared to the traditional oven drying method. With this aim, some food-borne pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Listeria monocytogenes*) were inoculated into the manti recipe prior to the production. Afterwards, the samples were dried with 17 different IR, UV-C and vacuum drying combinations, including the traditional oven drying method. In addition to the, total microorganisms and food-borne pathogens, some chemical analyses like Thiobarbituric acid (TBA) measurement as an indicator of lipid oxidation was also studied. In the results, application of 500 W IR drying method produced satisfactory results in terms of microbiological quality. Again, a bar-type IR drying method at constant temperature (150 ºC) seems to be a promising drying method for the industrial manti production. In conclusion, it might be suggested that IR drying method can be an alternative to the conventional method in industrial manti production.

Keywords: Manti; IR; UV-C; Vacuum; Drying; Pathogen bacteria

1. Introduction

Traditional foods are special products carrying cultural trends of certain localities and emerged from human experiences during hundreds of years without using modern technology (Gallegos-Infante et al 2010). Manti is one of the traditional foods with high nutritional value and widespread consumption in Turkey. It is a ground meat stuffed pasta product and marketed in fresh or mostly in dried forms. The manti process includes the following basic steps:

(1) meatball preparation with ground meat and spices for fillings, (2) preparation of a stiff dough, (3) sheeting and cutting the dough, (4) placing the fillings on the sheeted dough and enclosing and then (5) drying or cooking (Heldman & Lund 2007; Gokmen et al 2015; Sitti 2011).

Ground beef is the primary ingredient for microbial spoilage of manti. Therefore, in industrial production, heat treatment is included in the drying process. Drying is among the food preservation methods to decrease chemical, enzymatic and microbiological spoilage of the foods (Varlık et al 2004). Non-thermal food processing techniques are also used in contemporary industrial food preservation practices. For example, irradiation techniques are among these methods as an alternative to heat treatment. Again, utilization of new techniques, such as IR and UV-C, is essential for processing of foods at lower temperatures for higher quality product. Irradiation techniques are usually classified by wavelength. The main irradiation processes are performed by using infrared (IR) or ultraviolet (UV) spectra. The IR radiation is classified according to its wavelength as near IR (0.75-3 μm), mid IR (3-25 μm) and far IR (25-1000 μm) (Singh & Goswami 2003). The advantages of IR application include short processing time, energy efficiency, uniform product temperature, high-quality product, high degree of process control, high value of heat transfer coefficient and environmental friendly nature (Farkas 1990; Erdogdu & Ekiz 2011). The IR is used as a drying technique and tends to spread rapidly today. The UV radiation is also defined as a radiant energy form released from the sun. It is also divided into three groups; UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (200-280 nm). Having the highest germicidal activity, the UV-C is the mainly used form of UV radiation in the food industry (Gokmen et al 2014).

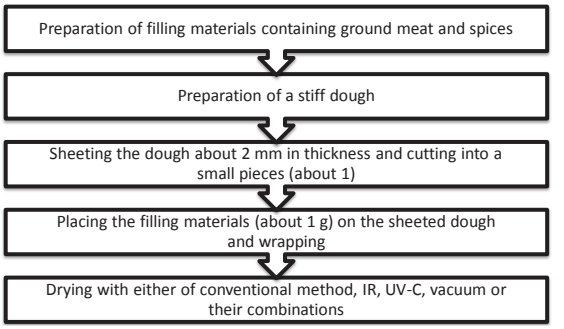
Consumer demands from food production have changed considerably in recent years, usually they prefer uniform, healthy and high-quality food products. During the industrialization of traditional foods like manti, quality criteria could not be sustained through the whole process (Gokmen et al 2015). Indeed, it is difficult to achieve standard sensory quality when it comes to traditional foods (Cayot 2007). Therefore, the purpose of this study was to investigate the effects of sole or combined applications of IR and UV-C radiations and vacuum drying in industrial manti production. Hence, these non-thermal processes may ensure sustainable quality and may provide invaluable information as compared to the traditional manti production methods.

2. Material and Methods

2.1. Material

2.1.1. Preparation of manti samples

Manti samples were prepared in the laboratory following the traditional processing steps (Gökmen et al 2015) as follows:



2.2. Methods

2.2.1. IR and UV-C drying system and treatment of manti samples

For this research, the IR oven dryer system was designed with four main subunits: IR lamps (250 W and/or 500 W, short and medium wavelength), UV-C lamps and vacuum unit (20 kpa) (Figure 1). An analytical balance, a thermocouple and a thermo-hygrometer were also mounted inside the oven system to measure the weight-loss, temperature and relative humidity respectively. Again, the IR and UV-C radiation intensities were measured using a pyrometer located inside the oven. Prior to drying applications, UV lamps and IR lambs were turned on

for about 15 and 5 minutes respectively to equilibrate the radiation. Stainless steel sample trays were used to hold the manti samples. The distance between the sample tray and the lamps was 25 cm. The manti samples were dried in this system until their moisture contents were reduced to about 12%. A total of 17 different combinations of drying methods UV-C, IR [250, 500 W and different wavelengths (short-wavelength: 0.76-2 μm, medium- wavelength: 2-4 μm)] and vacuum applications were used to treat the samples (Table 1). Traditional method was applied as dry hot air at 250 ºC, 30-35 min.

2.2.2. Ray intensity and energy consumption

Ray intensity and energy consumptions were determined with the aim of finding whether IR and UV-C applications had advantages over the traditional oven drying method. For this purpose,

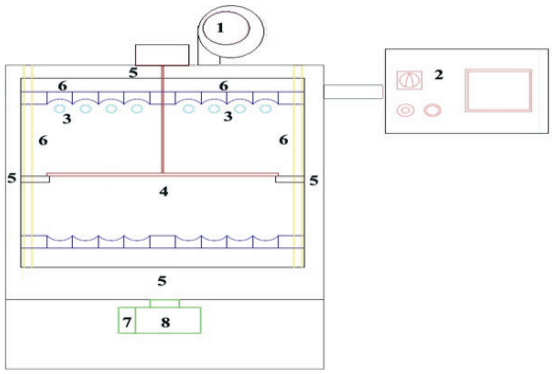


Figure 1- The designed IR dryer and its sections or components (1, the machine body; 2, heating plate; 3, lamps (IR and UV-C); 4, scales and tray; 5, suction fan; 6, calpe; 7, vacuum indicator; 8, vacuum pump)

Table 1- Mean ray density and energy consumption for the corresponded applications

Drying method	*Drying type*	*Mean ray density (W m^{-2})*	*Mean energy consumption (Turkish penny) (TL 100 g^{-1} manti)*	*Average investment costs of the dryers (TRY)*
FIR lamp*	250 W M Type IR**	1500	0.33	12.000****
	250 W M Type IR+UV-C	1100	0.38	
	250 W M Type IR+vacuum***	1200	0.38	
	250 W M Type IR+UV-C+vacuum	1150	0.44	
	500 W B Type IR**	2280	0.46	
	500 W B Type IR+UV-C	2500	0.53	
	500 W B Type IR+vacuum	2350	0.53	
	500 W B Type IR+UV-C+vacuum	2280	0.49	
MDIR lamp	250 W B Type IR	1500	0.33	
	250 W B Type IR+UV-C	1250	0.39	
	250 W B Type IR+vacuum	1500	0.39	
	250 W B Type IR+UV-C+vacuum	1200	0.43	
	500 W M Type IR	2700	0.47	
	500 W M Type IR+UV-C	2480	0.55	
	500 W M Type p IR+vacuum	2620	0.54	
	500 W M Type IR+UV-C+vacuum	2600	0.51	
Traditional method	250 °C for 30-35 min.	-	0.51	17.000

*, FIR Far-Infrared lamp; MDIR, Middle-Infrared lamp; **, M Mushroom Type IR lamp, B Bar Type IR lamp; UV-C, Ultraviolet-C, 254 nm λ; ***, vacuum: 20 KPa; W, watt, Formula 1 and 2 were used for calculation of energy consumption. Market conditions were researched for determine of dryer cost; Kr, kuruş; ****, the total cost of the laboratory-type IR dryer, including all lamps and vacuum system; −, the ray intensity was not measured for traditional method

the beam intensity was measured in W m^{-2} with a portable pyrometer (Apogee Instruments, Model Mp-200, USA). In addition, the current passing through the dryer was measured with an ammeter (Set Ac-Dc, Amp 06) and the energy consumption was calculated using the following equations;

W (Watt)= u (Volt) x *I (Amper)* x *t (time-hour)* (1)

Energy consumption= Power (kW) x *Time (hour)* x *Price (Turkish penny)* (2)

2.3. Chemical and microbiological analyses

2.3.1. Thiobarbituric acid (TBA) analysis

Manti sample (10 g) was homogenized with water. The mixture was transferred to a Kjeldahl flask and distilled by adding 2.5 mL 4 N HCl (Merck, Germany) and 1 mL antifoam chemical. Five mL of TBA (Merck, Germany) and 5 mL of distillate were incubated in water bath at 80-90 °C for 30 min in a Bain-Marie equipment. The measurement was made on a spectrophotometer at 538 nm and obtained absorbance value was multiplied by 7.8. The final value was presented as mg malondialdehyde (MDA) per kg of sample (Uzunlu 2011).

2.3.2. Microbiological analysis

2.3.2.1. Inoculation of food-borne pathogens

Food-borne pathogens [*Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Salmonella typhimurium* (*S. typhimurium*) and *Listeria monocytogenes* (*L. monocytogenes*)] were used to determine their survival in newly designed manti drying system. *L. monocytogenes* ATCC 7644, *S. enterica* subsp. *enterica serovar. typhimurium* ATCC 14028 and *E. coli O157:H7* ATCC 33150 and *S. aureus* ATCC 25923 were provided by the Agricultural Control and Protection Center, Kayseri, Turkey. These strains were maintained at -80 °C, and the all microbial processes were conducted under the aseptic conditions. Before the inoculation, they were incubated in nutrient broth at 35 °C for overnight. After the incubation, culture turbidity was adjusted to 0.5 McFarland standard. 10^2, 10^4 and 10^6 dilutions of adjusted cultures were used for inoculation of 25 g manti samples. Inoculated samples were treated with 17 different drying methods indicated in Table 1. After treatment, the samples were packaged in vacuum bags and stored at room temperature for 0 and 60 days before analysis. Before analysis, the manti samples were homogenized in isotonic solution with a ratio of 1:9. The homogenates were analyzed using suitable chromogenic solid medium for each organism. The chromogenic culture medias: *E.coli* 35 ºC Compact Dry EC chromogenic agar, blue colonies; *S. aureus*: 35 ºC Compact Dry X-SA chromogenic agar, light blue/ blue; *L. monocytogenes*: 37 ºC Compact Dry LS chromogenic agar, light blue/blue; *Salmonella typhimurium*: 41 ºC Compact Dry SL chromogenic agar, reddish purple or red colonies (AOAC 2012a; 2012b; 2012c; Pal et al 2007).

2.3.2.2. Total bacterial counts

The samples were prepared by homogenization of the manti samples with isotonic solutions at a ratio 1:10. For the inoculation of solid media, the sample solutions were diluted (10^0, 10^{-1}, 10^{-2} and 10^{-3}) and 0.1 mL of dilutions were spread on the plates in triplicates. Plate Count Agar (PCA) was used for total aerobic mesophilic bacteria. The plates were incubated at 35 °C for 24-48 h, and microbial counts were expressed in Colony Forming Unit (CFU) mL^{-1}.

2.4. Statistical analysis

SPSS version of 13.0 program was used for all statistical analyses with 95% confidence interval. Data were analyzed by using student's t test and compared by multiple one-way analyses of variance (MANOVA) (Norusis 1993).

3. Results and Discussion

In this research, the effects of infrared (IR), ultraviolet (UV-C) and vacuum-drying applications on the manti quality were investigated either in alone or with their combinations, as alternative to conventional oven-drying method. For this purpose, an IR dryer was

designed, and the samples were kept in drying system until their moisture contents were reduced to about 12% as in the case of industrial application. The effects of 17 different drying methods were observed considering the chemical and microbiological features of the manti samples, and the obtained results were summarized in following Tables.

3.1. Ray density and energy consumption

Ray densities of IR and UV-C lamps were measured by pyrometer after it had been reached to constant ray intensity. As seen in Table 1, while 500 W IR applications had the highest ray density which is about 2700 W m^{-2}, 250 W IR applications had the lowest one with 1100 W m^{-2} value. However, UV radiation was determined to have negative effects on the ray intensity. In a study about decontamination of cumin seeds by IR and UV-C methods, it was reported that while the ray intensity of IR was remaining stable in sole IR application, the use of UV radiation with IR reduced the intensity of IR (Erdogdu & Ekiz 2011). Similar results were also obtained in this study (Table 1). IR drying system showed better results than the traditional methods in terms of energy consumption and investment cost (Table 1). Moreover, among the drying methods in terms of energy consumption, there was no significant difference.

3.2. Thiobarbituric acid (TBA) analysis

Manti is a sensitive food against oxidation and microbial deterioration, because it contains ground beef and semi-dry dough. With this aim, TBA analyses were conducted in the manti samples, and the results given in Table 2. As seen in Table 2, high TBA concentration is considered a sign of food deterioration resulted from lipid oxidation, because the TBA concentration increases with the increasing lipid per oxidation in foods (Janero 1990). Especially for meat and meat products, TBA concentration should not be exceeded 5 mg L^{-1}, and it is also assured in Turkish Food Codex (Uzunlu 2011). The results showed that TBA values were increased after the radiation treatments. Additionally, the more the treatment combination increased, the more the TBA values of the manti samples increased. On the other hand, the highest value after two months of the storage was less than 3 mg L^{-1} (Table 2). In another study, it was reported that as the storage time of the manti samples increased, its TBA values was also increased (Uzunlu 2011). However, it has also been reported that the increase in TBA values in manti could not be directly correlated with the deterioration tested with sensory quality.

3.3. Microbiological analysis

In this study, for the determination of microbial quality, the effects and reliability of IR, UV-C and vacuum applications against some common food pathogens *E.coli*, *S. aureus*, *L. monoytogenes* ve *S. typhimurium* were investigated. Results of the treatments on four bacteria showed that even the sole application of 250 W IR was seemed to be efficient for the microbiological safety. Moreover, its combination with another treatment such as UV-C or vacuum made the application more assuring. Besides that, sole application of 500 W IR was enough for ensuring the food safety with no doubt, even if after 60 days of storage at room temperature. In another research, total mesophilic-aerobic bacteria count was determined in manti samples (Anonymous 2014). The results of the traditional or classical drying procedure applied in this study were the same that no microbial growth was observed. Therefore, the treatment applied in this study can be an alternative method in respect to classical method. The advantages of using IR were broadly mentioned in the literature (Sakai & Hanzawa 1994; İçier & Yıldız 2003; Sharma & Verma 2005). In these researches, mainly leafy vegetables were used as a sample and compared to IR usage with commercial heat drying methods (Sakai & Hanzawa 1994; İçier & Yıldız 2003). Their results showed that IR usage was more effective than that of the commercial method with respect to food quality. Moreover, they indicated that using IR drying system was cheaper than that of the commercial ones. In another study, Aslan (2012) indicated that IR application reduced the time needed for drying of the manti samples about 68% compared to traditional method, 250 °C heat treatment. Again, in this research, sole application of 500 W IR was enough to meet the microbiological criteria (Table 3).

Table 2- Average thiobarbituric acid (TBA) concentrations in stuffed pasta (manti) samples, dried with different methods during the storage periods

Drying types	*Thiobarbituric acid (mg L^{-1})*		
*FIR lamp**	*Storage times*		
	0. day	30. day	60. day
	0.19±0.03^{Aa}	0.29±0.01^{Ab}	0.47±0.07^{Cc}
	0.24±0.02^{Aa}	0.34±0.03^{Ab}	0.86±0.05^{Ac}
	0.15±0.01^{Aa}	0.27±0.01^{Ab}	0.65±0.04^{Ac}
	0.27±0.03^{Aa}	0.38±0.02^{Ab}	0.87±0.07^{Ac}
	0.15±0.02^{Aa}	1.05±0.03^{Bb}	1.60±0.09^{Ac}
	0.14±0.03^{Aa}	1.26±0.24^{Bb}	1.85±0.08^{Bc}
	0.16±0.01^{Aa}	0.78±0.03^{Ab}	1.19±0.06^{Ac}
	0.29±0.05^{Ba}	1.80±0.14^{Bb}	2.70±0.07^{Bc}
*MDIR lamp***			
	0.19±0.01^{Aa}	0.49±0.03^{Ab}	1.01±0.06^{Ac}
	0.14±0.07^{Aa}	0.73±0.05^{Ab}	1.28±0.16^{Ac}
	0.11±0.01^{Aa}	0.46±0.06^{Ab}	0.93±0.07^{Ac}
	0.15±0.02^{Aa}	0.59±0.03^{Ab}	0.86±0.03^{Ac}
	0.18±0.02^{Aa}	0.39±0.01^{Ab}	1.10±0.22^{Ac}
	0.18±0.02^{Aa}	0.46±0.01^{Ab}	1.41±0.10^{Ac}
	0.14±0.01^{Aa}	0.66±0.05^{Ab}	1.52±0.05^{Ac}
	0.12±0.01^{Aa}	0.66±0.05^{Ab}	1.49±0.07^{Ac}
Tradiational method (250 °C for 30-35 min.)	0.14±0.05^{Aa}	0.50±0.14^{Ab}	0.60±0.07^{Ac}

*, FIR Far-Infrared lamp; MDIR, Middle-Infrared lamp; **, M Mushroom Type IR lamp, B Bar Type IR lamp; UV-C, Ultraviyolet-C, 254 nm λ; W, watt; ^{ABC}, capital letters in the same column is the comparison of drying methods. There is no statistical difference between the drying methods represented by the same letters (P>0.05); ^{abc}, Lowercase letters in the same line is the comparison of storage times. There is no statistical difference between the storage times represented by the same letters (P>0.05).

Table 3- Microbiological results of the stuffed pasta (manti) samples dried with different methods during the storage periods

Application name	*Drying type*	*Pathogenic bacteria*	*Number of bacteria (cfu 25 g^{-1})**	
			Storage times	
FIR lamp	250 W M Type IR**	*S. aureus*	10	-
		L. monocytogenes	29	
	250 W M Type IR+UV-C	*S. aureus*	1	-
	250 W M Type IR+ vacuum***	*S. aureus*	8	-
	250 W M Type IR+UV-C+ vacuum	*S. aureus*	2	-
	500 W B Type IR**	-	-	-
	500 W B Type IR+UV-C	-	-	-
	500 W B Type IR+ vacuum	-	-	-
	500 W B Type IR+UV-C+ vacuum	-	-	-
	250 W B Type IR	*S. aureus*	2	-
MDIR lamp	250 W B Type IR+UV-C	*L. monocytogenes*	-	-
	250 W B Type IR+ vacuum	*L. monocytogenes*	3	-
	250 W B Type IR+UV-C+ vacuum	-	-	-
	500 W M Type IR	-	-	-
	500 W M Type IR+UV-C	-	-	-
	500 W M Type IR+ vacuum	-	-	-
	500 W M Type IR+UV-C+ vacuum	-	-	-
Traditional method	250 °C for 30-35 min.	*E. coli*	42	6

*, microbiological results were calculated for a sample of 25 g for manti. All-pathogenic bacteria were found in samples with a microbial load of 106 cfu 25 g^{-1} Un-named microorganisms have not been determined in the media; FIR, Far-Infrared lamp; MDIR, Middle-Infrared lamp; W, watt; **, M Mushroom Type IR lamp, B Bar Type IR lamp; UV-C, Ultraviyolet-C, 254 nm λ; ***, vacuum: 20 KPa

4. Conclusions

The effects of infrared (IR), ultraviolet-C (UV-C) and vacuum-drying applications on some quality features of manti samples were researched either in alone or combinations, as alternative to conventional method. Moreover, except for 500 W Bar Type IR; lamp type of IR has no effect on the microbiological properties and TBA values of manti. Also, as the power of the lamps increases, the TBA values and microbial qualities of manti samples have increased.

The results showed that traditional drying method needs higher initial investment cost and energy consumption compared to IR drying methods. Moreover, a combined drying system consisting of IR, UV-C and vacuum could be more beneficial in terms of qualitative properties of the manti. In conclusion, it might be suggested that although sole application of 500 W IR was enough to meet microbiological quality for manti, in general, the results showed that IR combined with UV-C and vacuum applications can be a better alternative to traditional methods in commercial manti drying practices.

Acknowledgements

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Abbreviations and Symbols	
IR	Infrared
UV-C	Ultraviyolet-C
Manti	Stuffed Pasta

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Activity Guided Isolation of Nematicidal Constituents from the Roots of *Berberis brevissima* Jafri and *Berberis parkeriana* Schneid

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ABSTRACT

Biological screening of different parts of the selected *Berberis* species (*B. brevissima* Jafri and *B. parkeriana* Schneid) showed that methanolic root extract possessed significant efficacy against *Meloidogyne javanica* (a root knot nematode). From root methanolic extracts of selected *Berberis* species four isoquinoline alkaloids; jatrorrhizine, dehydrocheilnthifoline, berberine and berberrubine were isolated. Structures of the isolated compounds were determined by using EIMS, ^{1}H and ^{13}C NMR, and other 2D spectroscopic techniques. Percentage juveniles mortality of *M. javanica* was determined at various concentrations (100, 200 and 300 µg mL^{-1}) using carbofuran as control. Berberine possessed the highest nematicidal activity (71.33%) followed by jatrorrhizine (59.50%). The *in vitro* results suggested that these compounds from *Berberis* species could be potential novel nematicides against *M. javanica*.
Keywords: Nematicidal activity; *Berberis brevissima*; *Berberis parkeriana*; *Meloidogyne javanica* and isoquinoline alkaloids

1. Introduction

All over the world, in the field of agriculture root knot nematodes (*Meloidognye* species) are mainly responsible for huge economic losses (Liu et al 2011). For several decades the use of various chemical nematicides is an important tool to control root knot nematodes. But due to its negative impact on environment and after use resistances, have either reduced or totally banned its use, therefore these nematicides must be replaced with safe and more effective chemical nematicides (Zuckerman & Esnard 1994). In the area of vegetables and fruits production, approximately 70 billion U.S. dollar crop damage is due to these root knot nematodes (*Meloidognye* species) annually (Reynolds et al 2011). Amongst the all probable strategies for controlling these pests, the biocontrol agents obtained from plant or microorganisms could be used to lower non-target contact of harmful pesticides and to face resistance growth (Isman 2006; Tian et al 2007). Different types of plants, constituents and

metabolites have been screened for efficacy against various plant nematodes (Hong et al 2007; Thoden et al 2009; Ntalli et al 2010; Bai et al 2011).

Berberis (Berberidaceae) posses more than 500 species and is the only genus of the family in the southern hemisphere (Bai et al 2011). The genus *Berberis* is full of isoquinoline alkaloids having high potential in the treatments of many ailments and insects control (Baird et al 1997; Wright et al 2000; Quevedo et al 2008). The hydro ethanolic extract of *Berberis* species (*B. aristata*, *B. asiatica*, *B. chitria* and *B. lyceum*) have shown very good antimicrobial efficacy against bacterial (eleven) and fungal (eight) strains (Küpeli et al 2002). In scurvy angina, sore throat and dysentery its leaves decoction has been used as antiscorbutic. Berries of the genus could be used as a tonic and be used in the form of a dye (Singh et al 2007). Various Chinese folk remedies have reported use of different species of the genus *Berberis* (*B. aquifolium*, *B. aristata* and *B. vulgaris*) for inflammations and rheumatic problems (Li et al 1989; Ju et al 1990; Teh et al 1990; Kondo et al 1992; Saied & Begum 2004). *B. aristata* have shown a very high anticancer efficacy against colon cancer cell line (HT29) (Seow et al 1992). In the current study, we have isolated four isoquinoline alkaloids through activity guided fractionation of methanolic crude extract of roots of *Berberis* species (*B. brevissima* and *B. parkeriana)* and studied their efficacy against root knot nematode *M. javanica.*

2. Material and Methods

2.1. General

Silica gel 60 (0.063-0.200 mm) was used for Column Chromatography (CC) while silica gel 60 PF254 was used for preparative Thin Layer Chromatography (TLC). The melting points of isolated compounds were determined by the melting point apparatus (Bibby Scientific Limited, Stone Staffordshire ST15 0SA, UK). UV spectra were taken by Thermo Spectronic UNICAM UV 300. IR spectra were recorded using JAESCO FT/IR-4200/A. Spectral characterizations of the compounds were performed by using Bruker AVANCE 500 and 400 MHz instruments. ^{13}C Nuclear Magnetic Resonance spectra (^{13}C NMR) were recorded at 100 MHz while ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectra at 500 MHz and 400 MHz using deutrated chloroform and methanol as solvents. EIMS (JEOL MSRoute) was determined by using direct insertion probe.

2.2. Plant material

B. brevissima roots (2.5 kg) were collected from Tirah (Khyber Agency, Khyber Pakhtunkhwa, Pakistan) and *B. parkeriana* roots (2 kg) from Dir (Lower) (Khyber Pakhtunkhwa). The species were identified by Prof. Dr. Jandar SHAH (Ex. Voice Chancellor Benazir Bhutto University, Sherengal, Khyber Pakhtunkhwa, Pakistan). The voucher specimens (No. Bot/10710 and 8719) were deposited in herbarium (Department of Botany, University of Peshawar).

2.3. Extraction and chromatography

The plants material were socked in 95% methanol for 7 days and the solvent was then evaporated at 40 ^{o}C (reduced pressure), using rotary evaporator. The residue obtained (*B. brevissima* root methanolic extract (BBR-MeOH= 225.7 g) and *B. parkeriana* root methanolic extract (BPR-MeOH= 186.5 g)) were dissolved in 6.5 L of 5% acidic water, filtered and left over night at room temperature. The yellow precipitate was filtered to obtained fraction A (*B. brevissima* root fraction A (BBR-FA= 86.3 g) and *B. parkeriana* root fraction A (BPR-FA= 77.5 g)). The filtrate was then extracted with CH_2Cl_2 (3x300 mL). The organic layer was separated and evaporated to afford fraction B (*B. brevissima* root fraction B (BBR-FB= 8.5 g) and *B. parkeriana* root fraction B (BPR-FB= 6.0 g)). The aqueous (acidic) layer was basified (pH 9-10) with concentrated aqueous NH_3 and then extracted with $CHCl_3$ (3×300 mL). Which was evaporated to afford fraction C (*B. brevissima* root fraction C (BBR-FC= 17.5 g) and *B. parkeriana* root fraction C (BPR-FC= 14.8 g)). The remaining aqueous layer was dried to obtained fraction D (*B. brevissima* root fraction D (BBR-FD= 53.5 g) and *B. parkeriana* root fraction D (BPR-FD= 45.8 g)).

2.3.1. Fraction A

On recrystalization of fraction A (BBR-FA and BPR-FA) almost pure berberine (3) (56 g) was obtained, mp 207-209 °C (lit 208-210 °C) (Suau et al 1998).

2.3.2. Fraction C

BBR-FC was further put to column chromatography (Silica gel, 180 g) and eluted with $CHCl_3$-MeOH with increasing polarity. The fractions so obtained were further subjected to preparative TLC using $CHCl_3$-MeOH-NH_3 (90:10:0.5 and 85:15:0.5) to give dehydrocheilanthifoline (2) (15.3 mg) and Jatrorrhizine (1) (21.6 mg) respectively. BPR-FC was also separated on CC (Silica gel, 150 g) eluted with $CHCl_3$-MeOH (10:90) followed by preparative TLC using $CHCl_3$-MeOH-NH_3 (10:90:0.5) to gave berberrubine (4) (7.5 mg).

2.4. Nematicidal assay

Pure culture of *M. javanica* was obtained from Department of Plant Pathology, The University of agriculture, Peshawar, Khyber Pakhtunkhwa and was maintained on tomato cultivar Rio-grande through single egg mass inoculation. For 60 days the tomato plants were grown inside the glass house. M. Javanica eggs were extracted (1% NaOCl solution) rinsed with on 25 µm aperture sieve (distilled water). Juveniles of second stage (J2s) were obtained from surface sterilized eggs placed in sterile water in a cavity block, which hatched after 3-4 days (Hussey & Barker 1973). Nematicidal assay of the various fractions and pure compounds were determined against M. javanica (J2s) using microwell assay (Naz et al 2012). All fractions and pure compound's stock solutions were prepared by dissolving them in 1% DMSO and was further diluted using distilled water. From the slandered solutions (300 µg mL^{-1}) final concentrations (100, 200 and 300 µg mL^{-1}) were prepared (Naz et al 2012). Second stage juveniles (100) were transferred to 24 microwell plate (Multiwell, TM 24, Becton Dickinson, USA) in final volume of 1 mL in various concentrations (100, 200 and 300 µg mL^{-1}) of the fractions and pure compounds, carbofuran (with DMSO; 1% v/v) was used as a positive control. At room temperature (25 °C), the experiment was performed twice and repeated four times for each concentrations. After 24 hours of incubation, total number of active or inactive J2s were recorded. Finally to find out the mobility or mortality after 24 hours, the J2s was transferred to distilled water. The J2s were considered as dead if they did not move even after mechanical prodding (Choi et al 2007). Percentages of J2s mortality was calculated for each well and the results obtained were subjected to ANOVA (Analysis of Variance) and means were separated through Fisher's projected least significance difference (LSD) test at P= 0.05 using MSTAT-C software (Gomez & Gomez 1984).

3. Results and Discussion

3.1. Activity guided isolated bioactive compounds

Four isoquinoline alkaloids were isolated through activity guided fractionation of methanolic crude extracts and their structures were characterized by various spectroscopic techniques. The values were compared with the literature data and the structures are given in Figure 1.

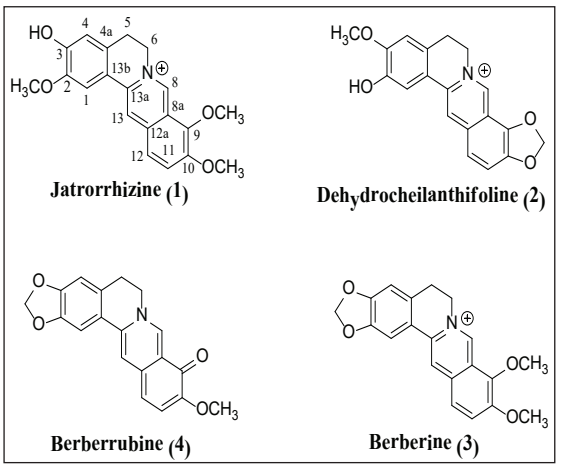

Figure 1- Structures of isolated nematicidal bioactive compounds

3.2. Compound characterization

3.2.1. Jatrorrhizine

Brown crystalline compound (MeOH); melting point: 281-282 °C (lit 280-282 °C) (Hsieh et al 2004). Molecular formula $C_{20}H_{20}N^{+}O_4$, EIMS m/z: 338.1387 (M^{+}). UV λ_{max} nm (MeOH) 226.0, 265.0, 349.0, 435.5; IR ν_{max} cm^{-1} 3340.1, 2942.8, 1600.6. The melting point, EIMS, UV, IR, ^{1}H and ^{13}C NMR data were in agreement with the literature (Hsieh et al 2004).

3.2.2. Dehydrocheilanthifoline

Brown amorphous compound (MeOH), melting point 269-270 °C. Molecular formula $C_{19}H_{16}N^{+}O_4$, deduced from the EIMS m/z 322.1074. UV λ_{max} nm (MeOH) 264.5, 359.0, 464.0; IR ν_{max} cm^{-1} 3361.8, 2924.5, 2358.5, 1601.6 (Santavy 1979).

3.2.3. Berberine

Yellow crystalline compound (MeOH), melting point 207-09 °C (lit 208-210 °C) (Suau et al 1998). Molecular formula $C_{20}H_{18}N^{+}O_4$, EIMS m/z: 336.1230 (M^{+}). UV λ_{max} nm (MeOH) 264.50, 349.0, 427.5; IR ν_{max} cm^{-1} 3047.9, 2925.5, 1596.8. The melting point, EIMS, UV, IR^{1}H and ^{13}C NMR data were in agreement with the literature (Hsieh et al 2004).

3.2.4. Berberrubine

Brown amorphous compound (MeOH), melting point 257-260 °C (lit. 255-259 °C) (Liu et al 2010). Molecular formula $C_{19}H_{15}NO_4$, EIMS m/z: 321.1001 (M^{+}), 306.3 (M^{+}-15), 292.3, 278.3. The EIMS, ^{1}H and ^{13}C NMR values were in close similarity to the reported one (Shamma & Rahimizadech 1986).

3.2.4.1. In vitro nematicidal efficacy of crude extracts and various fractions obtained from the berberis species against J2s mortality of M. javanica

The data obtained (Table 1) revealed significant ($P \leq 0.05$) effect on mortality of J2s at various concentrations i.e. 100, 200 and 300 µg mL^{-1}. The mortality of J2s was increased with the increase in concentration, the highest concentration (300 µg mL^{-1}) was most effective (54.10%). *Berberis brevissima* roots methanolic crude extract (BBR-MeOH) showed 22.33% J2s mortality and *Berberis parkeriana* methanolic roots extract (BPR-MeOH) 31.11%. Amongst the different fractions BBR-FA showed the highest mortality of 62.22% followed by BPR-FA (57.22%) and BBR-FB (54.00%). The BBR-FB and BPR-FB exhibited approximately 50% activity of the standard (carbofuran) but the differences were non-significant (Table 1).

Table 1- *In vitro* effect of different concentrations of plant fractions on juvenile mortality of root knot nematode *Meloidogyne javanica*[a]

Fractions	*Concentrations*			
	100 µg mL^{-1}	*200 µg mL^{-1}*	*300 µg mL^{-1}*	*Mean*
BBR-MeOH	18.33	23.00	25.67	22.33 g
BBR-FA	56.33	59.00	71.33	62.22 b
BBR-FB	41.67	48.67	51.66	47.33 d
BBR-FC	50.67	62.33	78.33	54.00 c
BBR-FD	39.00	43.33	45.67	42.67 e
BPR-MeOH	25.33	31.00	37.00	31.11 f
BPR-FA	35.67	40.67	44.00	40.11 e
BPR-FB	45.00	46.67	47.00	46.22 d
BPR-FC	54.00	58.00	59.00	57.22c
Carbofuran	89.67	89.67	96.67	88.22 a
Mean	44.27 c	49.07 b	54.10 a	

Data are means of five replicate per treatment using the combination of two experiments (Spring and fall, 2011); [a], means followed by the same letters do not differ significantly ($P \leq 0.05$) according to Fisher's protected LSD test. (LSD value for fractions= 3.31, LSD value for concentration= 1.82, LSD value for interaction= 6.62)

3.2.4.2. In vitro nematicidal activity of isoquinoline alkaloids from the two berberis spp. against second stage juvenile mortality of M. javanica

In vitro nematicidal efficacy of the four isolated alkaloids at various concentrations (100, 200 and 300 µg mL^{-1}) and its interaction were determined ($P \leq 0.05$). Increase in mortality of J2s was linear ($R^2 = 0.98$) dose dependent (Figure 2). Second stage juvenile mortality was 76.67% at a concentration of 300 µg mL^{-1}. Figure 3 indicated significant effect of the pure compounds at various concentrations. Amongst the tested compounds berberine (3) exhibited highest potential (97.3%) of the standard carbofuran at a concentration of 300 µg mL^{-1}. Amongst the isolated compounds jatrorrhizine (1) ranked second with efficacy of 59.50% followed by berberrubine (4) with J2s mortality of 49.17% (Figure 3). In the four isolated isoquinoline alkaloids dehydrocheilnthifoline (2) was less effective, nevertheless, showed significant mortality at the tested concentrations. The interaction of isolated compounds were studied at various concentrations and were lightly significant (Figure 4). The data showed that the percentage J2s mortality increased as the concentration of tested compounds were increased (Figure 4).

Literature survey indicated the antibacterial activity of the alkaloids of *B. thunbergii* DC and *B. vulgaris* (L) (Villinski et al 2003). The stem bark of *B. asiatica* L. showed high antimicrobial activity than the standard (Bhandari et al 2000), while the fresh and dried, aqueous as well as methanolic extracts of *B. asiatica* showed good activity against G-positive and G-negative bacteria (Shahid et al 2009). Berberine was suggested to be the main antimicrobial component of the plant. Alkaloids was suggested to have microbiocidal properties (Ghoshal et al 1996) whereas berberine has been found effective against many trypanosomes (Freiburghaus et al 1996), plasmodia (Omulokoli et al 1997) and many invertebrate pests (Rattan 2010). It was suggested that mechanism of action of berberine could be attributed to its ability to intercalate with the DNA synthesis of parasites (Phillipson et al 1987).

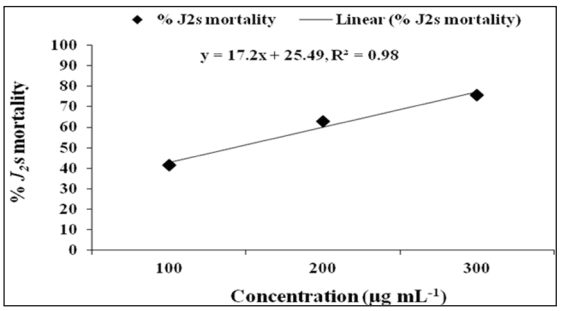

Figure 2- Effect of three different concentrations of pure compounds of *Berberis* spp., on % J2s mortality of *Meloidogyne javanica*

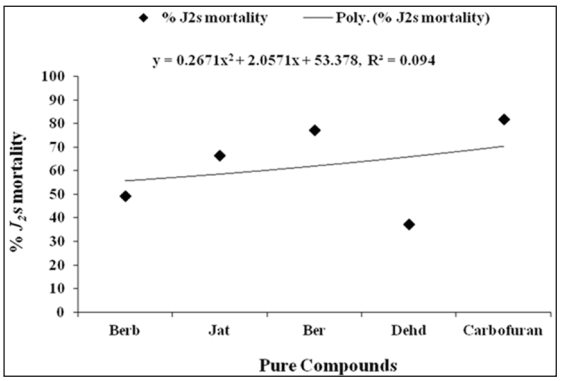

Figure 3- *In vitro* nematicidal effect of different pure compounds of *Berberis* spp., against % J2s mortality of *Meloidogyne javanica* (Berb, Berberrubine; Jat, Jatrorrhizine; Ber, Berberine; Dehd, Dehydrocheilanthifoline)

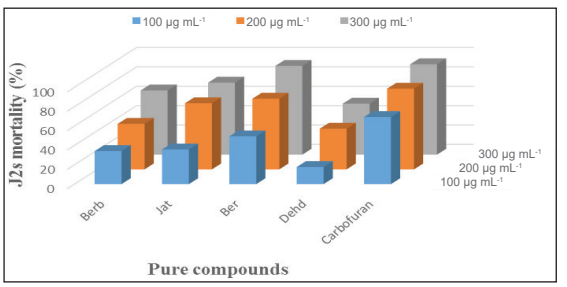

Figure 4- *In vitro* interaction effect between pure compounds of *Berberis* spp., and three different concentrations on % J2s mortality of *Meloidogyne javanica* (Berb, Berberrubine; Jat, Jatrorrhizine; Ber, Berberine; Dehd, Dehydrocheilanthifoline)

4. Conclusions

In the present study we have found that fractions of methanolic extracts of the two *Berberis* species have high potential against the root knot nematodes. Secondary metabolites of plants could be used as defense (toxic), which hinder reproduction and other physiological and biological functions of pests and parasites. These biomolecules could be used for enhancing the effectiveness and specificity in future nematicides design with specific or multiple target sites. These studies suggest that methanolic crude extracts and especially the isoquinoline alkaloids could be used as potential novel nematicides against *M. javanica*. Further research encompassing the isolation and identification of more nematicidal isoquinoline alkaloids from *Berberis* spp. may be carried out and tested against root knot nematodes as well as other plant parasitic nematodes.

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Identification of *Hordeum spontaneum* Genotypes Resistant to Net Blotch Disease

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ABSTRACT

Wild barley (*Hordeum spontaneum*) is a progenitor of cultivated barley and naturally grows in Turkey. *H. spontaneum* genotypes possess superior characteristics for biotic and abiotic stress tolerance factors. In this study, 3 virulent *Pyrenophora teres* f. *maculata* and 3 virulent *P. teres* f. *teres* isolates were tested under greenhouse conditions in order to find net blotch resistant *H. spontaneum* genotypes. A total of 104 *H. spontaneum* genotypes were used. Twenty-six *H. spontaneum* genotypes which corresponded to 25% of the genotypes (genotypes numbered 8, 13, 14, 16, 22, 24, 27, 31, 37, 44, 47, 54, 58, 62, 65, 66, 69, 74, 78, 81, 89, 94, 99, 102, 104 and 107) exhibited reactions classified in the resistant group to 3 virulent *P. teres* f. *maculata* isolates. Eight *H. spontaneum* genotypes which corresponded to 7.6% of the genotypes (genotypes numbered 24, 27, 29, 33, 44, 54, 89 and 94) exhibited reactions classified in the resistant group to 3 virulent *P. teres* f. *teres* isolates. Six *H. spontaneum* genotypes which corresponded to 5.7% of the genotypes (genotypes numbered 24, 27, 44, 54, 89 and 94) exhibited reactions in the resistant group to both 6 virulent *P. teres* f. *teres* and *P. teres* f. *maculata* isolates. In addition, a considerable number of genotypes exhibited resistant group reactions to one or two isolates of both forms of the pathogen. These genotypes could be used for developing net blotch resistant barley cultivars.

Keywords: Barley; Disease resistance; *Hordeum spontaneum*; Net blotch; *Pyrenophora teres*

1. Introduction

Wild barley (*Hordeum spontaneum,* syn: *Hordeum vulgare* subsp. *spontaneum*) is a progenitor of cultivated barley and naturally grows in Turkey (Kün 1996; Karakaya et al 2016). *Hordeum spontaneum* could hybridize with cultivated barley (*Hordeum vulgare*) and is an important plant for developing disease resistant barley cultivars. Wild barleys and barley landraces possess superior characteristics for abiotic and biotic stress tolerance factors (Ceccarelli & Grando 2000; Karakaya et al 2016; Çelik & Karakaya 2017). Because of these characteristics, it is advised to preserve *H. spontaneum* genotypes *in situ* and *ex situ* conditions for future research (Nevo 2012).

China, India, Central Asia, Near East, Mediterranean region, Ethiopia, Southern Mexico and Central and South America are eight main

regions in the world considered as plant gene centers. Turkey has a rich genetical diversity due to its location. Turkey is located at the intersection of the Mediterranean and the Near East gene centers, and it is on the historical migration and transportation routes of China, India, Central Asia and Ethiopia gene centers. In addition, Fertile Crescent region which includes Turkey's Southeastern Anatolia region is known as the region where barley, wheat, lentil, hard-seeded fruit and olive are cultivated for the first time (Vavilov 1951). In addition, Jakob et al (2014) reported that Levant, Turkey and east of Turkey are three main regions of wild barley (*H. spontaneum*) populations. Wild barleys including *H. spontaneum* are commonly grown under natural conditions in Turkey (Karakaya et al 2016).

Net blotch is caused by the fungus *Pyrenophora teres* that belongs to ascomycota. Anamorphic stage of the fungus is named as *Drechslera teres*. Two biotypes of the fungus are recognized. *Pyrenophora teres* f. *maculata* incites the spot form and *Pyrenophora teres* f. *teres* incites the net form of the disease (Karakaya & Akyol 2006; Liu et al 2011). The disease is commonly reported from different parts of the world and reduces the yield and quality of barley considerably (Mathre 1982; Liu et al 2011; Karakaya et al 2014).

In this study, 3 virulent *P. teres* f. *maculata* (*Ptm*) and 3 virulent *P. teres* f. *teres* (*Ptt*) isolates were tested under greenhouse conditions in order to find net blotch resistant *H. spontaneum* genotypes. A total of 104 *H. spontaneum* genotypes were used. An abstract of this study has been published previously (Çelik Oğuz et al 2017).

2. Materials and Methods

2.1. Experimental materials

In this study, 107 wild barley (*H. spontaneum*) genotypes that collected from various parts of Turkey and conserved by Field Crops Central Research Institute (Ankara, Turkey) were used. The seeds of these *H. spontaneum* genotypes were multiplied from single heads. Out of 107 genotypes, 104 provided the sufficient amount of seeds and were included in this study. No sufficient seeds were obtained from genotypes No: 4, No: 15 and No: 41. Three *Ptm* isolates and 3 *Ptt* isolates that were found to be the most virulent ones in the study by Çelik Oğuz (2015) were used in determination of seedling stage resistance of 104 *H. spontaneum* genotypes under greenhouse conditions.

2.2. Treatments

Sterile mixtures of soil, sand and organic substances (60, 20, 20; v/v/v, respectively) were placed in plastic pots with diameters of 7 centimeters and depending on the quantity of seeds of genotypes, 5-10 seeds were placed to the pots. The pots were maintained under greenhouse conditions. Inoculation was performed at growth stages 12-13 (Zadoks et al 1974). The inoculum was prepared from cultures grown on Potato Dextrose Agar maintained at 16-23±2 °C night/day with a 10 h/14 h dark/light period. In order to prepare inoculum, mycelia were harvested from Petri dishes using a no.12 brush and concentration of inoculum was adjusted to $15\text{-}20\times10^4$ mycelial parts/ml (Douiyssi et al 1998; Taşkoparan & Karakaya 2009; Usta et al 2014; Yazıcı et al 2015). One drop of Tween 20 was added to each 100 mL of inoculum (Aktaş 1995). Inoculum was sprayed onto barley seedlings using a hand sprayer and all leaves were covered with inoculum. The greenhouse temperature ranged between 18-23±1 °C night/day with a 10 h/14 h dark/light period. Plants were kept covered with nylon in transparent boxes with moist lids for 76 h following inoculation. Then, plants were maintained for another 48 h with the nylon uncovered and the ventilation of the boxes activated. There were three replications.

2.3. Evaluation of the disease

Plant evaluations were carried out seven days later following inoculation. For evaluation, scales developed for both forms of net blotch by Tekauz (1985) were used. Plant evaluations were based on lesion size, morphology, necrosis and chlorosis. Scale values of 1, 2 and 3 were considered as resistant group in this study. In the scale for the spot

form of net blotch, seven numerical classes were defined (1= R: resistant, 2= R: resistant to MR: moderately resistant, 3= MR: moderately resistant, 5= MR: moderately resistant to MS: moderately susceptible, 7= MS: moderately susceptible, 8= MS: moderately susceptible to S: susceptible, and 9= S: susceptible). In net form of net blotch scale ten numerical classes were defined (1= R: resistant, 2= R: resistant to MR: moderately resistant, 3= MR: moderately resistant, 4= MR: moderately resistant to MS: moderately susceptible, 5= MR: moderately resistant to MS: moderately susceptible, 6= MR: moderately resistant to MS: moderately susceptible, 7= MS: moderately susceptible, 8= MS: moderately susceptible to S: susceptible, 9= S: susceptible, and 10= VS: very susceptible). Resistant or moderately resistant genotypes have small net blotch lesions. Moderately susceptible or susceptible genotypes have chlorotic zones surrounding the necrotic areas and coalescence of these areas and death of leaves can occur.

2.4. Data analysis

Experiment was carried out using randomized block design with three replications. Data were square root transformed before statistical analysis. Separate two way analysis of variance was performed for each isolate and means of responses of *H. spontaneum* genotypes were separated by Least Significant Difference (LSD) test. Statistical tests were accomplished using JMP software (version 11; SAS Institute).

3. Results and Discussion

Seedling resistance reactions of 104 wild barley genotypes to 3 virulent *Ptm* isolates and 3 virulent *Ptt* isolates were determined. Analysis of variance revealed significant differences among the *H. spontaneum* genotypes ($P<0.01$). Response of the genotypes ranged between resistant (scale value 1) and susceptible (scale value 9) (Table 1). Thirty-nine, 9 and 2 genotypes exhibited moderately resistant, resistant-moderately resistant and resistant reactions to *Ptm* isolate GPS 263 PTM, respectively. Thirty-six and 3 genotypes showed moderately resistant and resistant-moderately resistant reactions to *Ptm* isolate 13-179 PTM, respectively. Fifty-three, 9 and 3 genotypes exhibited moderately resistant, resistant-moderately resistant and resistant reactions to *Ptm* isolate 13-167 PTM, respectively.

Twenty-six *H. spontaneum* genotypes which corresponded to 25% of the genotypes (genotypes numbered 8, 13, 14, 16, 22, 24, 27, 31, 37, 44, 47, 54, 58, 62, 65, 66, 69, 74, 78, 81, 89, 94, 99, 102, 104 and 107) exhibited reactions classified in the resistant group to 3 virulent *Ptm* isolates.

Nine and 1 genotypes showed moderately resistant and resistant reactions to *Ptt* isolate GPS 18 PTT, respectively. Thirteen, 6 and 2 genotypes exhibited moderately resistant, resistant-moderately resistant and resistant reactions to *Ptt* isolate UHK 77 PTT, respectively. Twenty-one and 8 genotypes exhibited moderately resistant and resistant-moderately resistant reactions to *Ptt* isolate 13-130 PTT, respectively.

Eight *H. spontaneum* genotypes which corresponded to 7.6% of the genotypes (genotypes numbered 24, 27, 29, 33, 44, 54, 89 and 94) exhibited reactions classified in the resistant group to 3 virulent *Ptt* isolates.

Six *H. spontaneum* genotypes which corresponded to 5.7% of the genotypes (genotypes numbered 24, 27, 44, 54, 89 and 94) exhibited reactions in the resistant to moderately resistant group range (Tekauz (1985) scale 1 to 3) to both 6 virulent *Ptt* and *Ptm* isolates. In addition, a considerable number of genotypes exhibited resistant to moderately resistant reactions to one or two isolates of both forms of the pathogen (Table 1).

Wild barleys are important resistance sources for controlling biotic and abiotic stress factors. Finding disease resistant wild barley genotypes facilitate disease resistance studies. In this current study, we determined *H. spontaneum* genotypes resistant to both forms of *P. teres*.

There are limited studies related to reactions of *H. spontaneum* genotypes to net blotch disease. In a study conducted by Kopahnke (1998), 770

Table 1- Seedling reactions of 104 *Hordeum spontaneum* genotypes to 3 virulent *Pyrenophora teres* f. *maculata* isolates and 3 virulent *Pyrenophora teres* f. *teres* isolates. Means not connected by same letter are significantly different (P<0.01)

Isolate	*GPS 263*		*13-179*		*13-167*		*GPS 18*		*UHK 77*		*13-130*	
Genotype	*PTM**		*PTM**		*PTM**		*PTT***		*PTT***		*PTT***	
1	5 cde	MR-MS	5 bcd	MR-MS	3 cde	MR	4 fghı	MR-MS	4 ıjk	MR-MS	6 bcde	MR-MS
2	3 efg	MR	5 bcd	MR-MS	5 abc	MR-MS	5 efgh	MR-MS	5 ghı	MR-MS	6 cdef	MR-MS
3	3 def	MR	5 bcd	MR-MS	5 bcd	MR-MS	6 abcd	MR-MS	5 fgh	MR-MS	6 abcd	MR-MS
4	Not enough seeds											
5	7 abc	MS	5 bc	MR-MS	5 abc	MR-MS	4 ghıj	MR-MS	4 ıjk	MR-MS	5 efgh	MR-MS
6	3 def	MR	5 bcd	MR-MS	3 cde	MR	4 hıjk	MR-MS	4 ıjk	MR-MS	4 hıjk	MR-MS
7	5 bcd	MR-MS	5 bcd	MR-MS	3 cde	MR	6 bcde	MR-MS	7 bc	MS	6 bcde	MR-MS
8	3 efg	MR	3 de	MR	2 gh	R-MR	4 ghıj	MR-MS	6 efg	MR-MS	5 defg	MR-MS
9	5 cde	MR-MS	5 bcd	MR-MS	3 efg	MR	6 bcde	MR-MS	8 ab	MS-S	5 defg	MR-MS
10	7 abc	MS	3 cde	MR	5 bcd	MR-MS	4 ghıj	MR-MS	4 ıjk	MR-MS	4 ıjk	MR-MS
11	5 cde	MR-MS	3 cde	MR	3 def	MR	6 abc	MR-MS	7 bc	MS	8 a	MS-S
12	3 efg	MR	3 de	MR	5 bcd	MR-MS	6 bcde	MR-MS	4 ıjk	MR-MS	5 efgh	MR-MS
13	3 efg	MR	3 de	MR	3 def	MR	3 defg	MR	4 jkl	MR-MS	3 lmn	MR
14	2 fgh	R-MR	3 de	MR	3 def	MR	4 fghı	MR-MS	3 lmn	MR	4 ghıj	MR-MS
15	Not enough seeds											
16	3 efg	MR	3 de	MR	2 fgh	R-MR	6 bcde	MR-MS	6 defg	MR-MS	4 ghıj	MR-MS
17	7 abc	MS	7 ab	MS	5 bcd	MR-MS	5 defg	MR-MS	6 cdef	MR-MS	5 fghı	MR-MS
18	7 abc	MS	5 abc	MR-MS	3 cde	MR	4 fghı	MR-MS	5 efg	MR-MS	5 fghı	MR-MS
19	5 abc	MR-MS	5 bc	MR-MS	5 abc	MR-MS	5 cdef	MR-MS	7 cd	MS	7 ab	MS
20	5 cde	MR-MS	5 bcd	MR-MS	3 def	MR	6 abc	MR-MS	5 efg	MR-MS	4 ghıj	MR-MS
21	7 abc	MS	3 cde	MR	3 def	MR	6 bcde	MR-MS	5 fgh	MR-MS	4 ıjk	MR-MS
22	3 fgh	MR	3 de	MR	3 def	MR	5 cdef	MR-MS	6 cdef	MR-MS	5 defg	MR-MS
23	5 cde	MR-MS	3 cde	MR	2 gh	R-MR	5 defg	MR-MS	2 no	R-MR	6 abcd	MR-MS
24	3 def	MR	2 e	R-MR	2 efgh	R-MR	2 m	R-MR	2 o	R-MR	3 klm	MR
25	5 bcd	MR-MS	5 bcd	MR-MS	5 bcd	MR-MS	5 efgh	MR-MS	6 defg	MR-MS	5 efgh	MR-MS
26	7 abc	MS	5 bc	MR-MS	3 cde	MR	4 fghı	MR-MS	4 ıjk	MR-MS	3 klm	MR
27	3 efg	MR	3 de	MR	2 efgh	R-MR	3 l	MR	3 lmn	MR	3 jkl	MR
28	5 bcd	MR-MS	5 bcd	MR-MS	3 cde	MR	5 defg	MR-MS	5 fgh	MR-MS	7 abc	MS
29	3 efg	MR	3 de	MR	5 bcd	MR-MS	3 l	MR	1 p	R	3 klm	MR
30	7 abc	MS	5 abc	MR-MS	3 efg	MR	4 ghıjk	MR-MS	6 defg	MR-MS	5 efgh	MR-MS
31	3 def	MR	3 de	MR	3 efg	MR	6 bcde	MR-MS	6 defg	MR-MS	6 bcde	MR-MS
32	3 efg	MR	5 bcd	MR-MS	3 cde	MR	5 efgh	MR-MS	6 cde	MR-MS	7 abc	MS
33	2 fgh	R-MR	3 de	MR	5 bcd	MR-MS	3 ıjkl	MR	3 klm	MR	3 lmn	MR
34	5 cde	MR-MS	5 bcd	MR-MS	5 ab	MR-MS	6 abcd	MR-MS	4 ıjk	MR-MS	4 ghıj	MR-MS
35	7 abc	MS	5 abc	MR-MS	5 abc	MR-MS	7 ab	MS	5 ghı	MR-MS	6 bcde	MR-MS
36	5 bcd	MR-MS	5 bc	MR-MS	5 abc	MR-MS	6 bcde	MR-MS	6 cdef	MR-MS	6 cdef	MR-MS
37	3 efg	MR	3 de	MR	3 def	MR	4 hıjk	MR-MS	3 mn	MR	3 lmn	MR
38	5 cde	MR-MS	3 cde	MR	3 cde	MR	6 abcd	MR-MS	6 cdef	MR-MS	6 abcd	MR-MS
39	5 bcd	MR-MS	5 bcd	MR-MS	3 def	MR	6 bcde	MR-MS	6 cdef	MR-MS	5 efgh	MR-MS
40	7 abc	MS	5 abc	MR-MS	5 abc	MR-MS	6 bcde	MR-MS	3 mn	MR	5 fghı	MR-MS
41	Not enough seeds											
42	3 efg	MR	5 bcd	MR-MS	3 def	MR	6 bcde	MR-MS	3 klm	MR	3 lmn	MR
43	7 abc	MS	5 bc	MR-MS	3 def	MR	6 bcde	MR-MS	3 lmn	MR	2 no	R-MR
44	3 efg	MR	3 de	MR	3 def	MR	3 ıjkl	MR	2 no	R-MR	3 lmn	MR
45	5 cde	MR-MS	5 bcd	MR-MS	3 def	MR	4 fghı	MR-MS	6 defg	MR-MS	3 lmn	MR
46	5 abc	MR-MS	5 bc	MR-MS	5 bcd	MR-MS	5 defg	MR-MS	7 cd	MR-MS	3 lmn	MR
47	3 efg	MR	3 de	MR	3 efg	MR	6 bcde	MR-MS	2 no	R-MR	2 mno	R-MR
48	7 abc	MS	5 abc	MR-MS	5 bcd	MR-MS	6 bcde	MR-MS	4 ıjk	MR-MS	3 lmn	MR
49	7 abc	MS	5 bc	MR-MS	3 def	MR	4 hıjk	MR-MS	5 fgh	MR-MS	4 ıjk	MR-MS
50	7 abc	MS	5 bc	MR-MS	3 def	MR	4 hıjkl	MR-MS	5 ghı	MR-MS	4 ıjk	MR-MS
51	5 cde	MR-MS	5 bcd	MR-MS	5 bcd	MR-MS	6 abcd	MR-MS	6 cdef	MR-MS	6 cdef	MR-MS
52	3 efg	MR	5 bcd	MR-MS	5 bcd	MR-MS	6 bcde	MR-MS	6 cde	MR-MS	6 bcde	MR-MS
53	3 efg	MR	5 bcd	MR-MS	5 bcd	MR-MS	6 bcde	MR-MS	5 fgh	MR-MS	4 ıjk	MR-MS
54	2 fgh	R-MR	3 de	MR	1 h	R	3 kl	MR	2 no	R-MR	2 mno	R-MR
55	3 efg	MR	5 bcd	MR-MS	5 bcd	MR-MS	6 bcde	MR-MS	5 efg	MR-MS	3 lmn	MR
56	5 cde	MR-MS	3 de	MR	3 def	MR	6 bcde	MR-MS	4 ıjk	MR-MS	3 jkl	MR
57	3 def	MR	5 abc	MR-MS	5 ab	MR-MS	6 bcde	MR-MS	4 jkl	MR-MS	6 bcde	MR-MS
58	3 efg	MR	3 de	MR	3 def	MR	4 fghı	MR-MS	6 cde	MR-MS	4 ıjk	MR-MS
59	2 fgh	R-MR	5 bcd	MR-MS	3 def	MR	4 hıjk	MR-MS	6 cdef	MR-MS	2 mno	R-MR
60	5 bcd	MR-MS	5 bcd	MR-MS	3 def	MR	6 abcd	MR-MS	9 a	S	6 abcd	MR-MS
61	2 fgh	R-MR	5 bcd	MR-MS	3 cde	MR	5 defg	MR-MS	5 fgh	MR-MS	6 cdef	MR-MS
62	1 h	R	3 de	MR	2 fgh	R-MR	6 bcde	MR-MS	7 Cd	MS	5 efgh	MR-MS
63	3 def	MR	5 bcd	MR-MS	3 cde	MR	6 bcde	MR-MS	4 ıjk	MR-MS	4 hıjk	MR-MS
64	3 efg	MR	5 bcd	MR-MS	5 bcd	MR-MS	4 fghı	MR-MS	4 jkl	MR-MS	3 klm	MR

Table 1- (continued)- Seedling reactions of 104 *Hordeum spontaneum* genotypes to 3 virulent *Pyrenophora teres* f. *maculata* isolates and 3 virulent *Pyrenophora teres* f. *teres* isolates. Means not connected by same letter are significantly different (P<0.01)

Isolate	*GPS 263*		*13-179*		*13-167*		*GPS 18*		*UHK 77*		*13-130*	
Genotype	*PTM**		*PTM**		*PTM**		*PTT***		*PTT***		*PTT***	
65	1h	R	3 de	MR	3 def	MR	4 fghı	MR-MS	3 klm	MR	3 klm	MR
66	2 fgh	R-MR	3 de	MR	3 efg	MR	6 bcde	MR-MS	4 jkl	MR-MS	6 cdef	MR-MS
67	5 cde	MR-MS	3 cde	MR	5 abc	MR-MS	6 bcde	MR-MS	6 defg	MR-MS	4 hıjk	MR-MS
68	8 a	MS-S	7 a	MS	7 a	MS	7 a	MR-MS	6 defg	MR-MS	7 abc	MS
69	3 def	MR	3 de	MR	3 efg	MR	5 efgh	MR-MS	7 bc	MS	4 ıjk	MR-MS
70	3 efg	MR	5 bcd	MR-MS	3 def	MR	4 ghıjk	MR-MS	6 cdef	MR-MS	4 hıjk	MR-MS
71	7 abc	MS	5 bcd	MR-MS	3 def	MR	4 ghıj	MR-MS	4 jkl	MR-MS	3 klm	MR
72	5 bcd	MR-MS	5 bc	MR-MS	5 abc	MR-MS	6 bcde	MR-MS	7 cd	MS	7 abc	MS
73	3 def	MR	5 bcd	MR-MS	5 bcd	MR-MS	6 bcde	MR-MS	6 cde	MR-MS	5 efgh	MR-MS
74	2 fgh	R-MR	3 cde	MR	3 cde	MR	6 bcde	MR-MS	7 bc	MS	5 defg	MR-MS
75	7 abc	MS	5 bc	MR-MS	3 efg	MR	4 fghı	MR-MS	5 fgh	MR-MS	4 ıjk	MR-MS
76	5 cde	MR-MS	3 cde	MR	3 def	MR	6 bcde	MR-MS	6 cde	MR-MS	6 cdef	MR-MS
77	7 abc	MS	5 abc	MR-MS	5 bcd	MR-MS	6 bcde	MR-MS	4 hıj	MR-MS	4 hıjk	MR-MS
78	3 efg	MR	3 cde	MR	3 cde	MR	6 bcde	MR-MS	6 defg	MR-MS	5 defg	MR-MS
79	3 def	MR	5 bcd	MR-MS	5 bcd	MR-MS	6 abcd	MR-MS	7 bc	MS	6 defg	MR-MS
80	5 bcd	MR-MS	5 bcd	MR-MS	2 fgh	R-MR	7 ab	MS	8 ab	MS-S	5 efgh	MR-MS
81	3 def	MR	2 e	R-MR	1 h	R	6 bcde	MR-MS	6 defg	MR-MS	5 defg	MR-MS
82	3 efg	MR	5 bcd	MR-MS	5 bcd	MR-MS	6 abcd	MR-MS	7 cd	MS	5 defg	MR-MS
83	3 def	MR	5 bcd	MR-MS	3 cde	MR	4 fghı	MR-MS	6 defg	MR-MS	4 ıjk	MR-MS
84	2 fgh	R-MR	5 bcd	MR-MS	3 cde	MR	4 ghıj	MR-MS	4 hıj	MR-MS	2 mno	R-MR
85	5 cde	MR-MS	3 de	MR	3 def	MR	5 defg	MR-MS	7 cd	MS	6 bcde	MR-MS
86	7 abc	MS	7 ab	MS	5 abc	MR-MS	6 bcde	MR-MS	5 efg	MR-MS	7 ab	MS
87	5 bcd	MR-MS	5 bcd	MR-MS	5 bcd	MR-MS	4 ghıj	MR-MS	6 defg	MR-MS	7 abc	MS
88	5 abc	MR-MS	5 bc	MR-MS	5 abc	MR-MS	6 abc	MR-MS	7 bc	MS	6 bcde	MR-MS
89	3 def	MR	3 de	MR	3 def	MR	3 jkl	MR	3 klm	MR	3 klm	MR
90	7 abc	MS	5 abc	MR-MS	5 bcd	MR-MS	6 bcde	MR-MS	2 no	R-MR	5 fghı	MR-MS
91	7 abc	MS	5 bcd	MR-MS	5 bcd	MR-MS	4 ghıj	MR-MS	5 fgh	MR-MS	4 ıjk	MR-MS
92	7 ab	MS	5 abc	MR-MS	5 bcd	MR-MS	4 fghı	MR-MS	6 defg	MR-MS	5 efgh	MR-MS
93	7 abc	MS	3 de	MR	3 efg	MR	3 ıjkl	MR	4 jkl	MR-MS	3 klm	MR
94	3 efg	MR	3 de	MR	3 def	MR	3 jkl	MR	3 klm	MR	2 o	R-MR
95	5 bcd	MR-MS	5 bcd	MR-MS	5 bcd	MR-MS	6 abcd	MR-MS	4 jkl	MR-MS	5 efgh	MR-MS
96	7 abc	MS	5 bc	MR-MS	5 bcd	MR-MS	6 bcde	MR-MS	4 ıjk	MR-MS	3 jkl	MR
97	7 abc	MS	5 abc	MR-MS	3 cde	MR	6 bcde	MR-MS	6 defg	MR-MS	5 defg	MR-MS
98	5 cde	MR-MS	5 bcd	MR-MS	3 def	MR	6 abc	MR-MS	5 fgh	MR-MS	5 fghı	MR-MS
99	3 efg	MR	3 de	MR	1 h	R	4 ghıj	MR-MS	1 p	R	4 ıjk	MR-MS
100	3 def	MR	5 bcd	MR-MS	3 def	MR	6 abcd	MR-MS	5 fgh	MR-MS	4 ghıj	MR-MS
101	3 def	MR	5 cde	MR-MS	3 efg	MR	6 bcde	MR-MS	3 klm	MR	3 jkl	MR
102	2 fgh	R-MR	2	R-MR	2 efgh	R-MR	5 defg	MR-MS	3 lmn	MR	4 ghıj	MR-MS
103	5 cde	MR-MS	5 bcd	MR-MS	3 cde	MR	6 abcd	MR-MS	4 jkl	MR-MS	4 ıjk	MR-MS
104	3 efg	MR	3 de	MR	2 fgh	R-MR	5 defg	MR-MS	3 klm	MR	2 o	R-MR
105	5 cde	MR-MS	7 ab	MS	5 bcd	MR-MS	7 a	MS	6 efg	MR-MS	4 ıjk	MR-MS
106	5 bcd	MR-MS	5 bc	MR-MS	5 abc	MR-MS	7 a	MS	5 fgh	MR-MS	2 mno	R-MR
107	3 def	MR	3 cde	MR	3 cde	MR	7 ab	MS	6 cdef	MR-MS	4 ıjk	MR-MS
CV%	9.41%		9.98%		9.58%		5.16%		4.46%		5.53%	

**P. teres* f. *maculata* scale values: 1= R: resistant, 2= R: resistant to MR: moderately resistant, 3= MR: moderately resistant, 5= MR: moderately resistant to MS: moderately susceptible, 7= MS: moderately susceptible, 8= MS: moderately susceptible to S: susceptible, 9= S: susceptible. ***P. teres* f. *teres* scale values: 1= R: resistant, 2= R: resistant to MR: moderately resistant, 3= MR: moderately resistant, 4= MR: moderately resistant to MS: moderately susceptible, 5= MR: moderately resistant to MS: moderately susceptible, 6= MR: moderately resistant to MS: moderately susceptible, 7= MS: moderately susceptible, 8= MS: moderately susceptible to S: susceptible, 9= S: susceptible, 10= VS: very susceptible

H. spontaneum and 300 *H. vulgare* accessions were evaluated for their resistance status to *P. teres* under greenhouse and field conditions. *H. spontaneum* genotypes exhibited different resistance reactions and 143 genotypes showed resistant reaction to all isolates. Fetch et al (2003) determined the diversity of 116 *H. spontaneum* genotypes for their reaction to six barley fungal pathogens. The genotypes were obtained from Israel and Jordan. At seedling stage, a high level of diversity was found. Resistance frequency of genotypes from Israel and Jordan was high for net blotch (68% and 72%, respectively). Two genotypes were found resistant to 6 pathogens. Similarly, in our current study variation was found among the *H. spontaneum* genotypes. In our study, six *H. spontaneum* genotypes showed resistant to moderately resistant reactions to all *Ptt* and *Ptm* isolates. Jana & Bailey (1995) determined the resistance status of *H. vulgare* subsp. *spontaneum* and *H. vulgare* subsp. *vulgare* genotypes from Jordan and Turkey to *P. teres* f. *maculata, P. teres* f. *teres* and *Cochliobolus sativus.* More *H. vulgare* subsp. *spontaneum* genotypes were resistant to *P. teres* f. *teres* (21.8% vs. 0.5%) than *H. vulgare* subsp. *vulgare*. An equal number of *H. vulgare* subsp. *spontaneum* and *H. vulgare* subsp. *vulgare* genotypes were resistant to *P. teres* f. *maculata*. A larger percentage of *H. vulgare* subsp. *spontaneum* genotypes (10.5%) had at least moderate resistance to *P. teres* f. *teres*, *P. teres* f. *maculata* and *C. sativus* compared to only 1.3% in *H. vulgare* subsp. *vulgare*. However, in our current study, 25% of the genotypes and 7.6% of the genotypes exhibited resistant group reactions to *P. teres* f. *maculata* and *P. teres* f. *teres*, respectively. This finding is hopeful, because *P. teres* f. *maculata* is more prevalent in Turkey than *P. teres* f. *teres* (Karakaya et al 2014). *H. spontaneum* accessions showed different resistance reactions, depending upon their origin. Sato & Takeda (1997) evaluated net blotch resistance in 175 *H. vulgare* subsp. *spontaneum* (*H. spontaneum*) accessions and 149 wild *Hordeum* accessions of thirteen species or subspecies. Most *H. spontaneum* accessions showed resistance to each of the four *P. teres* f. *teres* isolates (Japanese isolates K105 and Pt860514 and Canadian isolates WRS102 and WRS1581) tested. Some accessions from Russia and Afghanistan showed a high level of resistance and Morocco accessions were susceptible. *H. spontaneum* accessions susceptible to the Canadian isolate WRS102 but resistant to the other three isolates were found in Iraq. This suggested the geographical differentiation of resistance genes in *H. spontaneum*. All accessions of the other wild *Hordeum* species, especially some accessions of *H. marinum* subsp. *gussoneanum*, showed high levels of resistance. Sato & Takeda (1997) concluded that resistance genes may be useful candidates for incorporation into cultivated barley.

H. spontaneum is a rich source of genes for disease resistance. Many resistant barley genotypes were found in barley evolution centers (Afanasenko et al 2000). Suitable habitat conditions for *H. vulgare* subsp. *spontaneum* exist especially in the Levant and Turkey and genetic diversity was observed in these populations (Jakob et al 2014). Turkey is an important gene center of barley and wild barleys (Kün 1996). Karakaya et al (2016) examined a total of 40 naturally growing *H. spontaneum* field populations in Şanlıurfa, Mardin, Şırnak, Siirt, Diyarbakır, Gaziantep, Kilis and Hatay provinces of Turkey for the presence of diseases and their severities in 2015. Nine *H. spontaneum* populations were disease free. The following diseases were found: Scald incited by *Rhynchosporium commune*, powdery mildew incited by *Blumeria graminis* f. sp. *hordei*, both forms of net blotch incited by *Drechslera teres* f. *teres* and *D. teres* f. *maculata*, semi loose smut incited by *Ustilago nigra*, loose smut incited by *Ustilago nuda*, brown rust (leaf rust) incited by *Puccinia hordei* and barley stripe caused by *Drechslera graminea*. Scald was the most commonly encountered disease followed by powdery mildew and net blotch. The incidence and severity values of diseases varied. The authors reported a wide range of variation in terms of disease resistance status of naturally growing *H. spontaneum* populations.

4. Conclusions

The use of disease resistant cultivars is the desirable control method of diseases. For sustainable crop production, monitoring virulence changes in pathogen is necessary. New pathotypes could be more virulent than previous pathotypes. For this reason, a broad base of genetical source is necessary. Wild barleys and especially *H. spontaneum* are valuable sources for disease resistance. Useful traits including disease resistance could be transferred to barley cultivars. Nevo (1992) pointed out the importance of *H. spontaneum* for disease resistant barley breeding programs and for developing a gene pool for desired traits. Also, Nevo et al (1986) examined the *H. spontaneum* populations of Israel, Turkey and Iran in the Fertile Crescent and reported their genetic diversity as well as their adaptability. Turkey is an important gene center of *Hordeum* species (Kün 1996). *Hordeum spontaneum* populations are naturally growing in Turkey and heterogenous nature of disease resistance among the populations has been reported (Karakaya et al 2016).

With this study, novel wild barley (*H. spontaneum*) genotypes resistant to both forms of *Pyrenophora teres* have been identified. These genotypes could be used in obtaining disease resistant and high yielding barley cultivars.

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Isolation of *Staphylococcus hominis* from Cultured Gilthead Sea Bream (*Sparus aurata* L.) in Antalya Bay, Turkey

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ABSTRACT

In this study, a disease case caused with 15% mortality on gilthead sea bream (*Sparus aurata*) which was cultured in the Antalya Bay in the Mediterranean coast of Turkey was investigated in July 2015. Lethargy, loss of equilibrium, petechae on the operculums and the dorsal part of the body, pillar of the gills were recorded in the diseased fish. Internally, ascites, splenomegaly and hemorrhages on the intestinal tissue and muscle were observed. Occurrence of parasite was not detected. According to the results of phenotypical test and 16S rDNA sequencing analysis, the isolated bacterial species was identified as *Staphylococcus hominis*. Histopathologically, hyperplasia of primary lamellae, lamellar telangiectasia, edema and hemorrhages on the gill arch, numerous melanomacrophage centers (MMCs) in the spleen tissue, vacuoler degeneration, necrose and hemorrhages in the liver, multifocal necrosis, and numerous MMC in the kidney were determined and hemorrhages in the *tunica propria* region of the intestine were observed. It was determined that he isolates showed different susceptibility against antimicrobial agents.

Keywords: Gilthead sea bream; *Sparus aurata*; Cage culture; *Staphylococcus hominis*

1. Introduction

Mediterranean aquaculture production has shown a continuous growth over the years. The production mainly includes two species, gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). Because of economic importance of *S. aurata*, many studies, including nutrition (Andrew et al 2003), genetic (Alarcón et al 2004), biology (Noyo & Lamas 1996), infectious and noninfectious disorders (Mladineo 2006; García-Rosada et al 2007; Beraldo & Canavese 2011) were carried out.

A variety of infections affect farmed finfish species. These infections reduce the volume and quality of fish production. Bacterial infections, which are mostly affecting farmed sea bream include photobacteriosis, tenacibaculosis, pseudomonadiasis ('winter ulcer') and vibrosis (Toranzo et al 2005; Tanokhy 2013; Scarano et al 2014). The causative agents of the above mentioned infections are Gram-negative bacterial species. Among Gram-positive bacterial infections, *Staphylococcal* species, *S. iniae* (Zlotkin et al 1998), *S. agalactiae* (Evans et al 2002), *S. epidermidis* (Kubilay & Ulukoy 2004), *S. cobuii*, *S. lentus*, *S. schleiferi* and *S. warneri* (Yiagnisis & Athanassopoulou 2011) were isolated from sea bream.

S. hominis was isolated from unpolluted marine waters, crab and crabmeat (Gunn & Colwell 1983). Altuğ et al (2013) also reported *S. hominis* from unpolluted areas of the Sea of Marmara in Turkey. In addition, *S. hominis* were isolated from the diseased farmed sea bass fry (Yiagnisis & Athanassopoulou 2011).

This study describes the first record of *Staphylococcus hominis* from cultured gilthead sea bream (*Sparus aurata*) in the Antalya Bay. *S. hominis* was isolated as the causative agent of the disease and identified by 16 S rRNA sequence and its histopathology are determined.

2. Material and Methods

2.1. Fish sampling and clinical examination

This study was carried on a commercial firm in the Antalya Bay, Eastern Mediterranean Sea, Turkey during spring and summer seasons (from May to November) in 2015. Twenty fish samples were collected from each sampling. Totally 120 fish were randomly collected from floating cages. The water temperatures during this period fluctuated 24 °C between 28 °C. The weights of the fish ranged from 150 g to 240 g. In July, a disease outbreak with 15% fish losses occurred in the farm. The affected fish were brought alive and transported back to the Akdeniz University Faculty of Fisheries (AUSUF) research laboratory in plastic bags containing aerated sea water from the cage environment. After fish transporting, the fish were deep anesthetized with 2-Phenoxyethanol at 300 mg L^{-1} consantration in sea water for 15 min before necropsy and examined for the external signs on the body surface. Then, the samples were necropsied and internal findings of them were noticed. The procedures were reviewed and approved by the Akdeniz University Local Committee on Animal Research Ethics. Number: 2015.03.06.

2.2. Parasitological examination

Standard parasitological methods for examination of fish were applied (Collins 1993). Smear preparations on glass slides from skin and gill lamellae were prepared and examined under light microscope. Then, organs (stomach, intestine, liver, kidney, and spleen) were removed and placed in petri dishes containing sterilized saline water and examined under light microscope.

2.3. Bacteriological study

For bacterial isolation, samples taken from liver, kidney, and spleen of moribund gilthead sea bream were inoculated on brain heart infusion (BHI) agar with 1.5-% NaCl. Cultures were incubated at 26±2 °C for 72 h. After incubation, bacterial colonies were subcultured and pure cultures of the colonies were subjected to conventional tests including Gram-staining, motility, cytochrome oxidase and catalase for biochemical identification by following the procedures of Seeley et al (1991) and Austin & Austin (2012). 16S rDNA studies were also conducted on that pure cultures.

2.4. Antimicrobial susceptibility testing

In vitro susceptibility testing of the strains was carried out by using the disc agar diffusion method according to the CLSI (M42-A) (2006). The tests were performed in duplicates. The following antimicrobial agents (µg) were employed: ampicillin (10), bacitracin (0.04), chloramphenicol (30), erythromycin (15), flumequine (30), kanamycin (30), nalidixic acid (30), oxolinic acid (2), oxytetracycline (30), streptomycin (10), sulfamethoxazole (25), tetracycline (30) and trimethoprim (5).

2.5. Nucleic acid isolation

A typical colony from pure culture was grown in 5 mL of Nutrient broth (NB) with 1.5% NaCl at 26 °C for overnight. 1.5 mL of this culture was used for bacterial DNA extraction using DNA extraction kit (Thermo Scientific) in accordance with the manufacturer's Gram-positive bacterial DNA isolation instructions. Bacterial genomic DNA was eluted by 10 mM Tris-EDTA buffer up to volume 200 µL and stored at -20 °C until used.

2.6. PCR assay

Isolates from the diseased fish samples were identified by using Gram staining, colony morphology and biochemical tests. They were

further identified with 16S rDNA gene sequence analysis. The polymerase chain reaction (PCR) has provided for the detection. The universal primers, B27F (5'-AGAGTTTGATCCTGGCTCAG-3') and U1492R (5'-GGTTACCTTGTTACGACTT-3'), were used for amplification of the 16S ribosomal DNA gene (Liu et al 2013; Liu et al 2014). 5 µL of genomic DNA solution in Tris-EDTA buffer was added to 45 µL of a PCR mixture consisting 2X mix (Qiagen), 1 µL (10 nmol) of each primers and sterile Millipore-quality water added up to 50 µL. Amplification was carried out in a thermocycler (Kyratec SC-200), iniated by 10 min of denaturation at 95 °C and then carried out for 35 PCR cycles of which each consists of 45 sec of denaturation at 95 °C, 45 sec of annealing at 60 °C, and 2.5 min extension at 72 °C. The reaction was lasted with heating at 72 °C for 10 min after the last cycle. A negative control with all the reaction components except template DNA was included with each test run.

2.7. Molecular characterization, gel extraction and identification of the isolate by BLASTN

Twenty µL of the reaction mixture was analyzed by submarine gel electrophoresis in 1% agarose (Bioron) at 8 V cm^{-1}. 1 kb DNA ladder (GeneRuler) was used for molecular characterization of the PCR product. To extract amplicons from the gel extraction kit (Macherey-Nagel) was used by following the protocol of the manufacturer. After that PCR products were sequenced by Macrogen Inc. The sequences were subjected to BioEdit V7.2.5. program (Hall 1999) to assemble the revers and forward fragments then compared to 16S rDNA sequences in the GenBank database by using the BLASTN algorithm (Chu & Lu 2005; Liu et al 2013; Liu et al 2014).

2.8. Histopathological study

Tissue samples from gills and internal organs were fixed in 10% buffered formalin solution for routine histopathological examination (Culling 1963). The fixed tissue samples washed in tap water overnight and dehydrated in the ascending concentrations of ethanol. After dehydration, tissue samples were cleared in xylene and sectioned at 5 µm. Then, the sections were stained with hematoxylin and eosin (H & E).

3. Results and Discussion

3.1. Clinical findings

The moribund fish were lethargic. They showed petechae on the operculums and dorsal parts of their body surfaces. Other signs included hemorrhages on the intestinal wall and muscle, pale liver and enlarged spleen. The spleen was also in cherry red color. Yiagnisis & Athanassopoulou (2011) isolated *S. hominis* from a bacterial survey study of the wild and farmed marine fish species in Greece. The authors reported that affected fish had anorexia and the generalized hemorrhagic septicemia including visceral petechation. Our findings were similar to those reported by Yiagnisis & Athanassopoulou (2011).

3.2. Parasitological findings, bacterial isolation and identification

Ectoparasites or endoparasites were not detected in fish. As a result of bacteriological studies, whitish to creamy colored bacterial colonies on BHI agar with 1.5% NaCl were observed at 26±2 °C for 72 h. These bacterial colonies were subcultured and the strains from these colonies were tested biochemical tests. The isolate was nonmotile, Gram-positive, facultatively fermentative, cytochrome oxidase negative and coagulase (rabbit plasma) negative. 16S rDNA sequences of the isolated strains were compared with the GenBank database by using the BLASTN. The closest matches obtained with *Staphylococcus hominis* (GenBank accession number KF254627.1; maximal score 2636, E value 0.0, and maximal identity 99% (1429/1430)). Most *Staphylococci*, including *S. epidermis*, *S. hominis* and *S. warneri* from fish as coagulase-negative were reported (Ahmet et al 2011). *S. hominis* isolated in this study was Gram-positive, clusters, cytochrome oxidase and coagulase negative and facultatively fermentative. Our own biochemical test results for *S. hominis* agreed with those of Schleifer & Bell (2009) and Ahmet et al (2011) except mannitol and arabinose test results.

3.3. Results of antimicrobial susceptibility testing

Staphylococcus hominis showed varying susceptibility to the antimicrobial agents which were employed in this test. It was highly sensitive to chloramphenicol, tetracycline, streptomycin, oxytetracycline, kanamycin, trimethoprim and erythromycin (more than inhibition zone of 25 mm) except bacitracin (the inhibition zone <20 mm) while it showed intermediate resistance to ampicillin. Bacterial infections in fish culture are important, and for this reason, agents are widely used for treatment; however, a few reports on antimicrobial susceptibility of *S. hominis*. Uddin et al (2013) reported that the species isolated from black tiger shrimp (*Penaeus monodon*) showed resistance to ampicillin and erythromycin. But, *S. hominis* was found sensitive against the above mentioned antibiotics and also others in this study.

3.4. Results of histopathological examination

The histopathological examination of tissues of gilthead sea bream revealed that the gills demonstrated hyperplasia of the primary lamellae (Figure 1a), lamellar telangiectasia (Figure 1b), and the gill arch showed edema and hemorrhages (Figure 1c). The spleen showed numerous of melanomacrophage centers (MMCs) (Figure 2a), and activations of MMCs were detected. These centers were well defined, numerous and dark brown colored. Splenic congestion was also observed (Figure 2b). Basophilic areas with bacterial colonies were observed (Figure 2b). Focal empty shaped

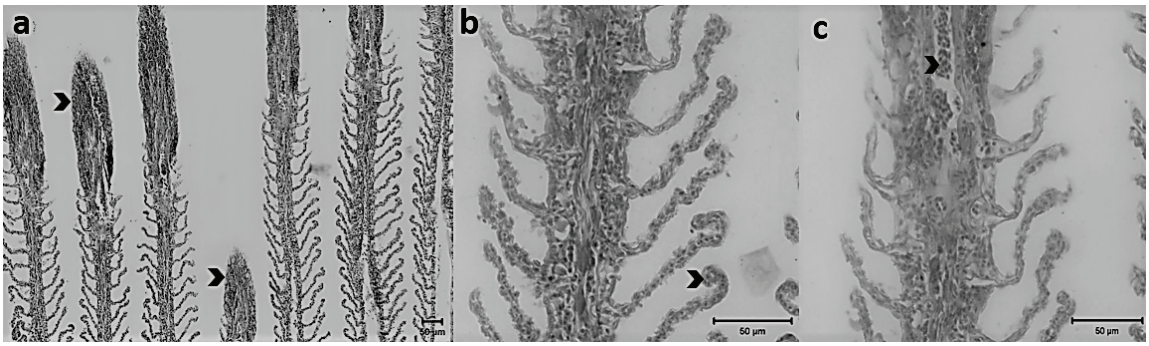

Figure 1- Gill of diseased fish (H+E). a, Hyperplastic area rich in secretory cells in the primary lamellae (arrowhead); b, Lamellar telangiectasia (arrowhead); c, the gill arch showing edema and hemorrhages (arrowhead) (H & E)

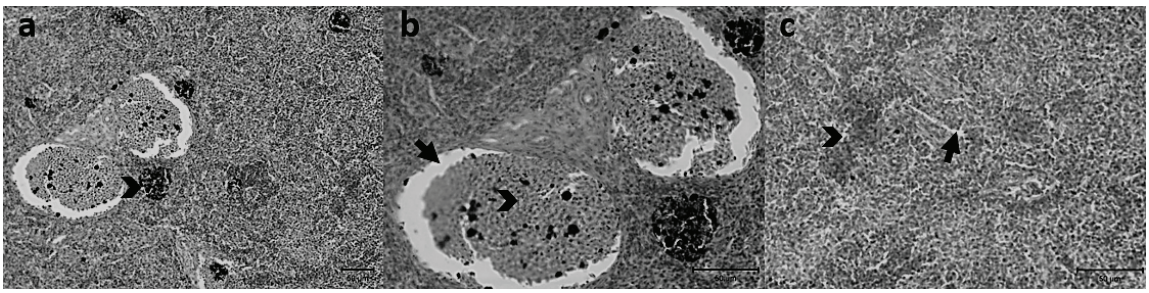

Figure 2- Spleen of diseased fish (H+E). a, activation of melanomacrophage centers (MMCs) (arrowhead); b, congested blood vessels (arrow) and basophilic areas with bacterial colonies (arrowhead); c, focal empty spaces (arrow) due to depletion, necrosis and lysis of hematopoietic elements and focal hemorrhages (arrowhead) (H & E)

spaces in the spleen due to depletion, necrosis, loss of hematopoietic elements and focal hemorrhages in the spleen tissue were evident (Figure 2c). The liver showed vascular degeneration of hepatocytes, hepatocytic necrosis and hemorrhage (Figure 3a). Multifocal areas of tubular and interstitial necrosis and hemorrhages, heavy depositions in the MMCs in kidney (Figure 3b) were observed. Hemorrhages (Figure 3c) in *tunica propria* of the intestine, villous atrophy and also depletion of the intestinal mucosa were evident. Histopathological examinations of tissues showed activations of MMCs, necrosis and lysis of hematopoietic elements and hemorrhages in the spleen, liver and kidney.

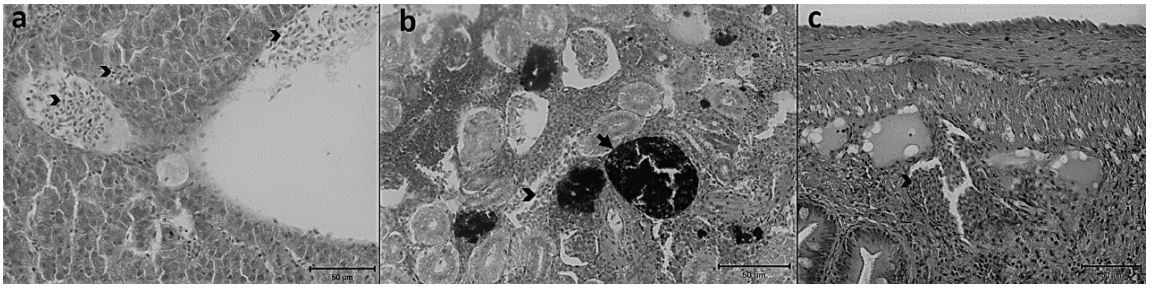

Figure 3- a, Hepatocytic necrosis and hemorrhages in the liver; b, hemorrhage (arrowhead) and heavy deposition in the MMCs (arrow) in the kidney; c, hemorrhages (arrowhead) in *tunica propria* of the intestine (H & E)

4. Conclusions

Staphylococcus hominis was first isolated and identified from the cultured gilthead sea bream (*S. aurata*) from Antalya Bay (Turkey) in this study. High water temperature above optimal growth temperature (22-25 °C) for gilthead sea bream and handling could be two reasons to begin disease outbreak. Histopathological changes were detected on tissues of affected fish. To better understand virulence mechanisms of *Staphylococcus hominis* on different fish species, including *Sparus aurata*, further studies should be carried on under controlled conditions.

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References

Cite references in the text as author‘s family name should be followed by the year of the publication in parentheses (Peter 2010; Basunia & Abe 2001). Use et al after the first author’s family name for citations with three or more authors (Lawrence et al 2001). For citations of the same authors published on the same year, use letters after the year (Dawson 2009a).

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Journal Articles

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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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