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Application of Bacteriocin-Like Inhibitory Substances (BLIS)-Producing Probiotic Strain of *Lactobacillus plantarum* in Control of *Staphylococcus aureus* in White-Brined Cheese Production

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ABSTRACT

The aim of this study was to investigate the antimicrobial activity of an autochthonous probiotic strain of bacteriocin-like inhibitory substances (BLIS)-producing *Lactobacillus plantarum*, previously isolated from a Tulum cheese and satisfied technological criteria as adjunct culture in cheese production, in reducing *Staphylococcus aureus* during production and ripening of white-brined cheeses. Cheeses were manufactured in two trials from pasteurized milk artificially contaminated with *S. aureus* to the mean level of 6.243 log MPN mL^{-1}. *Lb. plantarum* BG33 was added at 1% as adjunct to the starter culture. The study was also carried out with control group cheeses produced without the adjunct culture. *S. aureus* counts were monitored for up to 90 days by BAM's 5-tube MPN method and each positive tube of MPN (most probable number) method was confirmed by PCR amplification of a 400 bp fragment of the *nuc* gene, which encodes the thermostable nuclease of *S. aureus*. The capacity of *Lb. plantarum* BG33 to reduce *S. aureus* count was found as 0.9 log unit on the 18th day of ripening. After 39 and 59 days of ripening, *Lb. plantarum* BG33 lowered *S. aureus* count by 1.9 and 2.0 log units, respectively, when compared to control group cheeses in which it was lowered by 0.5 and 1.0 log units, respectively. As a result, the BLIS activity of *Lb. plantarum* BG33 throughout ripening of white-brined cheese could make it useful as bioprotective adjunct culture in white-brined cheese production to prevent *S. aureus* growth which is an important foodborne pathogen in respect of safe cheese production.

Keywords: *Lactobacillus plantarum*; *Staphylococcus aureus*; White-brined cheese; Bacteriocin; Bio-control

1. Introduction

Traditionally manufactured cheeses especially white-brined cheeses made from thermized or raw cow's milk with a soft or semi-hard texture and a salty and acidic taste, play an important role in nutrition of people in Turkey besides being the most popular part of dairy products export of Turkey (Hayaloglu et al 2002; Temelli et al 2006). *Staphylococcus aureus* is a common commensal of the skin and mucosal membranes of humans. White-

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brined cheese production requires an extensive manual processing of the curd by the cheese-maker. This represents a possible route resulting in the risk of *S. aureus* contamination in white-brined cheeses and hence they could be a major cause of staphylococcal food poisoning (Rilla et al 2004; Mercanoglu Taban et al 2017).

Although there has been an improvement in cheese production facilities in dairy industries, *S. aureus* is still one of the leading pathogen that contaminates cheeses (Le Loir et al 2003; Charlier et al 2009). The frequency of *S. aureus* contamination in cheeses and the impact of staphylococcal food poisoning on public health have focused on the researches to control this pathogen. Therefore, the behaviour of *S. aureus* during production and ripening of some cheeses has been well studied (Nunez et al 1988) and many studies have been documented regarding the control of this pathogen by the direct application of bacteriocins (primarily nisin) to the cheeses (Abdalla et al 1993; Cintas et al 1998; Rilla et al 2004; Trmčić et al 2010). The use of bacteriocin-producing starter cultures in cheese production has gained the greatest interest from a food safety and human health point of view in addition with the consumers' preference of eating foods including minimum levels of chemically-synthesized additives (Rilla et al 2004). Besides, many papers have been published in combined use of various hurdles, including bacteriocins (Capellas et al 2000; Al-Holy et al 2012) or bacteriocin-producing cultures (Arques et al 2005) to inhibit foodborne pathogens in cheeses. Although the potential of bacteriocin-producing starters or adjunct cultures to control *Listeria* spp. in cheese production has been evaluated by a considerable amount of researches (Buyong et al 1998; O'Sullivan et al 2002; Rodriguez et al 2005), little has been known about the efficacy of using bacteriocin-producing starter or adjunct cultures on the growth and survival of *S. aureus* in cheese production (Rodriguez et al 2000; Rodriguez et al 2005; Favaro et al 2015), which may result in a better and economic way to control this pathogen in addition to the necessity of their contribution to the typical sensory characteristics and nutritional value of cheeses. For example; Rodriguez et al (2000) showed a reduction on the counts of *S. aureus* in a semi-hard cheese made with a nisin-producing starter and Rilla et al (2004) designed nisin-producing dairy starters to specifically inhibit *S. aureus* in acid-coagulated cheeses.

Considering the fact that there has still been technological drawbacks in using them as starters in industrial cheese production due to their poor acidifying, proteolytic and lypolytic characteristics and thus it is not easy to select appropriate strains having both strong technological and antimicrobial activities as starters in cheese production (Sarantinopoulos et al 2002; Favaro et al 2015), the present study aimed to evaluate antistaphylococcal ability of using autochthonous probiotic plantaricin-like bacteriocin producing *Lactobacillus plantarum* BG33 as adjunct culture in white-brined cheese production. The use of antistaphylococcal adjunct culture in white-brined cheese production like in this study is important in respect of dairy technology as well as food safety since there is an increasing amount of public demand for high-quality cheeses that are free of both pathogens and artificial additives.

2. Material and Methods

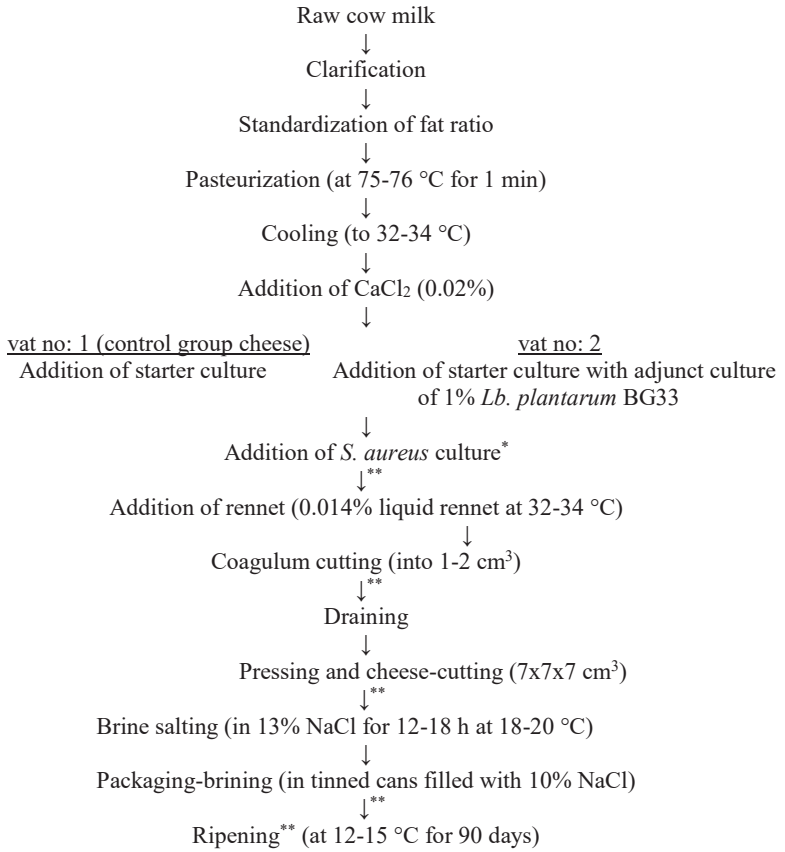
2.1. Cultures

Lb. plantarum BG33, previously isolated from traditional Turkish Tulum cheese and selected on the basis of its proven technological capability as adjunct culture in cheese production by Dr. M. Akcelik (Biology Department, Faculty of Science, Ankara University), were used as adjunct to the starter culture in white-brined cheese production in this study. It was cultivated routinely in MRS broth (De Man, Rogosa & Sharpe) (Merck, Germany) at 35-37 °C for 18-24 h and subcultivated twice in sterile reconstituted skim milk before use in white-brined cheese production. The synthesis of plantaricin-like bacteriocin (400 AU mL^{-1}) with low heat stability but high resistancy to lipase by *Lb. plantarum* BG33, was defined in the previous study,

including its antimicrobial and probiotic potential (Uymaz et al 2011). The reference strain of *S. aureus* ATCC 6538 was cultivated in tryptic soy broth (TSB; Merck, Germany) at 35-37 °C for 18-24 h and subcultivated twice in sterile reconstituted skim milk before use in white-brined cheese production. Frozen glycerol stock of each strain was made in sterile reconstituted skim milk supplemented with 30% glycerol and maintained at 80 °C. The initial number of strains used to inoculate pasteurized cow milk was determined by streaking on the appropriate media incubated at 35-37 °C for 18-24 h.

2.2. Cheese production

To study the antimicrobial ability of BLIS-producing *Lb. plantarum* BG33 to control *S. aureus* during production and ripening of white-brined cheeses, two independent vats of cheeses for each trial were conducted. Cheese production was carried out as given at Figure 1. White-brined cheeses were experimentally prepared from pasteurized cow's milk (at 75-76 °C for 1 min). After warming pasteurized milk to 32-34 °C, 0.02% $CaCl_2$ (Merck, Germany), commercial mesophilic homofermentative lactic acid bacteria (LAB) (mixture of *Lactococcus lactis* subsp.



*, Vat no:1 and vat no:2 were inoculated with *S. aureus* to the mean level of 6.243 log MPN mL^{-1}; **, steps of *S. aureus* counts (just after addition of *S. aureus* culture, coagulum cutting, pressing and moulding, and packaging-brining) and on the 1st, 6th, 18th, 25th, 39th, 59th, 80th, and 90th days of ripening.

Figure 1- The flow diagram of white-brined cheese production with adjunct culture, including the steps of *S. aureus* counts in this study

lactis and *Lc. lactis* subsp. *cremoris*, CHR Hansen R-708, Denmark) was added to the milk in each vat. Since vat no: 1 was served as control to determine the effect of production procedures for white-brined cheese on the growth of *S. aureus* added to milk at the start of the process, adjunct culture of *Lb. plantarum* BG33 was only added to the vat no: 2. In other words; control cheese from pasteurized milk was made without any LAB culture, just with *S. aureus* in vat no: 1. Then, vat no: 1 and vat no: 2 were inoculated with *S. aureus* to a final concentration of 6.243 log MPN mL^{-1}. Next, liquid rennet (CHR Hansen Naturen® Mandra 175, 175 IMCU mL^{-1}, Denmark) was used as a rate of 14 mL 100 L^{-1} milk to obtain coagulum within 90 min. After pressing, the cheese masses were divided into blocks of about 7x7x7 cm^3 and these blocks were salted in brine (13% NaCl for 12-18 h at 18-20 °C). The brined blocks were then placed in tinned cans filled with 10% NaCl and ripened at 12-15 °C for 90 days.

2.3. Staphylococcus aureus counts

In each trial, all cheeses were sampled at the production steps of just after addition of *S. aureus* culture, coagulum cutting, pressing-moulding, and packaging-brining and at the 1^{st}, 6^{th}, 18^{th}, 25^{th}, 39^{th}, 59^{th}, 80^{th}, and 90^{th} days of ripening. The counts of *S. aureus* were monitored by BAM's recommended 5-tube MPN method. Twenty five grams of each sample were mixed in sterile plastic bag for 1 min with 225 mL of 0.1% Butterfield's phosphate buffer in stomacher (Stomacher 400, the UK). One mL portions of decimal dilutions of each sample homogenate was inoculated into 5 tubes of tryptic soy broth (TSB) (Merck, Germany) containing 10% NaCl and 1% sodium pyruvate (Merck, Germany) and these tubes were incubated at 35-37 °C for 48 h. One loopful from each tube showing growth (turbidity) was spreaded onto the surface of prepared Petri plates on duplicate with Baird-Parker agar (Merck, Germany) and all plates were incubated at 37 °C for 48 h. At least 1 colony suspected to be *S. aureus* from each plate was transferred to TSB and was confirmed for *S. aureus* by polymerase chain reaction (PCR) amplification of a 400 bp region of the *nuc* gene.

2.4. PCR confirmation of S. aureus

The DNA isolation was performed as in the study of Mercanoglu Taban & Aytac (2009) with a high-pure PCR template preparation-HPPTP kit (Roche, Germany). The resulting template DNAs were subjected to PCR. Each PCR contained 4 mM $MgCl_2$ (with 1×PCR buffer, containing 10 mM Tris, 50 mM KCl, pH 8.3) (Roche, Germany), 200 µM of dNTP mix, each PCR primer at a concentration of 0.4 µM [based on the sequence of thermostable nuclease gene (*nuc*), F166: (5′- AGT TCA GCA AAT GCA TCA CA-3′) and R565: (5′-TAG CCA AGC CTT GAC GAA CT-3′) Cremonesi et al (2005)] (Roche, Germany), 0.04 µM 5 U μL^{-1} FastStart Taq DNA polymerase (Roche, Germany), and 3 µL target DNA. The final volume was adjusted to 50 µL by adding sterile ultrapure water. DNA amplification was performed in Primus 96 thermal cycler (THE-MWG, Germany) using the following conditions: initial denaturation for 5 min at 95 °C followed by 35 cycles of denaturation (95 °C for 30 s), annealing (56 °C for 30 s), and extension (72 °C for 30 s). A final extension step (72 °C for 7 min) was performed after the completion of the cycles. As positive control, PCRs containing template DNA extracted from the reference strain *S. aureus* ATCC 6538 was carried out. Some PCRs received ultrapure water instead of template DNA to provide negative control. Aliquots of the PCR products, along with a 100-bp GeneRuler DNA ladder plus (ready-to-use, Fermentas, Lithuania), were loaded into 1% agarose gel (Sigma-Aldrich, the USA) containing ethidium bromide (1 mg mL^{-1}-Invitrogen, the USA) and submitted to electrophoresis in Trisborate EDTA buffer for 40 min at 125 V. The amplified DNA fragments were visualized with InGenius gel visualization and analysis system (Syngene, the UK). The expected size of the *nuc* PCR product is 400 bp.

2.5. Statistical analysis

Comparison of means by "T test, Mann-Whitney, one way ANOVA, and Kruskal-Wallis" tests were performed using SPSS 11.5 program (SPSS Inc., the USA). Statistically significant comparative results are achieved when the significance level was $P<0.01$.

3. Results and Discussion

Pasteurized milk in vat no: 1 and vat no: 2 were inoculated with *S. aureus* to the mean level of 6.243 log MPN mL^{-1}. *S. aureus* count in pasteurized milk inoculated with 1% adjunct culture of *Lb. plantarum* BG33 in vat no: 2 was firstly increased only 0.4 log unit from pasteurized milk to 1-day-old cheese and then reduced by 0.9 log unit (to the mean level of 5.309 log MPN mL^{-1}) on the 18th day (432 hours) of ripening whereas it was increased 0.7 and 0.3 log units from pasteurized milk to 1-day-old and to 18 days (432 hours) of ripened control group cheeses, respectively. After 39 days (936 hours) of ripening, *Lb. plantarum* BG33 lowered *S. aureus* count by 1.9 log units with respect to control group cheeses in which it was lowered by only 0.5 log units (Figure 2). According to the analysis of variance, *S. aureus* counts in white-brined cheese were influenced ($P<0.01$) by addition of adjunct culture of *Lb. plantarum* BG33 during the ripening period. This is an important reduction unit when it is considered that there is always a risk of enterotoxin accumulation at high levels of *S. aureus* contamination in foods (Lindqvist et al 2002; Akineden et al 2008; Mercanoglu Taban et al 2017).

According to the results obtained in this study, the amount of plantaricin-like bacteriocin produced by *Lb. plantarum* BG33 was sufficient enough for the inhibition of high levels of *S. aureus* cells present in white-brined cheeses by 1.9 log units although Abdalla et al (1993) concluded that *S. aureus* shows reduced sensitivity to bacteriocins in food matrices. Sarantinopoulos et al (2002) also concluded that the complex environment of Feta cheese, which is very similar to white-brined cheese, thoroughly interferes with bacteriocin production levels of bacteriocinogenic starter or co-cultures and there is no guarantee for their in situ antimicrobial efficiency. This is also confirmed by the study of Uymaz et al (2011) who demonstrated a greater and broad inhibitory activity of *Lb. plantarum* BG33 against all the tested indicator strains, including *S. aureus*, by the agar overlay assays. In addition, it might be considered that the antimicrobial effect of this adjunct culture might show more inhibitory effect than the effect obtained in this study if the milk was contaminated with lower levels of *S. aureus*. Therefore; the high counts of *S. aureus* (mean level of 4.380 log MPN mL^{-1}) in white-brined cheeses produced by autochthonous probiotic strain

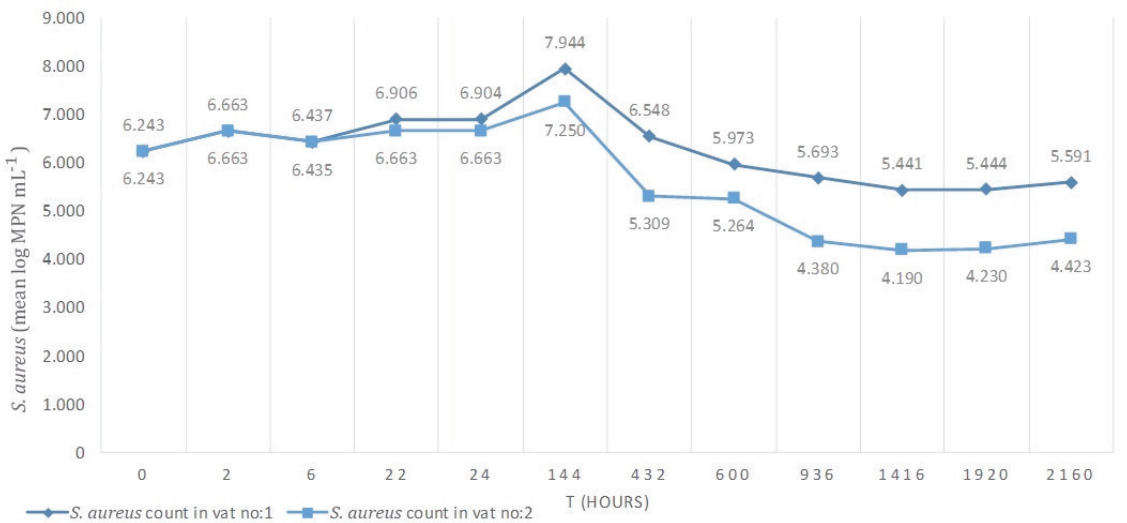


Figure 2- Survival of *S. aureus* (mean log MPN mL^{-1}) in control group cheeses (vat no: 1) and in white-brined cheeses produced with 1% adjunct culture of *Lb. plantarum* BG33 (vat no: 2)

of *Lb. plantarum* even at the 39th days (936 hours) of ripening can be based on the high inoculum level which was several log units above the levels that could be expected in naturally contaminated milk.

Beyond the direct use of bacteriocins as functional ingredients for the biopreservation of cheeses, nisin-producing lactococci have been of interest in the development of protective starter or adjunct cultures for cheese production due to their broad inhibitory activity. Therefore, most of the studies on the use of bacteriocin-producing cultures during cheese production related to them for the retardation of late gas blowing in Swiss style cheeses (O'Sullivan et al 2002). As an example; although Abdalla et al (1993) showed that *S. aureus* was not inhibited by nisin during production of white-brined cheeses from pasteurized milks and Cintas et al (1998) reported a very scarce inhibition of *S. aureus* by nisin A and pediocin PA-1, nisin-producing *Lc. lactis* ESI 515 which was also used as adjunct culture in cheese production was found to lower *S. aureus* count by 0.64 log units on the 30th day of cheese ripening (Rodriguez et al 2005), but a complete elimination of this foodborne pathogen was only achieved when nisin was added to process cheese spreads (Zottola et al 1994). In other words, Zottola et al (1994) showed significant reductions in numbers of *Clostridium sporogenes*, *Listeria monocytogenes*, and *S. aureus* when they used nisin-producing transconjugants of *Lc. lactis* ssp. *cremoris* JS102 and *Lc. lactis* ssp. *lactis* NCDO 1404 as starters in Cheddar cheese production. On the other hand in our study, 1.9 log unit reduction on *S. aureus* count was achieved by using adjunct culture of *Lb. plantarum* BG33 in white-brined cheese on the 39th days of ripening which was far beyond better than the reduction units obtained by transformant strain *Lc. lactis* CL1 of Rodriguez et al (2005) and by nisin-producing *Lc. lactis* TAB 50 of Rodriguez et al (2000) since these cultures showed 0.98 and 0.82 log units reduction on *S. aureus* counts in cheeses only after 30 days of ripening, respectively.

Likewise in our study, Rodriguez et al (2000) found that *S. aureus* count was firstly increased from pasteurized milk to 1-day-old semi-hard cheese, and then was decreased. On the contrary, El-Kholy et al (2014) evaluate the inhibition capacity of probiotic strains of *Lb. acidophilus* La-5 and *Bifidobacterium longum* ATCC 15707 on the growth of *S. aureus* and *Escherichia coli* O157:H7 during Domiati cheese production and storage and found that *Lb. acidophilus* La-5 reduced *S. aureus* and *E. coli* O157: H7 populations in Domiati cheese by about 3 and 1.88 logs after 14 days of storage, respectively, whereas *B. longum* ATCC15707 reduced *S. aureus* and *E. coli* O157: H7 populations in cheese by about 1.7 and 0.88 logs after 14 days of storage, respectively, compared with the control cheeses. Hence, we also showed almost the same reduction levels of *S. aureus* with the probiotic culture of *Lb. plantarum* BG33 in our study.

4. Conclusions

Considering the consumers' current demand for zero tolerance concerning the risk for foodborne pathogen contamination in dairy products, the use of antistaphylococcal starter cultures or adjunct cultures as an alternative to chemical additives in cheese production is a point of crucial importance both in respect of dairy technology as well as food safety. Therefore in this study, the use of BLIS-producing *Lb. plantarum* BG33 that was previously proven to have technological capability as adjunct culture in cheese production, to control growth and survival of *S. aureus* during white-brined cheese production and ripening was investigated. This study demonstrates the potential application of plantaricin-like bacteriocin-producing autochthonous probiotic strain of *Lb. plantarum* BG33 as adjunct culture in safe cheese production which is of crucial importance. Although this strain can slightly inhibit *S. aureus* growth in white-brined cheeses, due to the risk of enterotoxin production in cheese and of subsequent human intoxication, its inhibition potential of the expression of enterotoxin genes of *S. aureus* and antimicrobial potential in the control of other foodborne pathogens can also further be investigated.

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Resistance and Tolerance of Commercial Onion Cultivars to Stem and Bulb Nematode, *Ditylenchus dipsaci*

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ABSTRACT

Nematode resistance and tolerance reactions of 28 onion cultivars grown commercially in Turkey to stem and bulb nematode were studied at 20 °C, 70% RH and 16:8 h L:D photoperiod in growth chamber with 10 replications and at 27±4 °C and 16:8 h L:D photoperiod in greenhouse with 3 replications, respectively. *Ditylenchus dipsaci* multiplied in all cultivars tested. The lowest multiplication was determined in cv. Valenciana from Atatürk Horticultural Central Research Institute with 91 nematodes/pot and a multiplication rate of 0.5. Plant height of cultivars were significantly different in the first tolerance experiment with having an average plant hight of 33.5 and 103.1 mm for inoculated and inoculated plants, respectively ($P<0.05$). Onion shoot diameter was statistically greater in nematode inoculated plants with 7.4 mm than inoculated plants with 6.0 mm in second tolerance experiment ($P<0.05$). Plant weight was not found different in both tolerance experiments with nematode inoculation. There was not any statistically difference among cultivars for plant height, plant diameter, plant weight and nematode multiplication in the experiments. Plant diameter for cv. Betapanko in first tolerance experiment and plant height for the Banko type onion in the second tolerance experiment sustained significant negative correlations with nematode numbers. Plant weight for cv. Biotek Boran in second experiment, plant height for cv. Taraz in second experiment and, plant diameter and plant weight for cv. Taraz in first tolerance experiment sustained significant positive correlations with nematode multiplication. It could be recommended that Valenciana could lower nematode multiplication and Biotek Boran and Taraz could maintain a better plant development in nematode infested onion growing areas.

Keywords: *Ditylenchus dipsaci*; Stem and bulb nematode; Onion; Resistance; Tolerance

1. Introduction

Ditylenchus dipsaci originally described from *Dipsacus fullonum* (Kühn 1857). It has 30 biological races on more than 500 hosts (Sturhan & Brzeski 1991).

The onion race of *D. dipsaci* has a wide host range, though it mostly infects onion, garlic, pea and bean (Janssen 1994). Control of the nematode using host crop rotation under field conditions is difficult due to the variation in host preferences of different races. Because of the environmental concerns; use of the nematicides is not preferable and economical under field conditions. There are no registered nematicides for *D. dipsaci* control in onion in Turkey. Dipping onion bulbs in hot water

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at 44-45 °C for 3 h before sowing provides a level of control the nematode (Bridge & Hunt 1986). Early sowing of onion plants were shown to decrease the nematode population and plant damage (Mennan 2005).

Resistance to nematodes is defined as a genetic mechanism of plant to prevent nematode infection and/or reproduction. Besides, tolerant plants provides high yield under nematode multiplication (Cook & Evans 1987). Both mechanisms are useful for producers in nematode infested areas. Bergquist & Riedei (1972) reported lack of any available resistance source of onion to *D. dipsaci*. However, there is some hope of some results providing lower nematode multiplication under field conditions (Yavuzaslanoglu et al 2015). Some resistant oat, rye, bean and clover (Plowright et al 2002) showed some advantages in crop rotations given the nematodes wider host range. Yet, there are no studies reporting the tolerance of onion cultivars to *D. dipsaci*.

Aim of this study was to investigate resistance and tolerance reactions of some onion cultivars grown commercially in Turkey to *D. dipsaci* under controlled growth chamber and greenhouse conditions.

2. Material and Methods

2.1. Plant material and nematode source

Onion cultivars were provided by commercial seed suppliers in Turkey and Atatürk Horticultural Central Research Institute (AHCRI), Yalova, Turkey. Total of 28 cultivars were tested for *D. dipsaci* resistance a in growth chamber and for tolerance in a greenhouse. The population of *Ditylenchus dipsaci* used was originally collected from garlic in Karaman Province (N: 37.111592, E: 33.112628), Turkey. The nematodes were cultured on sterile carrot discs (Kühnhold et al 2006) and extracted in tap water.

2.2. Growth chamber experiments

The experiments were conducted in a growth chamber at 20 °C, 70% RH and 16:8 h L:D photoperiod. Plants were grown in 7x8 cm diameter plastic pots containing a mixture of sterile sand, field soil and organic matter (45: 45: 10 by weight) and watered as needed. Ten replicates were used for each cultivar tested being totally 280 plant.

An inoculum of 200 nematodes in 10 µL of 1% carboxymethylcellulose (Kühnhold et al 2006) was applied between two leaves of each onion plant at 4-5 weeks old. The plants were grown for 6 weeks after inoculation. The nematodes were extracted from the plants overnight (Hallmann & Subbotin 2018) and counted. Nematode multiplication rate was calculated dividing the final number by 200, i.e. the number applied per plant.

2.3. Greenhouse experiments

Greenhouse experiment was conducted with same set of cultivars used in the growth chamber evaluations. The tolerance testing in the greenhouse consisted of three replicates in 7x8 cm plastic pots using 70% and 30% sand and field soil respectively. Organic liquid fertilizer (Biovin, Konya Şeker, Konya, Turkey) with a ratio of 0.6 mL ha^{-1} was applied one week after planting. The onions were planted on 14 April 2017 and harvested on 4 September 2017 as two sets of the material. The temperature in the greenhouse was 27±4 °C during experiment. Nematodes were applied (200 nematodes in 1 mL tap water) into soil around the plant in each pot 5 weeks following planting. Plant height, plant diameter and fresh weight were recorded at the end of the experiment. Nematodes were extracted and counted from the plant and soil from each pot (Hallmann & Subbotin 2018).

2.4. Statistical analysis

Data on plants and nematodes for each cultivar was analyzed using ANOVA and LSDs calculated using all pairs Tukey's HSD test. Plant height, plant diameter, plant weight and nematode numbers for each cultivar in the study was compared using multivariate test. Statistical analyses were applied using JUMP 5.0.1a. Program (JMP 2009).

3. Results and Discussion

3.1. Growth chamber experiments

There were significant differences ($P<0.05$) in the nematode multiplication rates of the cultivars tested. Cultivars fell into four LSD groups (Table 1). The lowest nematode multiplication was in cv. Valenciana ex AHCRI with mean of 91 nematodes/plant (range 0-600) and multiplication rate of 0.5 (0-3). Other cultivars with low multiplication rates were Burgaz10 ex MTN Seed, Kantartopu, Naz, Seyhan, Panko, Banko type onion, Aki, Gence, Akgün12, Sampiyon, Hazar, Banka, Early White Grano and Valenciana ex MTN Seed with 216-776 nematodes/plant and multiplication rates of 1-3.8 (Table 1).

Table 1- Plant material and their origin and times tested with nematode numbers multiplied and multiplication rate (MR) in the resistance experiment

Genotype	*Origin*	*Times screened*	*D. dipsaci plant*$^{-1}$	*MR*	*LSD Group*
Kantartopu	AHCRI	2	272±833 (0-1280)	1.3±4.1 (0-6.4)	c
Betapanko	AHCRI	4	1108±493 (0-14900)	5.5±2.4 (0-74.5)	c
Valenciana	AHCRI	2	91±833 (0-600)	0.5±4.1 (0-3.0)	c
Pan88	AHCRI	3	1038±589 (0-8980)	5.2±2.9 (0-44.9)	c
Akgün12	AHCRI	1	424±949 (0-1960)	2.1±4.7 (0-9.8)	c
Banka	KUCUK CIFTLIK SEED©	2	618±802 (0-4120)	3.1±4.0 (0-20.6)	c
Daytona	INTFA AGRICULTURE©	2	1606±802 (0-8580)	8.0±4.0 (0-42.9)	bc
Banko type onion	INTFA AGRICULTURE©	1	374±949 (0-3460)	1.9±4.7 (0-17.3)	c
Panko	INTFA AGRICULTURE©	1	364±949 (0-2520)	1.8±4.7 (0-12.6)	c
Safa Karbeyazi	INTFA AGRICULTURE©	2	1018±905 (0-6580)	5.1±4.5 (0-32.9)	bc
Biotek Boran	INTFA AGRICULTURE©	1	836±949 (0-2420)	4.2±4.7 (0-12.1)	bc
Aki	INTFA AGRICULTURE©	2	383±802(0-3020)	1.9±4.0 (0-15.1)	c
Beyaz Bilek	INTFA AGRICULTURE©	2	2954±802 (0-21660)	14.8±4.0 (0-8.6)	bc
Ersoy	INTFA AGRICULTURE©	1	744±949 (0-3260)	3.7±4.7 (0-16.3)	bc
Seyhan	MTN SEED©	1	292±949 (0-1940)	1.5±4.7 (0-9.7)	c
Hazar	MTN SEED©	2	586±802 (0-7560)	2.9±4.0 (0-37.8)	c
Metan88	MTN SEED©	2	951±802 (0-4560)	4.8±4.0 (0-22.8)	bc
Burgaz10	MTN SEED©	1	216±949 (0-1040)	1.1±4.7 (0-5.2)	c
Karbeyazi	MTN SEED©	2	1412±867 (0-15340)	7.1±4.3 (0-76.7)	bc
Valenciana	MTN SEED©	2	776±802 (0-3460)	3.9±4.0 (0-17.3)	c
Gence	MTN SEED©	1	416±949 (0-1920)	2.1±4.7 (0-9.6)	c
Taraz	MTN SEED©	2	1130±802 (0-7840)	5.7±4.0 (0-39.2)	bc
MT300	MTN SEED©	2	2006±802 (0-23580)	10.1±4.0 (0-117.9)	bc
Sampiyon	MTN SEED©	1	424±949 (0-2680)	2.1±4.7 (0-13.4)	c
Early White Grano	MTN SEED©	1	624±949 (0-2620)	3.1±4.7 (0-13.1)	c
Naz	MTN SEED©	2	283±802 (0-2160)	1.4±4.0 (0-10.8)	c
Balkan	BALIKESIR SEED©	1	7330±1501 (0-4720)	36.7±7.5 (0-123.6)	ab
Burgaz10	PASA SEED©	1	13650±1501(0-26800)	68.3±7.5 (0-134.0)	a

Standard error was given as ± of mean, range of nematode numbers and multiplication rate was given in parenthesis. AHCRI, Atatürk Horticulture Central Research Institute, Yalova, Turkey; MTN Seed, Balıkesir, Turkey; Küçük Çiftlik Seed, Balıkesir, Turkey; İntfa Agriculture, Konya, Turkey; Balıkesir Seed, Balıkesir, Turkey; Paşa Seed, Balıkesir, Turkey

Mean nematode numbers of cvs Pan88 and Betapanko used for comparison were 1038 and 1108 nematodes/plant and multiplication rates of 5.2 and 5.5, respectively, and were included in the c LSD group.

Moderate nematode multiplication rates were found in cvs Ersoy, Biotek Boran, Metan88, Safa Karbeyazi, Taraz, Daytona, MT300 and Beyaz Bilek. These were included in the bc LSD group with multiplication rates of 3.7-14.8 (i.e., 744-2954 nematodes plant^{-1}). High multiplication rates were found in cvs Balkan and Burgaz10 ex Pasa Seed, 36.7 and 68.3 (7330 and 13650 nematodes plant^{-1}), respectively, and were included in the ab and a LSD groups.

Resistance studies for *D. dipsaci* are limited, possibility due to the wide range host of *D. dipsaci*, and resistance studies are mostly for commercial crops of the countries where this nematode is problem. Toynbee-Clarke & Bond (1970), Griffin & Waite (1971), Kühnhold et al (2006) and Mwaura et al (2015) conducted resistance testing studies on red clover, alfalfa, sugar beet and potato, respectively.

Onion has a large genome over which many repeats distributed (Fajkus et al 2016). It is difficult to identify resistance, and undertake resistance breeding onion, due to the complex nature of its genome and external pollination characteristics.

Pang et al (2009) identified cultivars resistant to *Pratylenchus penetrans* and *Meloidogyne hapla* among onions grown in Idaho. In the current study, none of the onion cultivars tested was completely resistant to *D. dipsaci*. However, low nematode multiplication was observed with cv. Valenciana from both AHCRI and MTN Seed, and some other cultivars. This is consistent with the degree of resistance already shown for cv. Valenciana ex AHCRI under field conditions by Yavuzaslanoglu et al (2015). Ogbuji (1979) conducted studies on onions with different skin color to determine *D. dipsaci* penetration rate. Lower nematode penetration and bloating in red skinned onion was recorded in both greenhouse and field studies. Given that cv. Valenciana has red skin, the findings of Ogbuji (1979) are consistent with those of the current study.

3.2. Greenhouse experiments

Plant height, plant diameter and weight were statistically significantly different between the two experiments. Therefore the data from the two experiments were analyzed separately. Mean plant height was 67.4 mm in the first experiment, and 40.0 mm in the second experiment. Plant diameter and weight was 5.8 mm and 0.31 g, 6.7 mm and 0.44 g for the first and second experiment respectively. While plant height was higher in the first experiment, plant diameter and weight was lower than in the second experiment. Mean nematodes pot^{-1} was 18 in the first experiment, and 110 in the second experiment (inoculated plants only), with mean multiplication rates of 0.008 and 0.6, respectively (Table 2, 3).

Mean plant height was significantly different between nematode inoculated and uninoculated plants in the first experiment; mean plant height was 34.0 mm (10.0-55.0 mm) in nematode inoculated plants, and 103.1 mm (30.0-180.0 mm) in uninoculated plants. There was not any statistical difference in the second experiment (Table 2, 3).

Plant diameter was not affected by nematodes in first experiment but it was significantly differed in the second experiment where it was observed a mean of 7.4 mm (1.0-15 mm) with inoculation and 6.0 mm (1.0-15 mm) without inoculation ($P<0.05$). Plant weight was not significantly different by nematode treatment in both experiments. There was no difference among cultivars by nematode treatment for plant height, diameter and weight in both experiments (Table 2, 3).

Plant height, plant diameter and plant fresh weight was used for tolerance evaluation in this study. Pang et al (2009) used plant dry weight and Ibrahim (2010) used plant height, number of leaves and bulb weight for evaluation onion yield. Islam et al (2007) reported a positive correlation between onion plant growth parameters including plant height, number of leaves and plant weight and bulb yield to evaluate tolerance under greenhouse conditions.

Table 2- Plant height (mm), plant diameter (mm), plant weight (g) and nematode numbers in first greenhouse tolerance experiment

Cultivar	*Origin*	*Plant height*		*Plant diameter*		*Plant weight*		*D. dipsaci*	
		+	-	+	-	+	-	+	-
Kantartopu	AHCRI	40.3	105.0	8.3	4.7	0.69	0.39	13.0	0.0
Betapanko	MTN seed	29.0	106.7	5.0	7.0	0.24	0.15	27.0	0.0
Valenciana	AHCRI	30.3	103.3	4.3	5.7	0.13	0.17	40.0	0.0
Pan88	MTN seed	20.7	70.0	2.0	5.0	0.03	0.08	0.0	0.0
Akgün12	MTN seed	33.3	140.0	5.0	7.7	0.30	0.58	27.0	0.0
Banka	İntfa agriculture	39.3	81.7	5.7	5.7	0.34	0.12	7.0	0.0
Daytona	İntfa agriculture	34.3	85.0	4.0	5.7	0.09	0.16	13.0	0.0
Banko type onion	İntfa agriculture	21.7	125.0	3.7	7.0	0.06	0.61	00	0.0
Panko	MTN seed	35.3	96.7	5.3	4.0	0.16	0.16	0.0	0.0
Safa Karbeyazi	MTN seed	39.0	105.0	5.6	8.0	0.31	0.22	13.0	0.0
Biotek Boran	AHCRI	32.0	111.7	6.0	6.0	0.19	0.29	40.0	0.0
Aki	MTN seed	36.5	123.3	6.0	5.7	0.31	0.37	50.0	0.0
Beyaz Bilek	Küçük Ciftlik seed	33.7	123.5	6.3	4.0	0.55	0.29	0.0	0.0
Ersoy	MTN seed	29.7	136.7	9.3	5.0	0.87	0.43	0.0	0.0
Seyhan	İntfa agriculture	32.3	75.0	7.3	6.0	0.45	0.60	13.0	0.0
Hazar	MTN seed	41.3	133.3	9.3	7.3	0.95	1.13	13.0	0.0
Metan88	İntfa agriculture	40.0	86.7	10.7	6.3	0.43	0.47	13.0	0.0
Burgaz10	MTN seed	33.7	110.0	5.3	5.0	0.23	0.29	0.0	0.0
Karbeyazi	İntfa agriculture	25.5	78.3	6.3	5.7	0.10	0.27	40.0	0.0
Valenciana	AHCRI	24.7	105.0	2.5	4.5	0.08	0.07	47.0	0.0
Gence	AHCRI	40.7	86.7	6.0	6.3	0.21	0.30	0.0	0.0
Taraz	MTN seed	31.3	65.0	7.0	4.7	0.22	0.06	40.0	0.0
MT300	MTN seed	27.0	96.7	4.7	4.3	0.10	0.24	40.0	0.0
Sampiyon	İntfa agriculture	38.0	116.7	5.7	6.3	0.22	0.40	0.0	0.0
Early White Grano	MTN seed	33.3	165.0	6.3	8.0	0.40	0.30	0.0	0.0
Naz	İntfa agriculture	36.7	84.0	7.7	2.7	0.38	0.14	7.0	0.0
Balkan	Balıkesir seed	39.5	102.7	7.5	5.3	0.32	0.25	70.0	0.0
Burgaz10	Paşa seed	39.0	140.0	7.0	7.0	0.48	0.07	27.0	0.0

(+; nematode inoculated, -; nematode un inoculated); AHCRI, Atatürk Horticulture Central Research Institute, Yalova, Turkey; MTN seed, Balıkesir, Turkey; Küçük Çiftlik seed, Balıkesir, Turkey; İntfa agriculture, Konya, Turkey; Balıkesir seed, Balıkesir, Turkey; Paşa seed, Balıkesir, Turkey

The common symptoms of the stem and bulb nematode infection are swelling of the plant tissue and misshapen, dwarf plants (Sikora & Fernandez 2005). In the tolerance experiment, symptoms of nematode infection were observed and associated with significantly reduced plant height and enlarged plant diameter.

For cv. Betapanko in the first experiment, there was a statistically significantly negative correlation between nematode number and plant diameter ($P<0.05$, $R= -0.82$), with plant diameter reduced 2.0 mm by nematode inoculation. There was no significant trend for the other plant parameters.

For cv. Banko type onion in the second experiment, there was as significantly negative correlation between nematode numbers and plant height ($P<0.05$, $R= 0.81$), with plant height reduced 5.7 mm by nematode inoculation.

Table 3- Plant height (mm), plant diameter (mm), plant weight (g) and nematode numbers in second greenhouse tolerance experiment

Cultivar	*Origin*	*Plant height*		*Plant diameter*		*Plant weight*		*D. dipsaci*	
		+	-	+	-	+	-	+	-
Kantartopu	AHCRI	48.5	45.0	11.0	4.5	1.05	0.33	120.0	0.0
Betapanko	MTN seed	42.0	43.0	5.7	5.3	0.22	0.25	80.0	0.0
Valenciana	AHCRI	37.0	48.7	5.0	4.7	0.11	0.26	240.0	0.0
Pan88	MTN seed	26.0	38.5	4.0	2.5	0.14	0.09	80.0	0.0
Akgün12	MTN seed	41.7	48.0	7.3	4.0	0.38	0.13	120.0	0.0
Banka	İntfa agriculture	38.0	43.3	7.0	6.0	0.31	0.26	180.0	0.0
Daytona	İntfa agriculture	30.0	42.0	5.0	7.0	0.25	0.70	100.0	0.0
Banko type onion	İntfa agriculture	31.7	37.3	5.0	5.0	0.13	0.21	27.0	0.0
Panko	MTN seed	42.5	47.5	8.0	5.0	0.55	0.27	180.0	0.0
Safa Karbeyazi	MTN seed	43.0	44.0	7.7	5.3	0.41	0.25	80.0	0.0
Biotek Boran	AHCRI	37.7	40.3	7.3	5.0	0.42	0.19	113.0	0.0
Aki	MTN seed	35.0	-	7.0	-	0.42	-	73.0	0.0
Beyaz Bilek	Küçük Ciftlik seed	38.0	32.0	9.0	5.5	0.84	0.41	30.0	0.0
Ersoy	MTN seed	40.0	34.0	10.0	9.0	0.74	0.65	20.0	0.0
Seyhan	İntfa agriculture	38.3	19.5	7.3	4.0	0.35	0.03	147.0	0.0
Hazar	MTN seed	32.0	39.3	6.5	3.0	0.59	0.16	200.0	0.0
Metan88	İntfa agriculture	38.5	33.0	5.5	6.0	0.10	0.39	150.0	0.0
Burgaz10	MTN seed	30.3	39.0	3.0	3.5	0.07	0.11	213.0	0.0
Karbeyazi	İntfa agriculture	-	33.5	-	9.0	-	0.79	-	0.0
Valenciana	AHCRI	42.3	39.3	6.7	7.3	0.42	0.50	80.0	0.0
Gence	AHCRI	41.3	39.7	7.7	7.3	0.38	0.51	73.0	0.0
Taraz	MTN seed	46.7	32.0	9.0	8.0	0.68	0.58	167.0	0.0
MT300	MTN seed	45.0	38.0	5.7	7.5	0.47	0.57	140.0	0.0
Sampiyon	İntfa agriculture	55.5	51.0	10.0	8.0	1.12	0.72	110.0	0.0
Early White Grano	MTN seed	39.7	44.0	10.7	9.5	1.36	0.86	73.0	0.0
Naz	İntfa agriculture	43.7	35.5	8.3	8.5	0.66	0.38	47.0	0.0
Balkan	Balıkesir seed	53.0	42.0	10.3	5.0	0.58	0.11	107.0	0.0
Burgaz10	Paşa seed	46.0	39.5	9.0	7.5	0.94	0.54	230.0	0.0

(+; nematode inoculated, -; nematode un inoculated). AHCRI, Atatürk Horticulture Central Research Institute, Yalova, Turkey; MTN seed, Balıkesir, Turkey; Küçük Çiftlik seed, Balıkesir, Turkey; İntfa agriculture, Konya, Turkey; Balıkesir seed, Balıkesir, Turkey; Paşa seed, Balıkesir, Turkey

For cv. Taraz there were significant positive correlations between nematode numbers and plant height ($P<0.05$, $R= 0.98$) in the second experiment and between nematode numbers and plant diameter ($P<0.05$, $R= 0.90$) and weight ($P<0.05$, $R= 0.98$) in the first experiment. Plant height, diameter and weight increased 14.7 mm, 2.3 mm and 0.16 g with nematode inoculation, respectively (Table 2, 3).

For cv. Biotek Boran, in the second experiment, there was a statistically significantly positive correlation between nematode numbers and plant weight ($P<0.05$, $R= 0.79$), with plant weight increased 0.22 g by nematode inoculation.

Significant relationship between nematode numbers and plant growth indicated that plant yield could be affected by nematode inoculation. Significant relationship between nematode numbers and plant growth were found for cvs Betapanko, Banko type onion, Taraz and Biotek Boran. While cvs Betapanko and Banko plant growth parameters

showed negative correlation to nematode numbers, plant growth in cvs Taraz and Biotek Boran was positively correlated to nematode numbers. Increased plant yield with increasing nematode numbers is considered to be an indicator of tolerance (Trudgill 1991).

A negative relationship between cv. Betapanko bulb yield and initial nematode populations was found under field conditions by Yavuzaslanoglu et al (2015), indicating the intolerance of this cultivar and its bulb yield decreased by up to 13% with nematicide treatment, which is consistent with the findings of the current study.

4. Conclusions

The main outcome of the study was that cv. Valenciana lowered *D. dipsaci* multiplication and the susceptible cultivars, Taraz and Biotek Boran, tolerated nematode infection with strong plant growth.

Results provide practical and ecologically friendly control option to growers by planting suitable cultivars in the nematode infested areas.

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House Dust Mite Species in Ordu Province, Turkey

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ABSTRACT

House dust mites inhabit human houses, causing allergies and respiratory disease. Of them pyroglyphid mites produce at least 23 allergen groups, affecting millions of people worldwide. We determined the house dust mite fauna in the city of Ordu situated on the Black Sea coast of Turkey. Dust samples were taken from a total of 53 houses in 2013-2015. The mite family Pyroglyphidae had the highest abundance in our samples, followed by family Chortoglyphidae. The most common species were *Dermatophagoides pteronyssinus* (Trouessart) and *Dermatophagoides farinae* Hughes (Astigmata: Pyroglyphidae). These two mite species are main allergen-producing species throughout the world, occurring in all sampled houses in the city of Ordu.

Keywords: Acari; House dust mites; Allergy; *Dermatophagoides pteronyssinus*; *Dermatophagoides farinae*

1. Introduction

House dust contains many organic and inorganic materials causing allergies. The most important one is the dust mites (Fassio & Guagnini 2018). The common families of mites observed in house dust belong to the families Pyroglyphidae, Glycyphagidae and Acaridae (Kosik-Bogacka et al 2010). However, actually, the term "house dust mite (HDM)" is usually used for *Dermatophagoides pteronyssinus* (Trouessart), *D. farinae* Hughes, and *Euroglyphus maynei* (Cooreman) (Astigmata: Pyroglyphidae). Furthermore, the most common and effective HDM allergens are Der p, from *D. pteronyssinus* exposure and Der f, from *D. farinae* exposure (Vidal-Quist et al 2015). The allergenic features of HDMs are caused by their feces and their body tissues. Over time, mite feces and body-tissue residues from their fragmentation after death accumulate in carpets, fabric-covered furniture, fuzzy toys, mattresses, and pillows. These allergens remaining suspend in air for a time and mix with air taken into the respiratory track, thereby stimulating immune-system elements (Zeytun et al 2018).

HDMs were first suspected as a source of allergen in 1928 and have been recognized as an important cause of allergic disorders since 1964 (Voorhorst et al 1964). To date, numerous faunistic studies in many countries including Turkey have been conducted on HDMs that are thought to play a role in the pathogenesis of several allergic diseases, including allergic rhinitis, allergic asthma, allergic dermatitis, and allergic conjunctivitis (Ree

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et al 1997; Chew et al 1999; Mariana et al 2000; Nadchatram 2005; Boquete et al 2006; Henszel et al 2010; Kosik-Bogacka et al 2010; Solarz 2010; Catanghal & Paller 2012; Sun et al 2013; Sun et al 2014; Gill & Kaur 2014; Heikal 2015; Yu et al 2015; Ziyaei et al 2017). There are also reports on HDMs in several cities in Turkey: İzmir (Gülbahar 2003), Konya (Aldemir & Baykan 2004), Kütahya (Akdemir & Gürdal 2005; Akdemir & Soyucen 2009; Akdemir & Yılmaz 2009), Malatya (Atambay et al 2006), Afyon-Usak, Isparta-Kütahya-Denizli (Çiftçi et al 2006), Eskişehir (Doğan et al 2008), Samsun (Çelik 2009; Çelik & Ozman-Sullivan 2009), Kayseri (Hasgül 2011; Kılınçarslan 2012), Muş-Bitlis (Aykut et al 2013), five regions of Anatolia (Kalpaklioglu et al 1997). In Ordu province, there are no records of mites in house dusts except for Akyazı et al (2018). However, Akyazı et al (2018) studied the seasonal changes in the populations of the HDM in five houses in the Ordu central district between 2013 and 2015. In this research, the mite species were listed resulted from surveys carried out in 53 houses in Ordu province except for the five above-mentioned houses.

Ordu is situated on the Black Sea coast of Turkey, which has a very humid climate. HDMs thrive in warm, humid environments (Arlian 1992). High humidity in coastal cities can facilitate mould growth and proliferation causing a range of respiratory and dermatological allergies (Bornehag et al 2004). Thus, our general hypothesis is that Ordu, being a coastal city, has a rich HDM fauna. This study was carried out to survey HDM fauna of the city of Ordu in 2013-2015.

2. Material and Methods

2.1. Study area and houses

This study conducted in randomly selected 53 houses in the city of Ordu province situated on the Black Sea coast of Turkey (Figure 1).

The mean annual temperatures (±SD) were 15.6 (±6), 16.11 (±6) °C and 15.47 (±6) °C, the mean annual relative humidities (±SD) were 67.70 (±4) %, 69.80 (±4) % and 69.86 (±3) % and the total rainfall estimates (±SD) were 978.4 (±49), 985 (±54) mm and 1059 (±50) mm in 2013, 2014 and 2015 in Ordu province, respectively.

During the sampling period of the study, the mean monthly temperatures (±SD) were 22.9±2.0 °C, 24±2.0 °C and 23.7±2.0 °C, the mean monthly relative humidities (±SD) were 67.7±2%, 68.8±1% and 70.5±3%, the mean monthly rainfall estimates (±SD) were 64±26, 86±30 and 46.2±25 mm in 2013, 2014 and 2015, respectively. The annual and monthly temperature, relative humidity and rainfall estimates were obtained from Ordu Meteorological Station.

2.2. Dust collection methods

Dust samples were obtained between July and August of each year (2013-2015). Because, the dust mite population was generally higher during these months in Ordu (Akyazı et al 2018). House dust samples were taken from beds (pillows, quilts, sheets, and mattresses), carpets, floor of bedrooms and furniture, carpets and floor of living rooms in each home. Samples were collected with a portable vacuum cleaner (Rowenta RO582301, 2200 W-Silent Force Extreme) for 2 minutes per 1 m^2 (Ozman-Sullivan & Celik 2010).

A new bag for each vacuuming was used. In addition, subtracting hoses and mouthpieces of the vacuum cleaners were cleaned before each vacuuming to prevent any possible contamination. After each vacuuming, the dust bag was taken out,

Figure 1- A map showing the city of Ordu in Turkey (from Google Maps)

placed in a plastic bag and brought to the laboratory for analysis (Wassenaar 1988). Dust samples were stored in a refrigerator at 4 ºC to prevent proliferation of mites, and the samples were examined within 24h.

One dust sample per house was taken for analysis. A total of 53 dust samples were collected from randomly selected 53 houses during the study.

2.3. Extraction and preparation of mite specimens

Mites were isolated from 1 g fine dust sample by a wet-sieving method adapted from Natuhara (1989). The mites within the samples were isolated immediately. Isolated mite specimens were stored in 70% alcohol. Specimens were cleared in Lacto-phenol and mounted in Hoyer on microscope slides and dried for 5-7 days in an oven at 50 °C according to the method of Krantz & Walter (2009).

The mean number of mites per gram of dust, percentage and incidence rates of each species detected during the sampling period were calculated as follows (Yu et al 2015).

The mean number of mites per gram of dust= Total number of isolated mite/Number of mite positive house (Zeytun et al 2015)

Percentage of each species (*%= (Number of each mite species/Total number of isolated mite*)×100) (Yu et al 2015)

Incidence of each species (*%= (Number of positive house for each species/Total number of sampled houses*)×100) (Yu et al 2015)

2.4. Identification of mite specimens

Mite species were identified under a light microscope (Leica DM 2500, Heerbrugg, Switzerland) equipped with phase contrast. Identification of mites at the species level was performed using the available keys, some relevant books and papers such as Fain et al (1990); Zhang (2003); Nadchatram (2005); Colloff (2009); Krantz & Walter (2009); Solarz (2010); Solarz et al (2016). Mite specimens were deposited in the Mite Collection at the Ordu University, Agricultural Faculty, Plant Protection Department, Ordu, Turkey.

3. Results and Discussion

A total of 53 dust samples from 53 houses were collected during the study and all of samples were found to be mite-positive. Çelik (2009) in Samsun, Aykut et al (2013) in Bitlis-Muş and Zeytun et al (2016) in Erzincan houses also found all examined dust samples to be positive for the mites. In other cities, the mite-holding rates of houses were 57.66% in Konya (Aldemir & Baykan 2004), 57.5% in Hatay (Gülkan 2004), 34.38% in Bursa (Güleğen et al 2005), 46.3% in Malatya (Atambay et al 2006), 23.1% in western Anatolia (Afyon, Uşak, Isparta, Kütahya, and Denizli) (Çiftçi et al 2006), 74.49% in the Aegean Region (Budak & Özbilgin 1988), 16.67% in Eskisehir (Doğan et al 2008), 18.05% (Akdemir & Gürdal 2005) - 31.7% (Akdemir & Yılmaz 2009) in Kütahya, 56% in Muş (Hasköy) (Aykut & Yılmaz 2010), 39.47% in Kayseri (Hasgül 2011), 94.44% (Zeytun et al 2015) and 98.5% (Zeytun 2015) in Erzincan. Kosik-Bogacka et al (2010) identified dust mites in 30% of urban and 53% of rural samples in West Pomerania in northwestern Poland. The infestation rate of homes in Singapore (Chew et al 1999), India (Patiala City, Punjab) (Gill & Kaur 2014) and Xishuangbanna, a tropical rainforest region in Southwest China, (Yu et al 2015) were 97%, 88%, 97.5%, respectively.

During the study, a total of 694 mite specimens in various development stages were collected; 89.19% adults (60.09% females, 29.11% males), 9.51% tritonymphs, 1.15% protonymphs, and 0.14% larvae (Table 1). Mean number of the mites per gram was found as 13.09 mites with minimal 3 mites g^{-1} dust and maximal 48 mites g^{-1} dust.

During the study, a total of 11 different species were detected. Seven of which are identified to species level with respective of predominancy of *Dermatophagoides pteronyssinus* (508, 73.2%), *Dermatophagoides farinae* (136, 19.6%), *Chortoglyphus arcuatus* (27, 3.89%), *Glycyphagus domesticus* (7, 1.01%), *Euroglyphus maynei* (2, 0.29%), *Lepidoglyphus destructor* (2, 0.29%), *Haplochthonius simplex* (2, 0.29%). However, 10 specimens were identified only to the genus level

as *Dermatophagoides* sp. (7, 1.01%), *Rhizoglyphus* sp.1 (1, 0.14%), *Rhizoglyphus* sp. 2, (1, 0.14%) and *Tyrophagus* sp. (1, 0.14%). All of isolated mites belonged to 5 families and 8 genera. The family Pyroglyphidae (94.09%) occupied the highest percentage of the total amount of mites collected, followed by Chortoglyphidae (3.89%), Glycyphagidae (1.30%), Acaridae (0.43%), and Haplochthoniidae (0.29%) families, respectively (Table 1).

D. pteronyssinus (Figure 2) was detected to be the most common (92.45%-49/53) and predominant (73.2% of total mites) species in the houses. While many researchers detected *D. pteronyssinus* as the most common species in house dust samples around the world, its rate was variable in different cities or countries (Çiftçi et al 2004; Gülbahar et al 2004; Gülkan 2004; Güleğen et al 2005; Atambay et al 2006; Boquete et al 2006; Doğan et al 2008; Çelik 2009; Aykut & Yılmaz 2010; Aykut et al 2013; Zeytun et al 2016; Soleimani-Ahmadi et al 2017; Wahongan et al 2017; Zeytun et al 2017a; Ziyaei et al 2017; Dutra et al 2018; Goutam 2018; Kaur & Dhingra 2018; Navarro-Locsin & Lim-Jurado 2018; Shafique et al 2018). In contrast, the most common mites were *Tarsonemus* sp., *Blomia* sp. and *Acarus siro* in Kayseri (Kılınçarslan 2012), and *T. putrescentiae* in Kütahya (Akdemir & Gürdal 2005). While the mite-holding rates of houses in Kayseri was 39.47%, the rate of *Dermatophagoides* sp. was just 8.2% (Hasgül 2011). Moreover, the most common species was *Acarus siro* (55.55%) in Erzincan (Zeytun et al 2015). However,

Table 1- Mite species found in house dust samples in Ordu city (Black Sea cost, Turkey) in 2013-2015 (TN, Tritonymph; PN, Protonymph; L, Larva)

Species	*House number (n: 53)*	*Incidence in houses (%)*	*Number of isolated mites*						*Percentage (%)*
			♀	♂	*TN*	*PN*	*L*	*Total*	
Astigmata									
Pyroglyphidae	53	100.00	392	188	64	8	1	653	94.09
Dermatophagoides pteronyssinus (Trouessart)	49	92.45	339	129	38	1	1	508	73.20
Dermatophagoides farinae Hughes	35	66.04	49	54	26	7	0	136	19.60
Dermatophagoides sp.	2	3.77	2	5	0	0	0	7	1.01
Euroglyphus maynei (Cooreman)	2	3.77	2	0	0	0	0	2	0.29
Acaridae	3	5.66	2	0	1	0	0	3	0.43
Rhizoglyphus sp. 1	1	1.89	0	0	1	0	0	1	0.14
Rhizoglyphus sp. 2	1	1.89	1	0	0	0	0	1	0.14
Tyrophagus sp.	1	1.89	1	0	0	0	0	1	0.14
Glycyphagidae	4	7.55	6	2	1	0	0	9	1.30
Lepidoglyphus destructor (Schrank)	1	1.89	2	0	0	0	0	2	0.29
Glycyphagus domesticus (De Geer)	3	5.66	4	2	1	0	0	7	1.01
Chortoglyphidae	7	13.21	15	12	0	0	0	27	3.89
Chortoglyphus arcuatus (Troupeau)	7	13.21	15	12	0	0	0	27	3.89
Oribatida									
Haplochthoniidae	1	1.89	2	0	0	0	0	2	0.29
Haplochthonius simplex (Willmann)	1	1.89	2	0	0	0	0	2	0.29
Total			417	202	66	8	1	694	100
			60.09	29.11	9.51	1.15	0.14		100

it is reported that *D. pteronyssinus* is the predominant species in studies carried out in 2016, 2017, and 2018 in Erzincan province (Zeytun 2015; Zeytun et al 2016; Zeytun et al 2017a, Zeytun et al 2018). Chew et al (1999) in Singapore and Mariana et al (2000) in Malaysia found that *Blomia tropicalis* was the most common mite followed by *D. pteronyssinus*. *D. pteronyssinus* was also the second common species (39.8%) in the southern part of Poland (Solarz 2010).

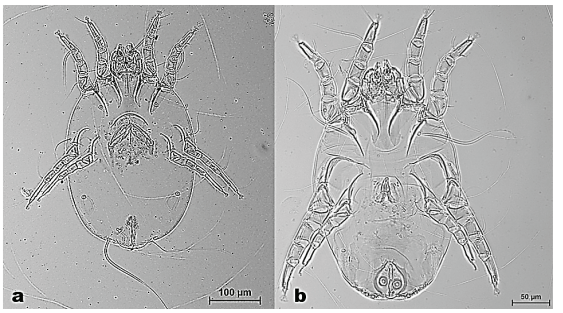

Figure 2- *Dermatophagoides pteronyssinus*; female (a) and male (b)

D. farinae (Figure 3) was the second most common species (66.04%) in Ordu homes such as in Izmir (Gülbahar et al 2004), Hatay (Gülkan 2004) and Erzincan (Zeytun et al 2017a) provinces in Turkey. Lower rates of *D. farinae* were detected in dust samples collected in Samsun (3.77%) (Çelik 2009), Bursa (4.16%) (Güleğen et al 2005), western Anatolia (0.7%) (Çiftçi et al 2006), Erzincan (3.67%) (Zeytun 2015), (2.1-7.5%) (Zeytun et al 2016) in Turkey. On the other hand, there was no *D. farinae* in any house in Malatya (Atambay et al 2006), Kütahya (Akdemir & Soyucen 2009), Eskişehir (Doğan et al 2008), Muş (Haskoy) (Aykut & Yılmaz 2010) and Afyon (Çiftçi et al 2004). From other countries, in India (Gill & Kaur 2014 (88.63%); Goutam 2018), Iran (98%) (Ziyaei et al 2017) and Philippines (95.8%) (Navarro-Locsin & Lim-Jurado 2018), *D. farinae* was also detected to be the second most common species (Gill & Kaur 2014; Ziyaei et al 2017). In contrast, the most common species was *D. farinae* in Korea (Ree et al 1997), Poland (Henszel et al 2010; Kosik-Bogacka et al 2010; Solarz 2010), Philippines (Los Banos, Laguna) (Catanghal & Paller 2012), China (Beijing) (Sun et al 2013; Sun et al 2014), Egypt (Shebin El-Kom Locality) (Heikal 2015), China (Xishuangbanna) (Yu et al 2015). Lower rates of *D. farinae* were detected in Malaysia (Klang Valley) (0.5%) (Chew et al 1999), Spain (Galicia) (5.2%) (Boquete et al 2006), India (Punjab) (7.1%) (Kaur & Dhingra 2018).

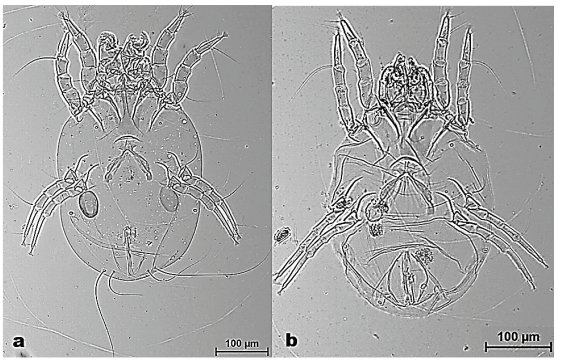

Figure 3- *Dermatophagoides farinae* females; dorsal (a), ventral (b)

Here, we report intermediate and heteromorphic males in *D. farinae* (Figure 4). According to Solarz et al (2016), heteromorphic males differ from normal homeomorphic by the thickening of the legs I and fusion of the epimera I to form a V or Y (with a sternum). The degree of thickening or fusion of epimera I may vary according to individuals in the same population. Mite species have been found in varying rates in numerous studies from our country and abroad.

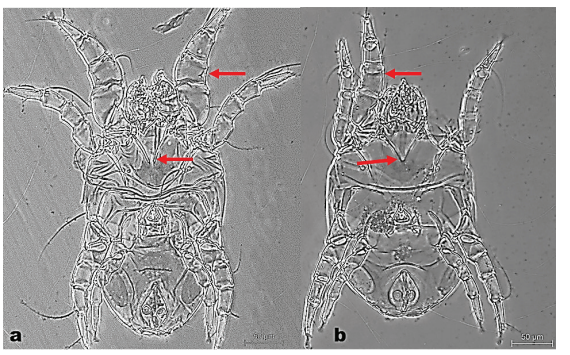

Figure 4- *Dermatophagoides farinae* male; intermediate (a) and heteromophic (b) form

Chortoglyphus arcuatus (Figure 5) was the third most common species (13.21%). It was found in varying rates in numerous studies from our country and abroad (Gülkan 2004; Çiftçi et al 2004; Atambay et al 2006; Boquete et al 2006; Çiftçi et al 2006; Doğan et al 2008; Aykut & Yılmaz 2010; Henszel et al 2010; Kosik-Bogacka et al 2010; Aykut et al 2013; Sun et al 2014; Heikal 2015).

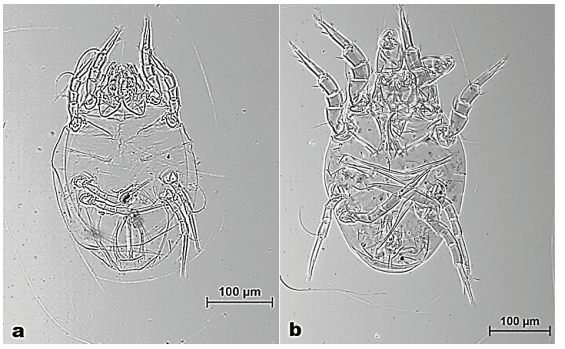

Figure 5- *Chortoglyphus arcuatus* female (a), male (b)

In our study, another species in dust samples was *G. domesticus* (5.66%) (Figure 6). Same species was found in Kütahya (2.58%, 23%, 3.33-3.48%) (Akdemir & Gürdal 2005; Akdemir & Soyucen 2009; Akdemir & Yılmaz 2009, respectively), Bursa (12.50%) (Güleğen et al 2005), Bitlis-Muş (1.3%) (Aykut et al 2013), Erzincan (0.06%) (Zeytun 2015) from Turkey and in Spain (10.4%) (Boquete et al 2006) and Egypt (Shebin El-Kom Locality) (1.44%) (Heikal 2015).

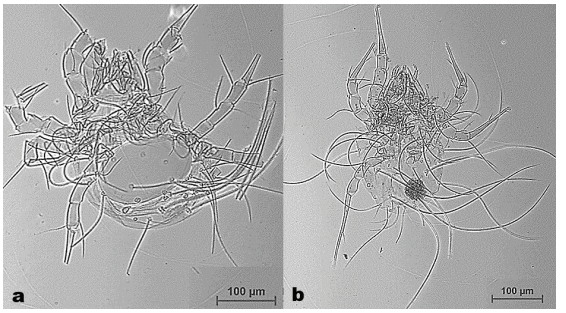

Figure 6- *Glycyphagus domesticus*; female (a), male (b)

The remaining mite species, *E. maynei* (Figure 7a), *H. simplex* (Figure 7b) and *L. destructor* (Figure 8) were detected only sporadically in Ordu homes. *E. maynei* which is of great medical importance was detected at varying rates in Turkey (Zeytun 2005; Aykut et al 2013; Zeytun et al 2015; Zeytun et al 2016) and abroad (Colloff 1987; Mehl 1998; Spieksma & Dieges 2004; Boquete et al 2006, Henszel et al 2010; Kosik-Bogacka et al 2010; Solarz 2010).

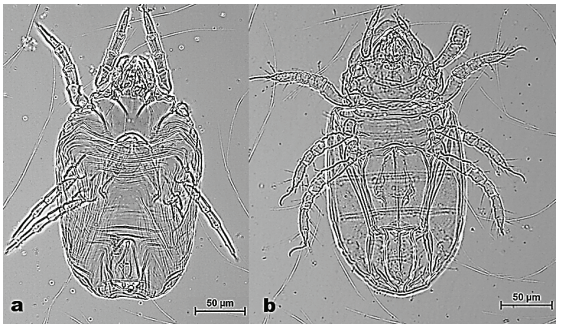

Figure 7- Females of *Euroglyphus maynei* (a) and *Haplochthonius simplex* (b)

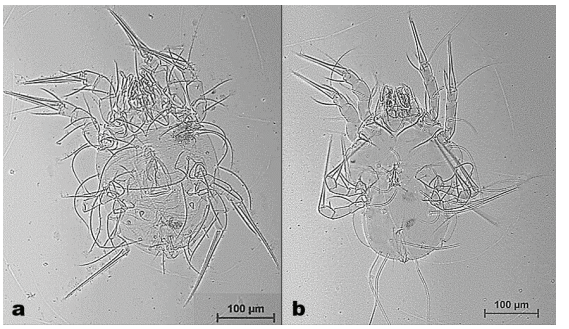

Figure 8- *Lepidoglyphus destructor;* female (a), male (b)

H. simplex, an oribatid mite species belong to the family Haplochthoniidae was also found in house dusts in Ordu similarly to Erzincan (Zeytun 2015; Zeytun et al 2017b). *Histiostoma* sp. was recorded in Samsun by Ozman-Sullivan K & Celik (2010). However, they didn't mention any species name. This species was also collected from house dusts in Okinawa Prefecture (6%) by Takeda et al (1998),

in Nagoya (Japan) (8.3%) by Suto et al (1992) and Sakaki & Suto (1995), in Brazil (0.36%) by Rosa & Flechtmann (1979), in Japan (26.2%) by Hatsushika & Miyoshi (1992).

L. destructor has been reported in varying rates in numerous studies in Turkey (Gülkan 2004; Akdemir & Gürdal 2005; Atambay et al 2006; Çiftçi et al 2006; Akdemir & Soyucen 2009; Aykut & Yılmaz 2010; Kılınçarslan 2012; Aykut et al 2013; Zeytun 2015; Zeytun et al 2015; Zeytun et al 2016), and abroad (Boquete et al 2006; Henszel et al 2010; Kosik-Bogacka et al 2010; Sun et al 2014; Yu et al 2015).

HDM species have been investigated in Turkey and the world by numerous researchers and noted different compositions of the dust mite fauna, as well as the abundance of each species. These differences may be due to the complex factors that involve geographical factors and household factors, affecting the distribution and abundance of mite species.

4. Conclusions

Our study shows that all surveyed homes in Ordu city were found to be positive for dust mites. The majority of homes sampled were infested with more than one dust mite species. Among the 53 sampled homes, 1 home had four mite species, 5 homes had three species, 34 homes had two species and 13 homes had one species. A total of 11 different species belonged to 5 families, 8 genera were determined in the dust samples collected from Ordu houses. The family Pyroglyphidae (94.09%) had the highest percentage of the total collected mites. All surveyed homes had *D. pteronyssinus* and *D. farinae*, which are two main allergenic dust mite species. They were also the most commonly seen species in the houses of Ordu province (Turkey).

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A Video Image Segmentation System for the Fruit-trees in Multi-stage Outdoors Orchard under Natural Conditions

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ABSTRACT

Segmentation is an important part of each machine vision system that has a direct relationship with the final system accuracy and performance. Outdoors segmentation is often complex and difficult due to both changes in sunlight intensity and the different nature of background objects. However, in fruit-tree orchards, an automatic segmentation algorithm with high accuracy and speed is very desirable. For this reason, a multi-stage segmentation algorithm is applied for the segmentation of apple fruits with *Red Delicious* cultivar in orchard under natural light and background conditions. This algorithm comprises a combination of five segmentation stages, based on: 1- L*u*v* color space, 2- local range texture feature, 3- intensity transformation, 4- morphological operations, and 5- RGB color space. To properly train a segmentation algorithm, several videos were recorded under nine different light intensities in Iran-Kermanshah (longitude: 7.03E; latitude: 4.22N) with natural (real) conditions in terms of both light and background. The order of segmentation stage methods in multi-stage algorithm is very important since has a direct relationship with final segmentation accuracy. The best order of segmentation methods resulted to be: 1- color, 2- texture and 3- intensity transformation methods. Results show that the values of sensitivity, accuracy and specificity, in both classes, were higher than 97.5%, over the test set. We believe that those promising numbers imply that the proposed algorithm has a remarkable performance and could potentially be applied in real-world industrial case.

Keywords: Background; Daylight; Machine vision; Natural condition; Texture; Color space

1. Introduction

Machine vision systems are used to perform different duties in agriculture and industry, among others. These systems have an automatic segmentation part that plays a main role in their accuracy. In environments with complex backgrounds including a wide variety of colors and textures, segmentation is the most sensitive part of a machine vision system (Rahimi-Ajdadi et al 2016; Rahimi-Ajdadi et al 2018). In these conditions, a wrong segmentation is caused when main object is considered as a background object that has to be removed, so the accuracy of machine vision systems is reduced (Slaughter et al 2008). Orchards are an example of environments

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with complex backgrounds. So in order to design a machine vision system to estimate the yield, spraying in proportion to the density of fruits, and fruit picking, segmentation operation has to be performed with high accuracy. There are various objects such as, leaves, different branches with different colors, cloudy or clear sky, among others. Therefore, the skill of programmer and the use of different segmentation methods such as color based segmentation, texture based segmentation and a combination of them, are very important often. Several researches have proposed different methods for in site-specific spraying, to combat weeds (Onyango & Marchant 2003; Montalvo et al 2013; Arroyo et al 2016; Sabzi et al 2017b; Sabzi et al 2018; Sabzi & Abbaspour-Gilandeh 2018; Sabzi et al 2018), plant segmentation in agricultural fields (Bai et al 2014; Hernández et al 2016; Sabzi et al 2017a), fruit segmentation (Liu et al 2016), determining the growth stage (Kataoka et al 2003) and in detecting plant diseases (Camargo & Smith 2009). Aquino et al (2017) proposed a segmentation method to count the number of 18 different cultivars of grape berry under artificial light. They used 152 images (126 images for training and 26 images for testing) to design a segmentation algorithm. The segmentation algorithm had two main steps: 1. general segmentation step to separate the grape cluster from the background, and 2. recognition each grape berry over each cluster. Results showed that the segmentation accuracy based on two classifiers including support vector machine (SVM) and artificial neural network (ANN) were 0.9572 and 0.8705, respectively. In another study, a segmentation method to detect immature green citrus in citrus gardens was proposed by Zhao et al (2016). They used a combination method of sum of absolute transformed difference (SATD) and color features. A total of 126 images, including 58 training image and 68 testing images, were used in the development of detection algorithm. Results showed that SVM classifier had a detection accuracy of 83%. Liu et al (2016) developed an apple segmentation algorithm based on artificial light with low brightness with 20 apple fruits. They used RGB and HIS color space components for training of ANN. They also consider the color and position of the pixels surrounding the segmented area in order to complete the segmentation. The estimation of number of citrus on trees based on image processing was studied by Dorj et al (2017). They stated that the estimation of performance is nowadays often done manually with low accuracy. For this reason, they used 84 images that were taken from 21 different trees. Their proposed algorithm had 6 main stages: 1- converting RGB color space to HSV color space, 2- thresholding, 3- identification of orange color, 4- noise removal, 5- applying watershed segmentation, and 6- final counting. The results showed that the determination coefficient between samples identified manually and with automatic algorithm was 0.93. Behroozi-Khazaei & Maleki (2017) studied the possibility of ripe grape clusters segmentation from leaves and the background based on color features using the combination of ANN and genetic algorithm. They used 129 images of ripe grape clusters that were directly taken from orchard. After training of proposed algorithm, results showed that their algorithm had an overall accuracy 99.4%. The probably of identification of cotton in the field based on an algorithm that was trained under supervised and unsupervised conditions was proposed by Li et al (2016). Their algorithm consisted of two main steps. The first step employed simple linear iterative clustering (SLIC) and density-based spatial clustering of applications with noise (DBSCAN) on Wasserstein distance and second step color and texture features were extracted from these regions based on texture. They used 42 images for training of the proposed algorithm. Results showed that the proposed algorithm had an average error within 4 years of 0.75 days.

It can be observed that most researches focus over processes of high quality images under artificial light also with a controlled background. In practice, in order to design a machine vision system being able to work in real orchard in picking of fruit, and estimating the number of fruits, a robust

segmentation algorithm is needed. Since in any orchard different objects with different color and texture exist, segmentation algorithm often needs to be a combination of different segmentation approaches. The aim of this study is to develop a new segmentation algorithm consisting of various color-based, texture-based, and threshold-based methods, for separation of apple fruits under real conditions.

2. Material and Methods

Given that in a number of orchard operations, such as estimating the number of fruits, the camera has to move across orchard, video processing was used. Video processing is harder than image processing since the frames are lower quality than common still images. The segmentation algorithm was trained under real environment and natural variable light conditions. Figure 1 shows a global outline of different stages to design a new algorithm for apple color segmentation in garden.

2.1. Data collection

Since weather conditions and light intensity change from sunny to very cloudy during day, segmentation algorithms should be trained under various light intensities to offer high accuracy in all possible conditions. For this reason, different videos were recorded in nine different states using a DFK 23GM021, CMOS, 120 f s^{-1}, Imaging Source GmbH, Germany, digital camera equipped with an appropriate lens (Computar CBC Group, model H0514-MP2, f= 5 mm F1.4, 1/2 inch type megapixel cameras, Japan). Table 1 shows light intensity values and the number of frames used in each time of filming. Filming was performed during different states of growing of apple cultivar *Red Delicious* in different orchards of Iran-Kermanshah (longitude: 7.03E; latitude: 4.22N) under natural conditions. The speed of filming was inside a range of 10 cm s^{-1} to 35 cm s^{-1}. Figure 2 shows two illustrative sample frames from different orchards.

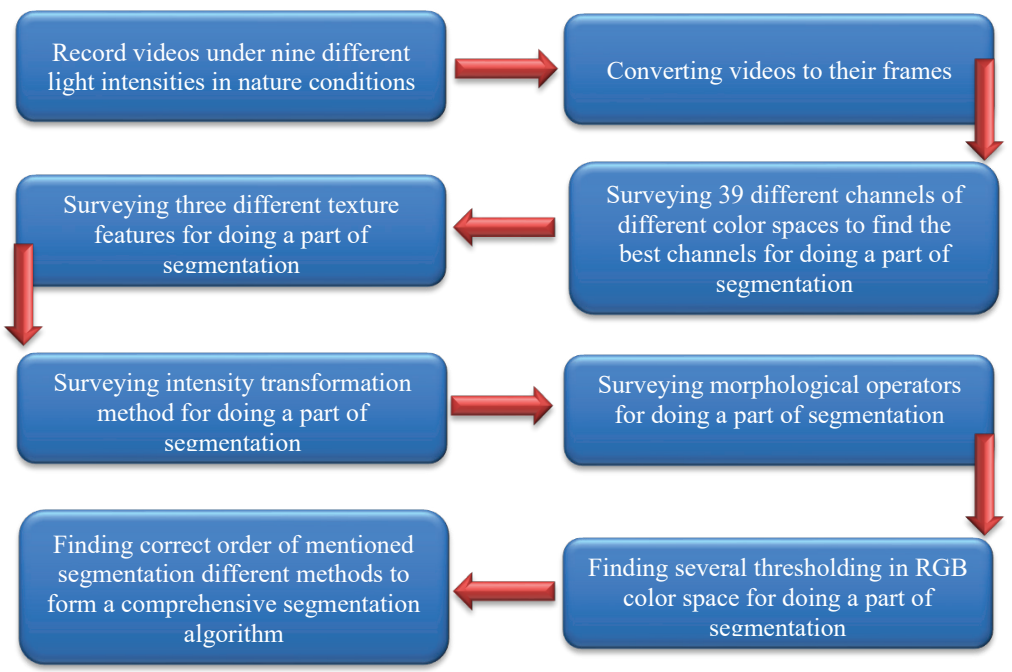


Figure 1- Different steps to form a comprehensive segmentation algorithm

Table 1- Light intensity and number of frames used in each video time state of filming

Video	*Light intensity (lux)*	*Number of video frames*
First	385	725
Second	628	439
Third	815	1264
Fourth	1120	694
Fifth	1386	496
Sixth	1593	583
Seventh	1793	1020
Eighth	1920	873
Ninth	2013	921

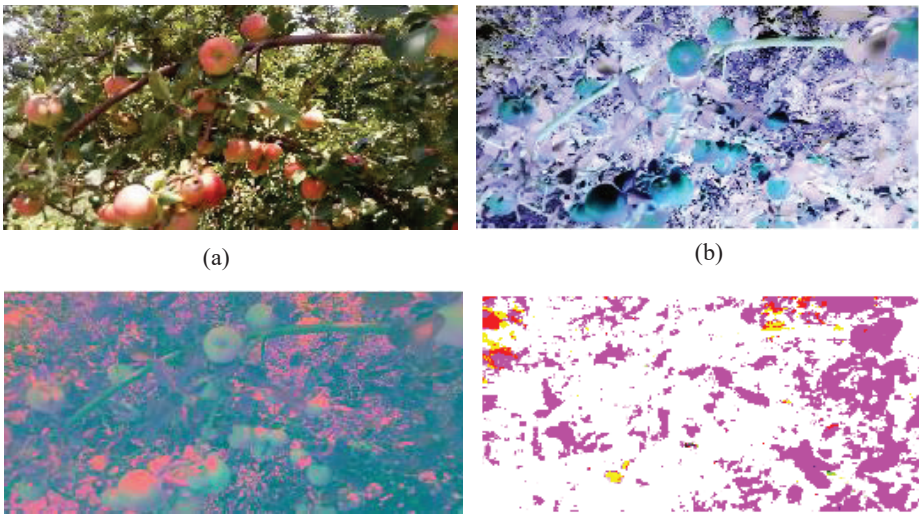
2.2. A survey of different color spaces

Difference researchers have surveyed the effect of the various color space in segmentation problems. They found that the finding of optimal color spaces increase the accuracy of segmentation (García-Mateos et al 2015). For this reason, A total of 13 different color spaces include: YIQ, L*a*b*, HSV, L*u*v*, YCbCr, CMY, HIS, JPEG-YCbCr, YDbDr, YPbPr, YUV, HSL, and XYZ that comprise a total of 13×3= 39 color channel, were investigated in this study. The aim of this survey is to find a suitable color space and channels for proper image segmentation. Figure 2 shows a sample video frame in different color spaces including RGB, CMY, L*a*b and L*u*v* color spaces. As it can be seen, each color space shows pixels with different colors. At this point, finding a color space that has minimum number of colors is very important since the possibility of main objects (apples) pixel elements is then reduced. On the other hand, background pixels need to be removed. Figure 3 shows that in L*u*v* color space there exists a minimum number of pixel color variations. Thus, we conclude that L*u*v* is a suitable color space to perform proper video frame segmentation.

2.3. The role of texture features in final segmentation

The use of texture features can offer useful information to detect background pixels in a segmentation process, since each kind of object can have different types of typical textures. These features have different outputs based on the texture of different objects. Texture is defined differently in different perspectives. One of the most common subdivisions is soft and hard texture. In fact, soft texture is the one with homogeneous object pixels, and hard texture is the one with heterogeneous object pixels. Because background of extracted frames from videos have different objects, and each object has a unique structure, using these features is useful for removing background objects from images. For this reason, local entropy, local standard deviation and local range were investigated to find the proper texture features for doing part of the segmentation work. Figure 4 shows the results of applying various local texture features over a sample video frame. As it can be seen, this method is very useful for pixel elements related to soil and plants that are dense.

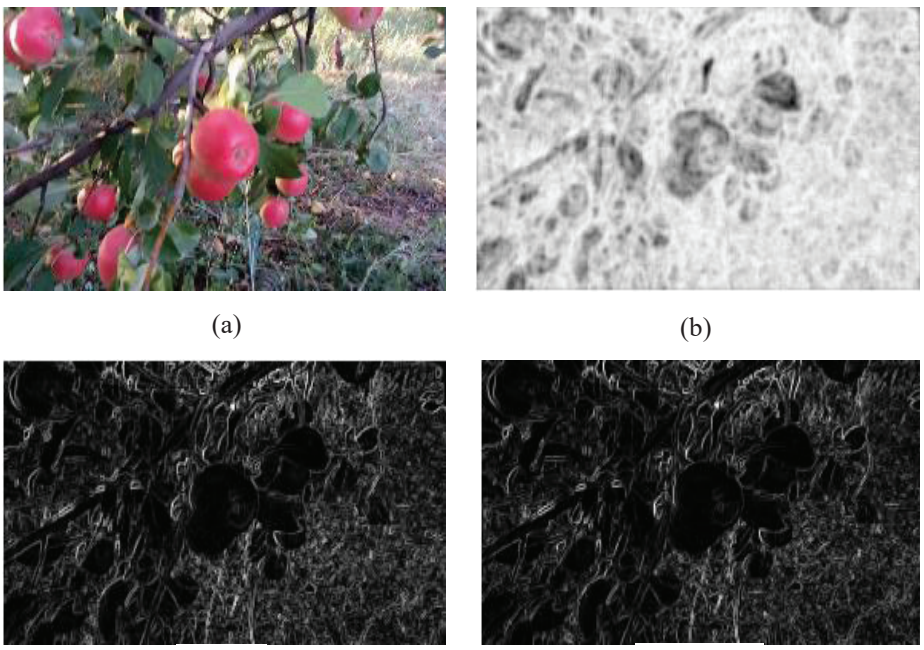
Figure 2- Two sample video frames from different apple orchards



(a) (b)

(c) (d)

Figure 3- A single sample video frame displayed in different color spaces. (a), RGB color space; (b), CMY color space; (c), L*a*b color space; (d), L*u*v* color space



(a) (b)

(c) (d)

Figure 4- Results of applying texture features over a sample video frame. (a), original frame; (b), result frame after applying local entropy feature; (c), result frame after applying local range feature; (d), result frame after applying local standard deviation feature

2.4. The use of intensity transformation method is segmentation

The use of intensity transformation method can be offer valuable information in segmentation, since changes in pixel intensity range on gray images lead pixels to either bright or dark modes. This is the case of the current problem, where apple pixels have more bright intensities than many objects in the background, thus allowing thresholding to remove some pixels not of interest in frames. Figure 5 shows the effect of applying the intensity transformation method over a single sample video frame. Based on this method, applying thresholding afterwards, part of the segmentation work was performed.

2.5. Partial segmentation based on morphologic operators

Morphologic operators play a key role in outdoor segmentation since in outdoor variable daylight conditions, different noise levels are present due to sunlight, and dust. Based on different morphologic operators such as the removing of object borders, filling holes and thickening removal of objects with a value less than the specified threshold, variable noise is conveniently removed (Gonzalez et al 2004). Thus, three morphologic operators of filling holes, labeling of connected components, and removal of objects with a thickness number less than the specified threshold, have been used in this study. The threshold used at different stages of the algorithm was set to 100, meaning that objects with a number of pixels less than 100 are to be deleted. Later threshold value was selected based on trial and error.

2.6. RGB color space thresholds to final stage in segmentation

After using different segmentation methods, some background pixels still remained in frames. After studying and working on various frames, 30 thresholds in RGB color space were used to complete the final segmentation of video frames.

3. Results and Discussion

As mentioned in previous sections, comprehensive segmentation algorithm is a combination of several segmentation methods. The selection of optimal state of each segmentation method guarantees the accuracy of segmentation algorithm.

3.1. An optimal color space partial video frame segmentation

Since the color of some pixels of background and apples are similar, the use of a simple threshold is not possible for performing complete segmentation. But it can be proven that thresholding can help solve a large part of the problem, by removing pixels which are clearly outside the color range of apple fruits. After studying different color spaces, L*u*v* color space was selected as a suitable color space to do part of segmentation. In fact, among all color spaces, L*u*v* color space showed how most of pixels in different frames were displayed with only 4 main colors: white, purple, red, and yellow. For this reason, with lower error, background pixels are removed optimally. Equation 1 shows the exact threshold used in L*u*v* color space:

(a) (b)

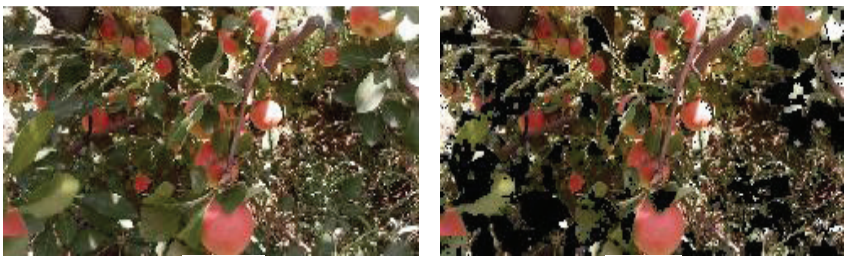
Figure 5- The effect of applying the intensity transformation method over a sample video frame. (a), original frame; (b), original frame after applying the intensity transformation method

$$Luv1(i, j)>90, Luv3(i, j)>90, Luv2(i, j)>90 \quad (1)$$

This equation implies that if all components of a pixel in L*u*v* space are more than 90, they belong to background and have to be removed from image. Figure 6 shows the result of applying L*u*v* color space threshold over a sample frame. As it can be seen in Figure 6, without deleting pixels of the main objects (apples) various background pixels were conveniently removed.

3.2. An optimal texture feature partial video frame segmentation

Since background includes different objects such as trunk of the trees in front of the sun, tree trunk in the shade, leaf in the sun, narrow branch, leaf in the shadow, petiole, thick branches in the sun, thick branches in the shadow, clear sky, cloudy sky, soil, green plants, yellow plants, dense leaves, mountains, wheat, straw, broken branches of trees, dried leaves on trees, flowers and weeds in the orchard, it often has sharper textures that produce higher texture values. On the other hand, apples skin show low values in all texture descriptors due to soft nature. So, based on this difference of texture value, a part of segmentation can be done based on texture features. Among next three texture features, local entropy, local standard deviation and local range, local standard deviation was selected to do part of the segmentation. In fact, any texture feature capable of removing the higher number of pixels possible from background with no damage to foreground (apple) pixels is a suitable texture feature. This is done by converting each texture structure image to a binary image, so that each pixel with value of 1 is considered as the background and will be consequently removed from frame image. Figure 7 shows the result of applying the second method of segmentation, the local standard deviation feature, over a sample frame. Figure 7b clearly shows that most part of background pixels were removed from image after thresholding.



(a) (b)

Figure 6- Result of applying L*u*v* color space segmentation threshold over sample video frame. (a), original frame; (b), same frame after applying L*u*v* color space thresholding segmentation

(a) (b)

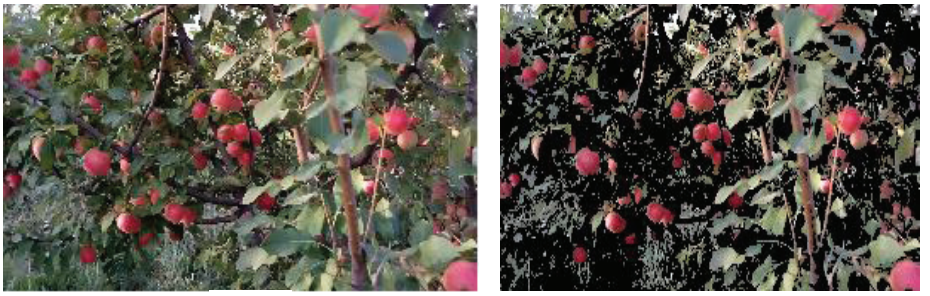
Figure 7- Result of applying the second method of segmentation (local standard deviation feature) over a sample frame. (a), original frame; (b), sample frame after applying the threshold of local range feature

3.3. An optimal threshold based on intensity transformation for partial video frame segmentation

Figure 8 shows the result of applying intensity transformation method over a sample frame. Figure 8b shows that most background pixels were eliminated from image frame without deleting apple foreground pixels, meaning that the method is remarkable robust. Frames were at first turned into gray images, and then pixel intensity range changed from {0, -1} to {0, -0.4}. Since images were in *uint8* data class, pixels were first multiplied by 225. Second, a threshold of value 75 was applied to perform part of segmentation. This threshold was obtained under a trial and error approach. This threshold assigns pixels greater than 75 to the background of frame image and thus should be removed. It is interesting to note that despite the different light intensities, with this thresholding method most of the darkest pixels belonging to the background, such as thick branches in the shadow, leaf in the shadow and petiole, were removed. This proves that this threshold was robust and fixed with high accuracy.

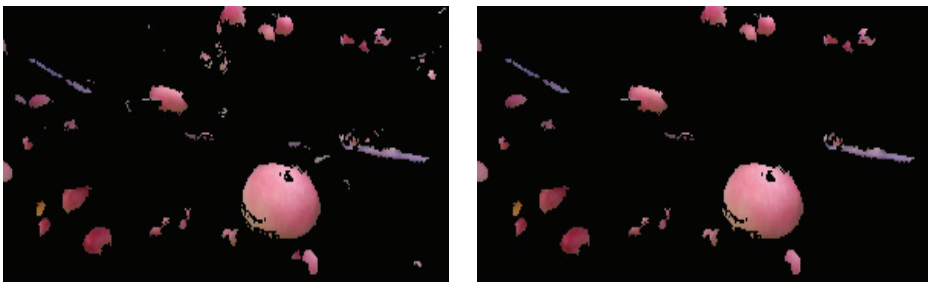
3.4. Optimal morphologic operators for partial video frame segmentation

Figure 9 shows the result of applying morphologic operators over a sample frame that was partially segmented by other segmentation methods. Figure 9a shows that some objects have the number of pixels less than 100. These objects have to be removed since they have not useful information and they reduce processing speed. Figure 9b shows the resulting frame after removing noise objects. The use of this operators cause that the speed of processing of segmentation algorithm rises. Since videos are taken in real conditions in garden and



(a) (b)

Figure 8- Result of applying intensity transformation method over a sample frame. (a), original frame; (b), result frame after applying the threshold using the intensity transformation method



(a) (b)

Figure 9- Result of applying morphologic operators over a sample frame that was partially segmented by other segmentation methods. (a), original image; (b), result frame after using morphologic operators

different objects in the background may exist, in each stage of segmentation a lot of pixels include noise. If noise is not removed, accuracy of results will be degraded for obvious reasons.

3.5. Best ordered combination of partial video frame segmentation methods

The order in which the segmentation methods are applied in the complete multi-stage segmentation algorithm is very important. In fact, each segmentation method can remove only part of background pixels from image, so the correct order in complete multi-stage algorithm of segmentation can cause removal of most background pixels. Figure 10 shows the effect of three different ordered segmentation methods in complete algorithm. As it can be seen in Figure 10b, the best order of segmentation is based on color, texture and intensity transformation methods. In other ordered methods, some noise still remains after final stage. This noise causes the use of additional segmentation steps thus increasing processing time. For online applications, time restrictions are very important, so processing time is a limitation factor.

3.6. Final color thresholds stage to complete multi-stage segmentation procedure

We tested that there were still few noise pixels after application of the different stages of segmentation. In order to remove noise, 30 thresholds in RGB color space were used as a final stage. Table 2 shows 11 RGB color thresholds definitions among 30 color thresholds to complete the segmentation process, which should be applied in an ordered fashion. For instance, first row in Table 2 means that if the first, second and third component of RGB color space are between 225 and 235, 170 and 180 and 135 and 145, respectively, and also the absolute difference between the second and third component of RGB color space is less than

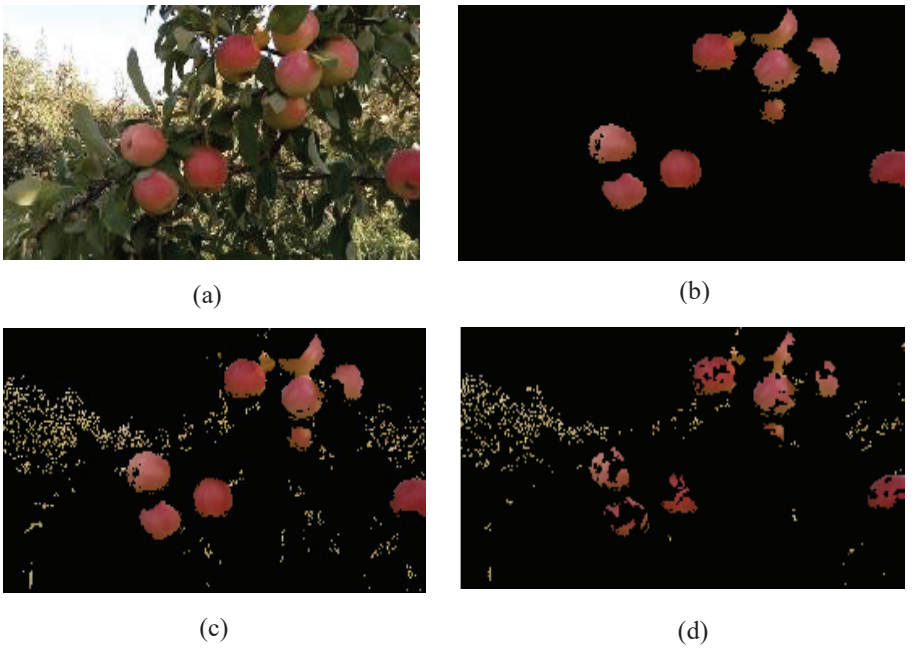


Figure 10- Results of three different ordered segmentation stages in complete multi-stage algorithm. (a), original image; (b), segmented image based on color, texture and intensity transformation ordered stages; (c), segmented image based on intensity transformation, texture, and color ordered stages; (d), segmented image based on texture, color, and intensity transformation ordered stages

40, then that pixel belongs to background and has to be removed from image. Figure 11 shows two sample frames before and after segmentation based on segmentation by the complete multi-stage segmentation algorithm here proposed. Figures 11b and d show how background pixels were completely removed from image.

3.7. Accuracy and speed of algorithm

Table 3 shows confusion matrix and the accuracy in detection of fruits in image for the here proposed multi-stage video frame segmentation system, over the test set. This table shows that 1.69% of apple samples were incorrectly detected as background objects (from 23359 apple pixels, 394 where misclassified

Table 2- Definition of 11 RGB color thresholds to complete final segmentation stage

1	FR(i,j)>225 & FR(i,j)<=235 & FG(i,j)>170 & FG(i,j)<180 & FB(i,j)>135 & FB(i,j)<145 & abs(FG(i,j)-FB(i,j))<40;
2	FR(i,j)>250 & FR(i,j)<=255 & FG(i,j)>200 & FG(i,j)<210 & FB(i,j)>170 & FB(i,j)<180 & abs(FG(i,j)-FB(i,j))<35;
3	FR(i,j)>220 & FR(i,j)<=245 & FG(i,j)>170 & FG(i,j)<200 & FB(i,j)>150 & FB(i,j)<180 & abs(FG(i,j)-FB(i,j))<30;
4	FR(i,j)>155 & FR(i,j)<=160 & FG(i,j)>130 & FG(i,j)<165 & FB(i,j)>110 & FB(i,j)<150 & abs(FG(i,j)-FB(i,j))<20;
5	FR(i,j)>178 & FR(i,j)<=185 & FG(i,j)>125 & FG(i,j)<140 & FB(i,j)>105 & FB(i,j)<125 & abs(FG(i,j)-FB(i,j))<25;
6	FR(i,j)>195 & FR(i,j)<=220 & FG(i,j)>185 & FG(i,j)<210 & FB(i,j)>135 & FB(i,j)<155 & abs(FR(i,j)-FG(i,j))<15;
7	FR(i,j)>195 & FR(i,j)<=215 & FG(i,j)>165 & FG(i,j)<195 & FB(i,j)>120 & FB(i,j)<140 & abs(FR(i,j)-FG(i,j))<35;
8	FR(i,j)>215 & FR(i,j)<=230 & FG(i,j)>155 & FG(i,j)<170 & FB(i,j)>130 & FB(i,j)<150 & abs(FG(i,j)-FB(i,j))<30;
9	FR(i,j)>195 & FR(i,j)<=210 & FG(i,j)>160 & FG(i,j)<185 & FB(i,j)>125 & FB(i,j)<150 & abs(FR(i,j)-FB(i,j))>50 & abs(FR(i,j)-FB(i,j))<70;
10	FR(i,j)>215 & FR(i,j)<=220 & FG(i,j)>183 & FG(i,j)<190 & FB(i,j)>135 & FB(i,j)<140 & abs(FR(i,j)-FG(i,j))<35;
11	FR(i,j)>220 & FR(i,j)<=235 & FG(i,j)>195 & FG(i,j)<215 & FB(i,j)>150 & FB(i,j)<180 & abs(FR(i,j)-FG(i,j))<30;

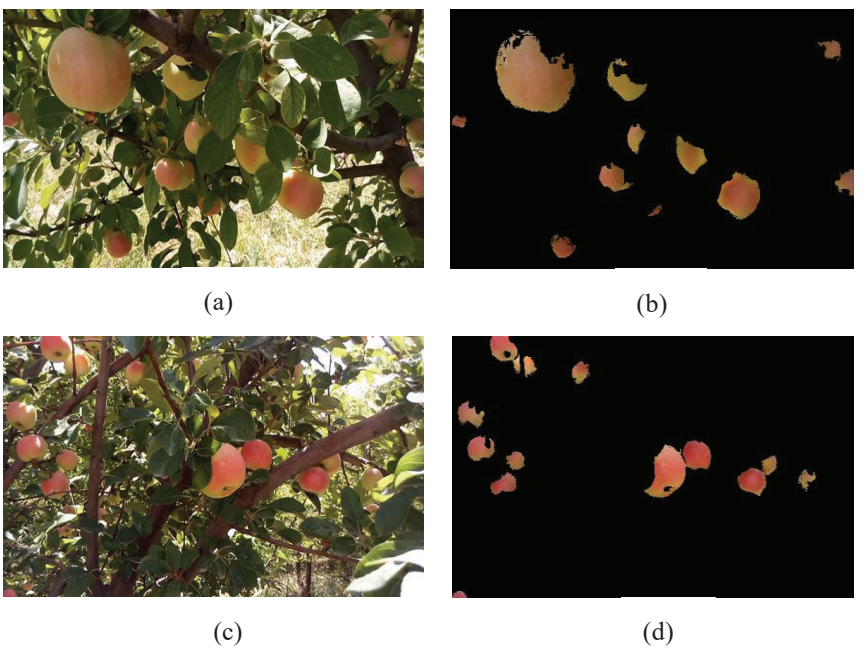


Figure 11- Two sample frames before and after segmentation based on the multi-sage segmentation algorithm. (a and c), original frames; (b and d), segmented frames after use of the multi-stage segmentation algorithm

as background and removed from image) and also 1.91% of apple samples incorrectly detected as background objects (from 16336 background pixels, 313 where misclassified as apple pixels and kept in image). These misdetection values show a well-balanced and homogeneous of classification errors among the two classes, since the number of apple color pixels misclassified as background objects is similar to the number of background color pixels misclassified as apple samples. Three commonly used criteria for analyzing the performance of a binary classification algorithm are sensitivity, accuracy and specificity. By definition, sensitivity expresses the wrong assignment of samples of the relevant class and specificity indicates the wrong assignment of the samples of other classes into the relevant class. Finally, accuracy is the percentage of total samples correctly classified in their corresponding classes (Wisaeng 2013). Table 4 shows the results of the algorithm performance in terms of sensitivity, accuracy and specificity, over the test set. As it can be seen in Table 4, all these three criteria have the value higher than 97.5% in both classes, implying a remarkable accuracy in segmentation and potential application under real conditions in industry. The speed of data processing in online application is important. For this reason, algorithms with high speed and accuracy are most useful. In this study, a laptop with processor Intel Corei3CFI, 330M at 2.13GHz, 4GB of RAM, Windows 10 and MatLab 2015b was used. The average processing time per video frame was 0.795 seconds, including image reading and full multi-state segmentation. Since the proposed method in this study is new and there is no similar case, there is no direct comparison of the results obtained in this study with the results of other researchers. However, comparing the results of this study with other studies reveals the importance of the method used here. For this reason, the proposed segmentation method is compared with two similar previous studies by Hernández-Hernández et al (2016) and Aquino et al (2017). These two studies focused on the segmentation of corn in agricultural land, and on counting the number of grape cubes with an artificial background, respectively. Table 5 shows the results of comparing our method with two

Table 3- Confusion matrix and the accuracy of the detection of the proposed multi-stage video frame segmentation system, test set

Classes	*Apple*	*Background*	*All data*	*Misdetection (%)*	*Correct detection (%)*
Apple	22965	394	23359	1.69	98.22
Background	313	16023	16336	1.92	

Table 4- Performance of the multi-stage frame image segmentation algorithm classification in terms of sensitivity, accuracy and specificity (%), test set

Class	*Sensitivity (%)*	*Accuracy (%)*	*Specificity (%)*
Apple	98.31	98.22	98.66
Background	98.08	98.22	97.60

Table 5- Compares the success rate of the different methods for segmentation

Method	*The number of samples*	*Accuracy rate (%)*
Proposed model	39695 (testing data)	98.22
Hernández-Hernández et al (2016)	182	97.00
Aquino et al (2017)	152	95.72

other methods. As it can seen, our method has higher accuracy. We believe that one of the most important reasons for high accuracy in our method is the use of a combination of several segmentation methods, while two other methods used only one single segmentation method. When using only one segmentation method, thresholding may be rather sensitive.

4. Conclusions

In current study, a segmentation algorithm to segment fruits over trees in outdoors garden under natural condition was proposed. This algorithm is formed from a combination of different methods. Due to different challenges in nature/real conditions in open field, the use of a combination of different methods may obtain higher accuracy. In fact, we believe that when only one segmentation method is used, threshold set value is very sensitive, since it may be altered by the pixels of main foreground objects. Also, in order to achieve methods that are able to work in real time, video input should be used instead of static still pictures, thus producing lower quality images. The order of segmentation methods in comprehensive algorithm plays also an important role in accuracy, so that an unsuitable order reduces segmentation accuracy. To conclude, we want to state that the accuracy of the final multi-stage segmentation algorithm in detecting background and apple objects was rather remarkable in 98.22%, over the test set.

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Modelling Indoor Environmental Conditions in a Commercial Broiler House

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ABSTRACT

Turkey's poultry industry has experienced significant growth in recent years, resulting in the construction of many new production facilities. It is important to maintain optimum environmental conditions for a profitable production. In this study, temperature, relative humidity and air velocity distribution inside a broiler house were analysed. Computational Fluid Dynamics (CFD) simulations (numerical method) and direct measurements (experimental method) were used to determine the appropriate indoor environmental conditions. Simulated values were validated by comparison with the measured values using the normalised mean square error (NMSE). The measured and predicted parameters of temperature, relative humidity and air velocity at birds' height, human height, and roof height upon comparison gave average NMSE values of 0.139, 0.181 and 0.090, respectively. The results showed a good agreement between simulated and measured values as obtained NMSE values were less than 0.25. In conclusion, CFD simulation can be used as an alternative method for the analysis of poultry house indoor environment. A better understanding of indoor environment conditions in poultry house provides useful information for manufacturers and end users for better management decisions.

Keywords: Broiler house; CFD modelling; Temperature; Relative humidity; Air velocity

1. Introduction

In recent years, Turkey's poultry industry has become the fastest emerging and growing industry. The industry constitutes a 2% share of the world poultry production market, with about 1.900 million tons per year and aims to be the world leading chicken industry in the next decade (USDA 2016). This can be accomplished through not only planning the appropriate genetic breeding programs (Leeson & Summers 2010), but also ensuring availability of suitable environmental conditions (indoor and outdoor) for birds.

During planning and designing of poultry houses, the birds' optimum environmental conditions (indoor) and regional climatic data (outdoor) are important factors to be considered for effective poultry production. Environmental conditions inside poultry houses greatly affect the productivity of the poultry facility. Chickens are very sensitive animals which are affected by temperature (Song & King 2015), light (Deep et al 2010), relative humidity (Xiong et al 2017) and air exchange fluctuations (Furlan et al 2000) during all rearing phases. Therefore, it is very important to consider environmental conditions and ensure proper design

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of ventilation system in the construction of a poultry house. High summer temperatures and extreme winter conditions greatly affect chicken production and these climatic factors also need to be considered for planning and designing of the poultry house.

The main reasons for the high death rates of broilers are high summer temperatures and humidity values. These deaths accompany considerable economic losses for poultry facilities. In recent years, there have been great efforts to improve the welfare of birds by establishing mechanical ventilation systems in poultry houses (Vizzier Thaxton et al 2016). Despite this solution, the problems arising from the thermal stress are yet to be resolved. Therefore, visualisation of air distribution within the poultry houses comes into prominence.

In agricultural structures two common methods exists to determine the appropriate environmental conditions, which are direct measurements of the conditions using electronic devices and Computational Fluid Dynamics (CFD) techniques (Bustamante et al 2013). The CFD software is often used for the analysis of fluid-related materials nowadays.

It is possible to determine indoor environmental conditions using various equipment and instruments. However, for large production facilities, it is not always easy to collect data using aforementioned instruments and methods as they are time consuming, labour intensive, costly and prone to human error. In addition, since measurements are conducted at regular intervals (grids) the results may not necessarily be representative of the entire house conditions, thus making the accurate prediction of stress areas inside the building difficult.

Because of these drawbacks encountered during direct measurement, CFD techniques can be used as an alternative to determine indoor environmental conditions. The CFD method allows solving of numerically difficult, long and complex equations by means of a computer and analysing the distribution of desired parameters associated with the flow (Bates et al 2005). The CFD simulations not only save time and labour, but also enable visual analysis of the poultry house indoor environmental conditions.

Although CFD techniques are widely used in different fields of engineering (such as mechanical, environmental, aerospace engineering), there are few detailed studies performed for the determination of indoor environmental conditions of poultry houses (Bustamante et al 2013; Bustamante et al 2015; Rojano et al 2015). On the other hand, validation of simulation models with experimental data is essential for reliable predictions.

Based on these facts, the distribution of air temperature, relative humidity and air velocity inside the broiler house were investigated using two methodologies, namely, direct measurement by instrumentation and CFD simulation program. Firstly, air temperature, relative humidity and air velocity were measured at certain number of fixed points and at different heights of a broiler house (experimental method) in Samsun province, Turkey. Then, the collected data were analysed, and findings were compared to those obtained from CFD techniques (numerical method). Finally, the CFD simulation results were used to reconstruct indoor environmental conditions such as stagnant and stress zones of broiler house in the form of visual simulations.

2. Material and Methods

2.1. The building

Measurements and simulations were carried out at a commercial broiler house located in Samsun province (Northern Hemisphere, Latitude 41°70', Longitude 36°30') in northern Turkey. The house was lean concrete-floored with a breeding area of 1260 m^2 (90 m×14 m), designed for 22.000 broilers yielding a stocking density of 14 to 17 birds m^{-2}. Basic geometric dimensions of the house are as follows: length, 90 m; width, 14 m; sidewall height, 2.70 m; maximum height, 4.40 m. Sawdust is used as a litter material. Feeding, watering, lighting, heating and ventilation are controlled automatically. The broiler house is equipped with eight big (diameter 1.38 m)

and five small exhaust fans (diameter 0.92 m). At the time of data collection (4th week), only two big fans located in the centre were operational. The house has sixty-six air inlets measuring 32.50×52.50 cm, placed along with the side walls. The heating system comprised an external heat source with a dual-tube system and outlet air temperature of 75 °C.

2.2. Experimental method

The vertical plane was defined based on three measurement positions from the floor, that are, birds' height (BH), human height (HH) and roof height (RH), placed at 0.25 m, 1.80 m and 2.50 m, respectively (Figure 1). To examine distribution of inside air temperature, relative humidity and air velocity, measurements were taken at fourth weeks of growing seasons (May 2016). A total of fifty-nine measurements were taken from different locations in the building including forty-two at BH, fourteen at HH and three at RH (Figure 2).

To monitor indoor temperature and relative humidity distribution, fifty-nine relative humidity and temperature data loggers (KC 320B, NDI Instrument and Hand Tools, Cheltenham Australia) with a resolution of ±0.1% and ±0.1 °C respectively, were positioned as indicated in Figure 3. Measurements were automatically recorded at an interval of 30 minutes by data loggers. Outdoor air temperature and relative humidity were monitored by a data logger that was placed beneath the outside eaves of the building for protection against direct sunlight and rain.

In addition to above-mentioned fifty-nine measurement points, air velocity measurements were also taken from each of the air inlet points and fan areas located in the building. Air velocity was measured using airflow anemometer (DCFM8906, General Tools & Instruments, New York, USA). The instrument gives the instantaneous value of the measured air velocity. For the correct measurement (to minimize human error) the operation has been replicated three times (10 s intervals) for each point. The average of three obtained velocity values was considered as the final result (Awbi 2003). During the experiment, a traverse was run across the fan inlet measuring air velocity using a hand-held anemometer at 9 specific coordinates as highlighted by Wheeler & Bottcher (1995). The mean air velocities were obtained at each air inlet from 25 measurements per inlet (ASHRAE 2009). A thermal camera (Testo 875-2i, Testo AG, Lenzkirch, Germany) was used to measure the surface temperatures of each wall, roof, heater and broilers.

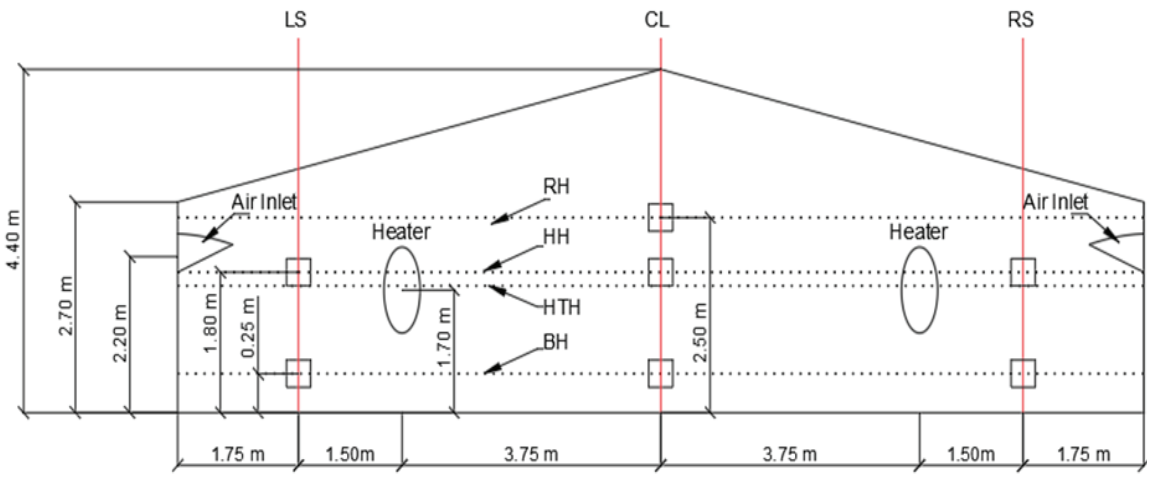

Figure 1- Broiler house geometry and sensor locations

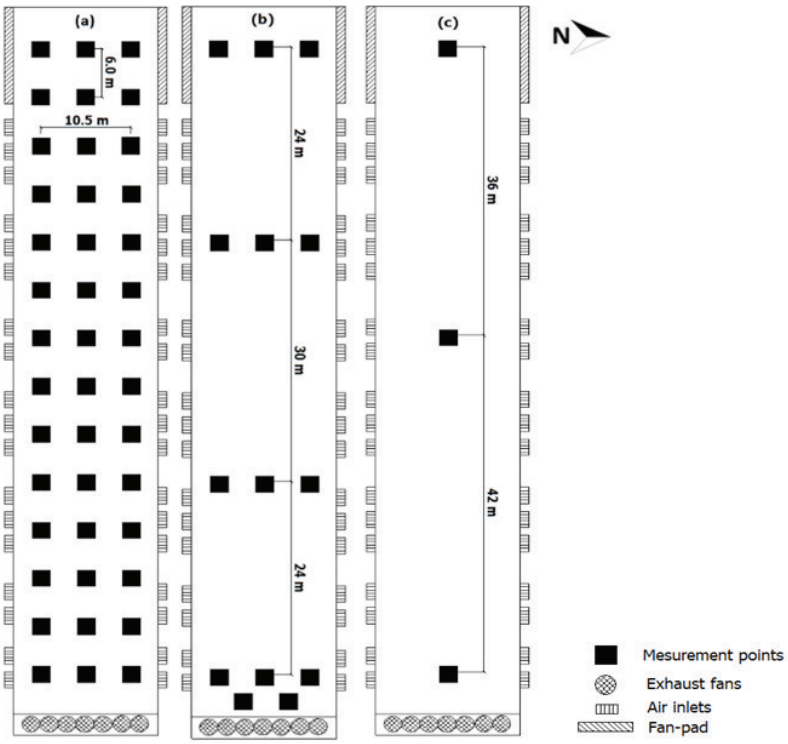

Figure 2- Broiler house measurement points (a), birds' height; (b), human height; (c), roof height

Figure 3- Measurements levels and general views of the broiler house

2.3. Numerical method

In numerical analysis, CFD software was used to determine environmental conditions (air temperature, relative humidity and air velocity) within the house. Although several types of CFD software are available in the market, Ansys Fluent, based on finite volume method, is the most popular amongst these. The finite volume method is a discretization method which transforms the partial differential equations from conservation laws to discrete algebraic equations (differential volumes) over finite volumes (or elements or cells) (Eymard et al 2000). The continuity, conservation of momentum (Navier-Stokes law), and the energy equation solved by the software. Detailed information about computational methods can be obtained from Versteeg & Malalasekera (2007).

In this study, standard k-ε turbulence model (Launder & Spalding 1974) was used to represent turbulent flow, predicted air temperature, relative humidity and air velocity distribution in the house. This model is widely preferred for turbulent poultry house simulations, due to its robustness and reliability (Blanes-Vidal et al 2008). AutoCAD (version 2016, Autodesk, San Rafael, Cal., USA) software was used to create 3D geometry of the broiler house at a scale of 1:1. The geometry was then imported into the software Ansys® Fluent (version 13, Fluent Inc., Lebanon, NH, USA) for development of a mesh. Boundary conditions were defined as follows, for air inlets "velocity-inlet", for fans "pressure outlet" and for walls, roof and floor "wall". The surface temperature of walls, roof, heater and broiler, air relative humidity and air velocity were set as the initial boundary conditions for CFD model according to field measurements as shown in Table 1. The production of total heat by broilers that were 4th weeks of age and having 1624 g of body weight was estimated using the CIGR (2002) equation.

2.4. Validation

The CFD simulation results were validated by comparing with the measured values at the same locations where the sensors were installed. The model fit between measured and predicted values was evaluated by calculating the normalized mean square error (NMSE) where values of NMSE less than 0.25 are accepted as good indicators of fit according to ASTM (2002). NMSE is calculated with the Equation (1) that reads as follows:

$$NMSE = \frac{1}{N}\sum_{i=1}^{n}\frac{(C_{pi} - C_{mi})^2}{(C_{pm} \times C_{mm})} \quad (1)$$

Table 1- Initial boundary conditions

Boundary elements		*Values*
Surface temperature	Walls	East: 23.20 °C, West: 23.70 °C
		North: 24.60 °C, South: 23.40 °C
	Roof	24.90 °C
	Heater	75.00 °C
	Broiler	33.40 °C
Air temperature	Outside the building	11.60 °C
	Inside the building	27.70 °C
Mass fraction water vapor	Outside the building	0.00765
Pressure	Fans	-20 Pa
Velocity	Inlets	3.34-3.51 m s^{-1}
Heat flux	Floor	137.91 W m^{-2}

Where; C_{pi} and C_{mi}, predicted and measured values, respectively; C_{pm} and $C_{mm,}$ average predicted and measured values, respectively; N, the total number of data.

3. Results and Discussion

CFD simulation and experimental results of indoor air temperature, relative humidity and velocity distribution are shown in Table 2. Good agreement between simulation results (predicted) and experimental data (measured) were found in the broiler house, since all calculated NMSE values were less than 0.25 according to the ASTM (2002) standards.

3.1. The indoor air temperature distribution: simulation vs. experiment

Considering the measurement line BH, higher temperatures were observed along the central line (CL) as compared to the temperatures observed on the left (south side, LS) and right (north side, RS) side of the CL. The presence of two working fans in the middle part of the building affected air movement and temperature distribution in the building. It was observed that birds tended to gather along the CL mostly closer the fan areas, which may have caused the observed increase in air temperature along the CL, with highest temperatures closer to the fans due to increased sensible heat produced by birds (Figure 4a). The CL temperature averaged 25.70 °C while the LS and RS regions recorded relatively lower temperature with an average of about 24.52 °C These observed results coincide with the temperature gradients found by Xin et al (1994) and Saraz et al (2012).

At HH, temperature near the side walls appeared to be warmer by about 2 °C than at the CL of the building due to the presence of heaters at this height (HTH) (Figure 4b).

Data recorded at RH showed that the indoor temperatures at CL registered relatively little lower temperatures with an average of 24.10 °C than air temperatures near the walls which had an average temperature of 25.40 °C. This may be attributed again to the presence of heaters near the side walls (Figure 4c).

The fresh cold air entering through side wall inlets must be kept away from the birds as much as possible. Therefore, the fresh air coming in through the inlets should first be directed towards the ceiling to mix with warm air before it reaches the birds. As shown in Figure 4b-c, incoming air from inlets closer to the fans is immediately drawn out by

Table 2- Average ± standard deviation indoor air temperature (°C), air relative humidity (%), air velocity (m s^{-1}) and NMSE values

Data	*Height of the birds (0.25 m)*		*Height of the human (1.80 m)*		*Height of the roof (2.50 m)*	
	Measured values	*Predicted values*	*Measured values*	*Predicted values*	*Measured values*	*Predicted values*
	Indoor air temperature (°C)					
Average	24.41±0.95	24.97±1.02	24.18±0.71	23.63±0.95	24.23±0.90	23.60±1.11
NMSE	0.049		0.124		0.243	
	Indoor air relative humidity (%)					
Average	69.10±2.39	70.18±2.69	70.48±1.85	71.73±1.66	69.98±0.90	72.50±1.44
NMSE	0.108		0.198		0.236	
	Indoor air velocity (m s^{-1})					
Average	0.98±0.44	1.05±0.43	1.05±0.46	1.19±0.51	1.26±0.53	1.19±0.48
NMSE	0.036		0.198		0.037	

the fans taking away with it the hot air. However, temperature is relatively high as the effect of the fans are minimal compared to the fan region. This is due to low static pressure in the region further away from the fans.

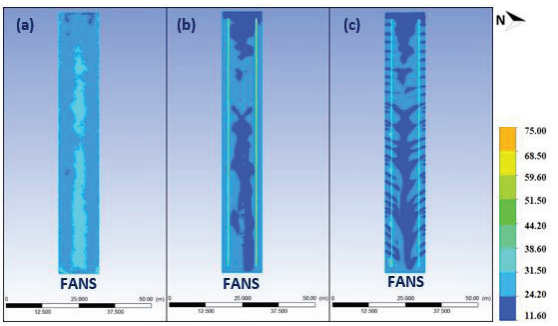

Figure 4- Simulated air temperature (°C) distribution at different measurement positions (a), birds' height; (b), human height; (c) roof height

Lindley & Whitaker (1996) and Reece & Lott (1982) indicated that optimum inside temperature of broiler house should be 32.00-33.00 °C in 1-2 weeks and 21.00-24.00 °C in 3-7 weeks. Considering previous studies, it can be concluded that the temperature of broiler house at 4th week was close to the optimal temperature.

3.2. The indoor relative humidity distribution: simulation vs. experiment

The production of moisture at BH is largely due to birds' respiration and manure exhalations. As shown in Figure 5a, indoor relative humidity at BH had maximum values (greater than 73.00%) along the CL of the building. The main reason of this can be attributed the preference by birds to gather in the center section of the building. As seen in Figure 5a, the areas close to the fans had relatively small relative humidity values due to the presence of the fans which draw away humidity. On the contrary, the section further away from the fan recorded high concentrations of relative humidity as there is limited fan effect in this region.

The relative humidity distribution at HH and RH demonstrated a similar distribution pattern with that of air temperature (Figure 5b-c) Within the regions where air temperature is found to be higher, relative humidity is lower and vice versa. The operation of exhaust fans created a negative static pressure in house which resulted in the withdrawal of moisture from the building.

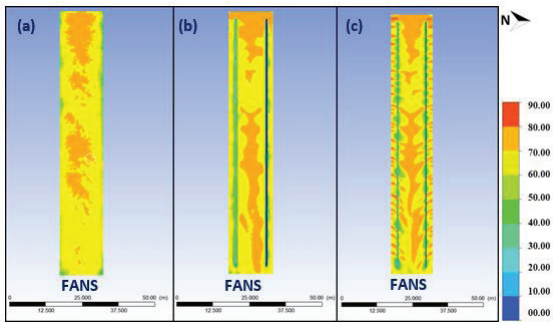

Figure 5- Simulated air relative humidity (%) distribution at different measurement positions (a), birds' height; (b), human height; (c) roof height

Winn & Godfrey (1967) recommended that the ideal relative humidity for broilers should remain between 50.00% and 70.00% during rearing periods. In this study, the relative humidity level of broiler house at 4th week was close to the optimum relative humidity level.

3.3. The indoor air velocity distribution: simulation vs. experiment

As illustrated in Figure 6a-b, air velocity at BH and HH is high within the proximity of fans and remained low within the first meter of the building creating stagnant (dead) areas. The fans draw the air from the building, creating a negative pressure (static) situation within the building. The negative pressure within the building pulls the air from outside the higher static pressure difference between the outside and inside, the higher the speed of air entering the house, thus the further the air shoot in.

As seen in Figure 6c, the speed of air flow at roof height is higher at inlets closer to the fans shown

by red and yellow color streaks than inlets further away from the fans shown by the dark and light blue patches (dead zone). This can be explained by the lower recorded airflow speed (3.34 m s^{-1}) at inlets further away from the fans as compared to the higher speed (3.51 m s^{-1}) closer to the fans. This may be further aggravated by the presence of heaters that intercept incoming air through the inlets thus causing incomplete airflow mixing in this section. A closer look at all the wind speed measured heights reveal that roof height recorded the highest mean wind velocity and this can be attributed to the positioning of the fans particularly fans' height above the ground.

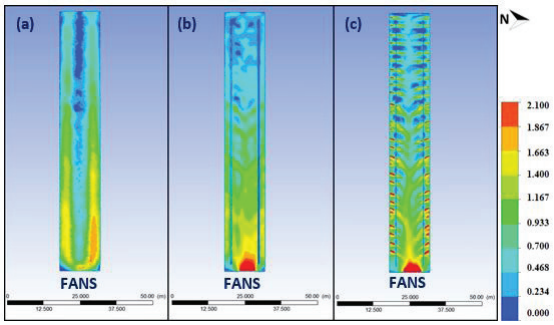

Figure 6- Simulated air velocity (m s^{-1}) distribution at different measurement positions (a), birds' height; (b), human height; (c) roof height

Dozier et al (2005) cited that birds' age is a factor that contributed to the observed air velocity range of 1.50-3.00 m s^{-1}. In the current study, measured air velocity values of broiler house at 4th week were under the ideal air velocity values.

4. Conclusions

From the previous results and discussions in this study, the following recommendations were arrived at;

The presence of stagnant areas in the region opposite the fans can be corrected by shutting off inlets closer to the fan region and opening inlets further away from the fans which create high static pressure in this region thus allowing better air mixing.

In line with stress areas particularly in the zone further from the fans there is need to introduce a system that enables homogenous air movement and mixing to avert air stagnancy.

CFD provides valuable information to manufacturers and end users on improvement of building designs, the requisite modifications for undertaking more informed decisions and thus more effective broiler production.

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Seed Quality of Oilseed Rape Varieties with Different Size and Colors After Three and Fifteen Months Storage

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ABSTRACT

During two years, germinability and initial growth of seedling of nine foreign varieties and four domestic varieties were studied. The seed is grouped by size (small, medium and large), color (dark transitional and light color of seed coat). Then it was preserved and at three and fifteen months of age in the laboratory and in the field, germinability, dormant seed and growth of shoot and radicle root were examined. In addition to the age, other factors (variety, size, color, period of storage) influenced significantly (P≥0.05 to P≥0.00) on seed germinability and growth of seedling.

Higher germinability is achieved from large seeds up to 3.6%. Depending on the seed coat color, the germinability varied to 11%. The seed coat color has had a high impact on maintaining germinability for fifteen months. Seeds with darker coat have also shown higher potential for storage and use in subsequent seeding periods, as determined by the application of the aging test on seed. Significant interdependence (r) was obtained between the germinability test and the growth of seedling in laboratory conditions and in field conditions. Between the germinability and the growth of the shoot and the root, a significant interdependence was established, depending on the varieties (P≥0.05 to P≥0.00).

Keywords: Age; Dormancy; Germinability; Seed; Seedlings

1. Introduction

Oilseed rape (*Brassica napus* L.) is the most important source of vegetable oil in Europe, the second most important in the world, with the production of over 35 million hectares (http://faostat.fao.rg/). The tendency to increase the area of this agricultural crop is also recorded in Serbia (Statistical Yearbook of Serbia 2017). In addition to growing it for oil, this plant species is also cultivated for green fodder and green fertilizing, but on smaller areas. By planting this plant species in the autumn period (August-September), it often happens that the crop does not sprout simultaneously, after that period the autumn frosts soon occur causing some seedling to freeze and planted crops do not have the required number of plants. For this situation, the

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reasons may be numerous such as insufficient and untimely rainfall, sowing in poorly prepared land in combination with low precipitation or deep sowing, etc. However, the seed that is used to plant crops can be the cause of uneven germinability. In open market countries like Serbia, farmers are offered a variety of varieties. Our producers have reported that the offered seed differs by color, size andage and that this may be the reason for the non-realization of the projected number of plants. On the other hand, it is known that in different cultivars, in a certain percentage, there will be dormant seeds that will germinate after the time of maturation and give a normal seedling, but the delayed germination and underdeveloped seedling in the period of stronger minus temperatures can be destroyed. According to Adkinset al (2002), through the process of breeding of agriculturally important species, dormant seeds have decreased or completely lost. On the other hand, there are no significant differences in species such as forage and ornamental grasses between sorts and populations from the natural environment in the presence of dormant seeds (Stanisavljević et al 2012).

Seed size is an important indicator of the physical quality of seeds.In research on different plant species, there are very contradictory results on the relationship between seed size and germinability, and the initial growth of seedlings. For example, in the species, *Triticum aestivum* according to Zareian et al (2013) the size of the seed does not have a significant effect on seed germinability, in contrast to Kakhki et al (2008) indicating a significantly higher germinability of large seed.The seed coat is an outer shell of each harvested seed and in many species, it differs in color. On the seed of the genus Brassica, this is very pronounced (Rahman & McVetty 2011)

After the release of seeds from dormancy, maximum germinability is achieved, and then the aging process begins, which inevitably results in reduced germinability, which depends on the plant species, the way of keeping the seed, etc. (Bewley 1997).

Therefore, the aim of these trials was to determine the agronomic value of seeds of domestic and foreign varieties and/or rapeseed hybrids of different size, color and age. It also recommends to producers that the seed is better for use. And it points to the growers the association of seed color and bulk with seed germinability and the initial growth of seedlings end thepossibility of improvement of management by cropping.

2. Material and Methods

2.1. Materials

During 2015 A1 and 2016 A2 (Table 1) (Factor of years-A) from the vicinity of the city of Pančevo (44°52’ N, 20°41’ E and 74 m asl), the seed of nine winter hybrid oilseed rape varieties (*Brassica napus*) were taken: RWA Prima (B1), Euralis neptune (B2), Bayer (B3), Umberto (B4), Travijat (B5), Balgira (B6), RWA Faxtor (B7), KVS Gordon (B8), Hybrirock (B9) and, four domestic varieties: Jasna (B10), Slavica (B11), Zlatna (B12), Zorica (B13) of oilseed rape (www.nsseme.com/about/?opt=oilcrops&cat=about) (the variety factor-B). Of all varieties according to the mass of 1000 seeds, the seeds are grouped into three sizes; small to 3.8 g C1, medium 3.8 g up to 4.1 g C2, and a large of more than 4.1 g C3, the seed size factor-C, (Stanisavljević et al 2018). Also from each variety, the seeds are grouped by color: light (D1), transient (D2) and dark (D3), the color factor (D). They were

Table 1- Seed harvesting time in seed germination incubator (experiment I) and containers (experiment II)

Day month year, harvesting seed	*25 June 2015*	*6 July 2016*
Day, month, year for an experimentI and experiment II	22 September2015 age of seeds about 3 months-E1	29 September 2016 age of seeds about 3 months-E1
	16 September 2016 age of seeds about 15 months-E2	19 September 2017 age of seeds about 15 months-E2

placed in double-layered paper bags and stored in warehouse conditions without controlling the temperature and humidity of the air. After seed storage for three months E1 (autumn sowing period) and seed storage of fifteen months E2 (autumn sowing period in the next year) the seed was divided into three subsamples from which III experiments were performed.

2.2. Experiment I

From the first subsample examined in laboratory conditions were: germination, dormancy seeds and the growth of seedlings (the shoot in cm and radicle (root) in cm).Seeds were chilled for five days at 5 °C and were exposed in the seed germination chambers to alternating temperatures 20/30 °C, 8 h in light (1250 lux) during 16 hours in the dark. Seed germination (%) and dormancy seeds (%) were determined for each of the factors (four replicates of 100 seeds on filter paper); final count was done on the 14th day (ISTA 2016.). The tetrazolium test was applied on dormancy seeds in order to separate dead seeds from hard ones (ISTA 2008). In addition, seedling vigor was determined in germinated seeds: the length of the shoot (cm), the length of the root (cm), (Stanisavljević et al 2011).

2.3. Experiment II

From the second sub-sample of the seed according to complete randomized block design, it was sown at optimum depth in containers filled with soil in which the optimal soil moisture content was maintained in the period after the three-autumn sowing (E1) and after fifteen months-autumn seeding next year (E2). After 12 days, the seedlings (germination %) were washed out from the soil and each measured (shoot in cm and root in cm).

2.4. Experiment III

From the third sub-sample, the seed was subjected to the aging test: of all the varieties (B1-B13) of the seed of different sizes (C1-C3), color (D1-D3) and the age of three months (E1), fifteen months (E2) and immediately after gathering (E0): in four replications/100 seeds each, the seed was placed in dishes and in a water bath at a temperature of 41 ^{o}C and a relative air humidity of 100% for 72 hours which is optimal for evaluating the seed of the rapeseed (Komba et al 2006).

2.5. Statistical analysis

Data from all experiments are mean values. Measurements were performed on three replicates ± standard deviation (SD). The obtained results were subjected to variance analysis (ANOVA; F test). Tukey multiplex test (P≥0.05) was used to assess the impact of the mean of treatment. For the interdependence between the Experiments I and II (Germinability, Shoot andRoot growth), the simple correlation coefficients (r) were calculated. Germination data and dormancy percentages were arcsine transformed [sqr (x/100)] before the variance analysis. Minitab 16.1.0 software was used for data processing.

3. Results and Discussion

3.1. The influence of factors

Using ANOVA, F test we found that the effect of the years did not significantly affect the studied properties. This can be explained by similar climatic conditions in which seed maturation (not shown) occurred. Therefore, in the further presentation of the results, the average from the two tested years was taken. The other factors (B, C, D, E) affected significantly (P≥0.05 to P≥0.00) the germinability and seedling growth (not shown).

3.2. Experiment I

Generally for the average of all varieties, from large seed (C3) in relation to small seed (C1), higher germinability was achieved by 3.6% after three months of seed storage (E1). After fifteen months for germinability, the same tendency was found with a difference of 2.9% (Table 2). The greater difference in germinability was achieved by the influence of varieties (C1 4%, and 5% for C2 and C3) after three months (E1) and, for 5% from seed C1, and 7% from seeds C2 and C3 in the period E2. The same tendency was observed for the growth of

the shoot (C3 7.25 cm C1 6.67 cm, with a difference of 0.58 cm) and root (C3 6.55 cm C1 5.70 cm, with a difference of 0.85 cm) and E1 period and in E2 shoot (C3 7.17 cm C1 6.62 cm with a difference of 0.57 cm), and root (C3 6.52 cm C1 5.70 cm) with a difference of 0.82 cm.

Depending on the seed volume in the E1 period, the difference in the percentage of dormant seeds was only 0.5%, and after fifteen months 0.1% (the average of all varieties) with variation due to the influence of varieties by 4% C1, 5% C2, and 3% C3 in the E1 period. After fifteen months, dormant seeds decreased > 1%, which affected a very similar germinability with an E1 period (Table 2).

For germinability between domestic varieties and foreign (hybrid varieties), a similar variability for the E1 period was found. After fifteen months (E2) the seed of all sizes of domestic varieties, had minimal higher variability (C1 CV= 3.27% to C3 CV= 3.45%) from foreign varieties (C2 CV= 1.59% to C3 CV= 1.80%). For the growth of the shoot foreign varieties showed higher variability in both periods of testing (E1 and E2), while there was no clear legality for the growth of the root (Table 2).

Depending on the seed coat color, germinability varied by 4% (D1 92% to D3 88%) in the E1 period and by 9% (D3 95 to 84% D1) in the E2 period. However, germinability from light seed in the E2 period decreased by 8% compared to E1 period whereas as contrast dark seed germinability in the E2 period increased by 7% in relation to the E1 period.

The difference in germinability caused by the influence of the variety in the E1 period ranged from 4% (D3) to 7% (D1) and in the E2 period from 6% (D3) to 8% (D1, D2) (Table 3). The germinability of domestic varieties of all colours had higher variability (CV= 1.59% D3 E1 to CV= 3.56% D1 E2) from foreign (CV= 2.43% D3 E1 to CV= 5.20% D2 E2), while there was no clear legality in the growth of seedling (Table 3).

From the group of light seeds (D1) 2.6% of dormant seeds were found and from dark (D3) 9.3% in the E1 period. After fifteen months (E1) from the D1 group, there was no dormant seed, while from D3 0.8% were found whereas B4 had 3% of dormant seed (Table 3).

3.3. Experiment II

In general between the germinability and the seedling growth (the shoot+root), significant correlative interdependence but of different strength (varieties B2, P≥0.001 to B7 and B2 P≥0.05) was established in all varieties (Table 4).

3.4. Experiment III

The application of the aging test on all varieties has shown that darker seeds have shown a stronger vitality which indicates a higher potential for storing darker seeds (Table 6). On the other side, the aging test did not detect the differences between the varieties in the potentials for use in the following period, not even at one factor (C, D, E), but clearly detected differences between seeds of different ages in all varieties (Table 5 and 6). According to Avcıet al (2017), the results showed that there was a significant difference in seed vigor of sorghum cultivars grown at same ecological conditions end the significant relationship between accelerated aging and laboratory emergence (r= 0.967).

After sowing in containers in the E1 and E2 period in field conditions on the 12th day the seedlings were harvested from the humid soil, which represented field germinability. As in laboratory conditions (Experiment I), the growth of seedlings was measured. By calculating the correlation coefficient (r) between the germinability and the growth of seedlings, significant interdependence was established in all varieties as follows: varieties B8, B10, B12; P≥0.001 to variety B7; P≥0.05 (Table 4).

Germination of seeds is a complex physiological-biochemical process controlled by many mechanisms (Bewley 1997; Liu et al 2013; Gu et al 2016).From an agricultural point of view, high germinability generally means, the realization of the projected number of plants with a smaller amount of seed, i.e., cheaper cropping. The second requirement for agriculture is as strong as possible the initial growth

Table 2- Influence of varieties on germination and dormancy of seeds and the growth of seedlings from seed of different size after three-E1 and fifteen months-E2

	C1 small				*C2 medium*				*C3 large*			
	G%	*D%*	*S cm*	*R cm*	*G%*	*D%*	*S cm*	*R cm*	*G%*	*D%*	*S cm*	*R cm*
B1	88±2.88 ab	8±0.84 ab	6.31±0.07ab	5.63±0.07 ab	91±1.92ab	6±0.77 ab	6.66±0.04 b	5.93±0.09 ab	94±3.22a	6±0.99 b	7.21±0.05 ab	6.33±0.07 b
B2	91±2.64 a	5±0.92bc	6.69±0.06 ab	5.91±0.04 a	91±2.72ab	7±0.84 ab	7.89±0.05 ab	6.31±0.07 a	92±2.52ab	6±0.61 b	7.35±0.07a	6.69±0.08 ab
B3	87±2.69 b	9±1.02 a	6.71±0.04 ab	5.51±0.05 ab	93±2.31a	5±0.91b	6.82±0.07 ab	5.91±0.05 ab	94±2.73a	6±0.84 b	7.33±0.09 ab	6.28±0.04 b
B4	86±2.44 b	6±0.99bc	6.59±0.08 ab	5.45±0.06b	89±2.11b	8±0.84 a	6.69±0.05 ab	5.99±0.11 ab	90±2.94 ab	7±0.66 ab	6.99±0.04b	6.31±0.06 b
B5	91±2.72 a	7±0.79b	6.71±0.06 ab	5.99±0.06a	91±2.52ab	6±0.52 ab	6.99±0.04 a	6.49±0.07a	92±2.66ab	6±0.56 b	7.41±0.07 ab	6.80±0.03 a
B6	89±2.68ab	8±0.81ab	6.79±0.04 ab	5.71±0.04 ab	90±2.88 b	5±0.81b	6.81±0.05 ab	6.19±0.04 a	93±1.72ab	7±0.59 ab	7.08±0.06b	6.52±0.04 ab
B7	87±2.94b	4±0.80c	6.08±0.05b	5.41±0.05b	90±3.13b	7±0.94 ab	6.59±0.04 b	5.69±0.06 b	90±2.41ab	5±1.04 ab	7.02±0.07b	6.44±0.05 ab
B8	88±2.34 ab	7±1.12b	6.82±0.06 ab	5.82±0.07a	94±2.23a	6±0.68 ab	6.89±0.04a	5.87±0.08ab	95±2.99 a	5±0.80 b	7.19±0.08 ab	6.52±0.07 ab
B9	90±2.55ab	9±0.75 a	6.88±0.03a	5.61±0.04 ab	90±2.74 b	6±0.65 ab	6.86±0.05 ab	5.77±0.10 b	91±2.56b	8±0.53a	7.39±0.10a	6.64±0.04 ab
CV%	2.04	24.7	3.95	3.59	1.74	15.6	5.61	4.35	1.95	15.62	2.23	2.79
$\overline{X}$	88.6	7.00	6.62	5.67	91.0	6.22	6.91	6.02	92.3	6.22	7.22	6.50
B10	91±2.82a	6±0.55bc	6.89±0.04a	5.97±0.07 a	92±2.79ab	8±0.64a	6.98±0.08 a	6.37±0.05 a	93±1.88ab	7±0.39ab	7.40±0.05ab	6.79±0.05 a
B11	92±2.61 a	7±0.77b	6.91±0.06a	5.89±0.06a	92±2.15ab	8±0.48a	7.01±0.05a	5.99±0.08ab	93±2.63 ab	7±0.65 ab	7.31±0.07ab	6.59±0.06 ab
B12	88±2.84 ab	8±0.81ab	6.71±0.04ab	5.77±0.04 ab	93±2.62a	6±0.62 ab	6.82±0.07 ab	5.97±0.07 ab	94±2.02a	6±0.77 b	7.41±0.08a	6.71±0.04a
B13	87±2.72 b	5±0.88bc	6.56±0.05 ab	5.48±0.04 ab	90±2.71b	7±0.82 ab	6.65±0.03 b	5.68±0.06b	90±2.72 b	6±0.80 b	7.11±0.09 ab	6.51±0.05 ab
CV%	2.35	19.9	2.44	3.72	1.37	13.2	2.42	4.71	1.87	8.88	1.90	1.87
$\overline{X}$	89.5	6.50	6.77	5.78	91.8	7.25	6.87	6.00	92.5	6.50	7.31	6.65
$\overline{X}$ g.	88.8	6.8	6.67	5.70	91.2	6.5	6.90	6.01	92.4	6.3	7.25	6.55
E2B1	86±3.04 b	0±0.52b	6.33±0.03c	5.59±0.07d-f	88±2.12 c	0±0.00 b	6.43±0.05 bc	5.69±0.05 a-c	89±2.09 b	1±0.58 a	7.20±0.04 ab	6.31±0.05 f
B2	90±2.92 a	1±0.89ab	6.71±0.07ab	5.92±0.04ab	92±2.36 a-c	1±0.81ab	6.71±0.06 a-c	5.99±0.03 a	93±2.70 ab	1±0.58 a	7.29±0.07 ab	6.66±0.04 a-c
B3	89±2.74 ab	2±1.02a	6.71±0.04ab	5.53±0.06 e-g	91±2.72 a-c	0±0.00 b	6.71±0.05 a-c	5.63±0.06 a-c	93±1.62 ab	1±0.50 a	7.31±0.06 a	6.25±0.03 f
B4	91±2.91 a	0±0.00b	6.48±0.08bc	5.48±0.04 f-g	92±2.77 a-c	1±0.81ab	6.58±0.06 ac	5.58±0.04 bc	93±2.79 ab	0±0.00 b	6.87±0.07 b	6.29±0.06 f
B5	91±1.98 a	0±0.00b	6.69±0.07ab	5.96±0.06ab	93±3.12 ab	0±0.00 b	6.75±0.05 ab	5.99±0.05 a	95±2.92a	1±0.81a	7.39±0.03 a	6.81±0.05 a
B6	90±2.04 a	0±0.00b	6.72±0.05ab	5.68±0.05 c-e	91±2.99 a-c	0±0.00 b	6.72±0.08 a-c	5.78±0.06 a-c	92±2.65 ab	0±0.00 b	7.02±0.06 b	6.48±0.07 de
B7	89±2.72 ab	2±0.75a	6.00±0.10d	5.39±0.04 g	90±2.06 bc	2±0.82 a	6.00±0.05 d	5.48±0.04 c	91±3.21 ab	1±0.00 a	6.89±0.05 b	6.4±0.04 ef
B8	89±2.91 ab	0±0.00b	6.75±0.11ab	5.84±0.06 ac	91±2.55 a-c	0±0.00 b	6.75±0.09 a-b	5.94±0.07 ab	92±2.12 ab	0±0.00 b	7.11±0.08 ab	6.49±0.05de
B9	90±2.84 a	0±0.50b	6.79±0.07a	5.59±0.04 d-f	92±2.77 a-c	0±0.00 b	6.79±0.06 a-b	5.69±0.05 a-c	93±2.88 ab	1±0.58 a	7.33±0.04 a	6.62±0.06 b-d
CV%	1.69	159	3.98	3.54	1.59	163	3.82	3.23	1.80	75.0	2.71	2.91
$\overline{X}$	89.4	0.556	6.58	5.66	91,1	0.444	6.60	5.75	92.3	0.667	7.16	6.48
B10	93±2.91 a	2±0.68a	6.86±0.05a	5.99±0.05 a	95±1.58 a	1±0.81 ab	6.89±0.07 a	6.08±0.03 a-c	96±2.79 a	1±0.50 a	7.36±0.05 a	6.77±0.08 ab
B11	92±2.59 a	1±0.62ab	6.89±0.03a	5.90±0.07 ab	93±2.65 ab	0±0.00b	6.89±0.08 a	5.99±0.05 a	95±2.23 a	1±0.81a	7.30±0.06 ab	6.58±0.04 cd
B12	88±2.62ab	0±0.50b	6.65 ±0.07ab	5.75±0.06 b-c	89±2.16 a-c	2±0.98 a	6.65±0.07 a-c	5.85±0.06 a-c	90±2.52 b	0±0.00 b	7.31±0.05 a	6.63±0.03 b-d
B13	87±2.09ab	0±0.00 b	6.49±0.08bc	5.50±0.04 e-g	88±2.31 c	0±0.00 b	6.59±0.04 cd	5.59±0.05 bc	90±2.90 b	0±0.00 b	7.08±0.07 ab	6.52±0.05 c-e
CV%	3.27	128	2.80	3.70	3.62	128	2.34	3.64	3.45	115	1.71	1.61
$\overline{X}$	90.0	0.750	6.72	5.79	91.3	0.750	6.76	5.88	92.8	0.500	7.26	6.63
$\overline{X}$ g.	89.6	0.6	6,62	5.70	91.2	0.5	6.65	5.79	92.5	0.6	7.19	6.52

Tukey multiplex test (P≥0.05)

Table 3- Influence of varieties on the germination and dormancy of seeds and growth of seedlings from the seeds of different color after three-E1 and fifteen months- E2

	D1 light				*D2 transient*				*D3 dark*			
	G%	*D%*	*S cm*	*R cm*	*G%*	*D%*	*S cm*	*R cm*	*G%*	*D%*	*S cm*	*R cm*
B1	90±2.66 cd	2±0.74 cd	6.29±0.07 cd	5.63±0.07 b-d	90±2.32 ab	8±0.65 ab	6.44±0.04 bc	5.53±0.09 ab	88±2.82 ab	9±0.79 b-d	6.19±0.06 ab	5.03±0.07 de
B2	95±2.53 ab	4±0.69 ab	7.03±0.06 a	5.91±0.04 ab	89±2.55 ab	8±0.64 ab	6.89±0.05 a	5.91±0.07 a	88±2.64 ab	10±0.59 a-c	6.33±0.07a	5.31±0.08 b
B3	91±3.09 b-d	3±0.92 bc	6.35±0.04 cd	5.51±0.06 cd	88±2.61 ab	6±0.50 b	6.41±0.07 c	5.51±0.05 ab	91±2.55 a	8±0.63 cd	6.29±0.08 ab	5.01±0.04 de
B4	91±2.99 b-d	2±0.66 cd	6.63±0.08 b	5.45±0.05d	89±3.01 ab	7±0.73 ab	6.39±0.05 c	5.59±0.11 ab	87±2.72 ab	10±0.61 a-c	6.00±0.03b	4.99±0.06 e
B5	95±2.02 ab	3±0.61 bc	6.99±0.06 a	5.99±0.04a	93±2.77 a	6±0.59 b	6.71±0.04 a-c	6.09±0.07a	88±2.78 ab	10±0.48 a-c	6.39±0.06 ab	5.51±0.03 a
B6	91±2.61 b-d	4±0.71 ab	6.41±0.04 c	5.71±0.06 a-d	90±2.89 ab	5±0.49 bc	6.61±0.05 bc	6.09±0.04 a	90±2.02 a	8±0.71 cd	5.98±0.10b	5.23±0.04 bc
B7	89±2.11 d	1±0.89 d	6.19±0.05 d	5.41±0.05d	89±3.01 ab	9±0.54 a	6.43±0.04 bc	5.29±0.06 b	87±2.50 ab	12±0.94 a	6.01±0.05b	5.20±0.05 bc
B8	91±2.51 b-d	2±0.82 cd	6.33±0.06 cd	5.82±0.06ab	90±2.55 ab	6±0.68 b	6.49±0.04a-c	5.47±0.08ab	90±2.68 a	7±0.76 d	6.21±0.08 ab	5.31±0.07 b
B9	94±2.05 a-c	5±0.77 a	6.89±0.03 9	5.61±0.07b-d	90±2.93 ab	8±0.77 a	6.56±0.05 ab	5.37±0.10 b	89±2.80 ab	10±0.39 a-c	6.40±0.09a	5.32±0.04 b
CV%	2.40	43.9	4.96	3.59	1.55	18.9	2.53	5.36	1.59	16.1	2.71	3.35
$\overline{X}$	91.9	2.89	6.57	5.67	89.8	7.00	6.55	5.65	88.7	9.33	6.20	5.21
B10	95±2.66 ab	2±0.66 cd	7.04±0.04 a	5.97±0.09 a	92±2.19 ab	5±0.55 bc	6.68±0.08 bc	6.07±0.05 a	90±1.81 a	9±0.36 b-d	6.35±0.09ab	5.51±0.05 a
B11	96±2.61 a	2±0.71 cd	7.01±0.06 a	5.89±0.04ab	92±2.66 ab	5±0.68 bc	6.71±0.05c	5.59±0.08ab	91±2.59 a	8±0.69 cd	6.30±0.06ab	5.31±0.06 b
B12	90±2.12 cd	3±0.59 bc	6.41±0.04 c	5.77±0.05 a-c	90±2.81 ab	6±0.52 b	6.42±0.07 d	5.57±0.07 ab	89±2.41 ab	9±0.71 b-d	6.39±0.09a	5.19±0.04bc
B13	89±2.13 d	1±0.64 d	6.28±0.05 cd	5.48±0.06 cd	87±3.01 b	6±0.80 b	6.41±0.03 d	5.38±0.06b	86±2.12 b	11±0.80 ab	6.09±0.06 ab	5.14±0.05 cd
CV%	3.80	40.82	5.93	3.72	2.62	10.50	2.47	5.20	2.43	13.6	2.13	3.11
$\overline{X}$	92.5	2.00	6.69	5.78	90.3	5.50	6.56	5.65	89.0	9.25	6.28	5.29
$\overline{X}$ g.	92	2.6	6.60	5.70	90	6.5	6.54	5.65	88	9.3	6.23	5.24
E2B1	81±2.44bc	0±0.50	5.21±0.07de	4.77±0.07ab	86±2.65 de	0±0.00 b	6.41±0.04 b	5.56±0.05 a-c	94±2.09 ab	1±0.58 c	7.41±0.04 a	6.52±0.05 a
B2	87±2.60 a-c	0±0.00	5.60±0.06ab	5.18±0.04a	91±2.31 a-c	2±0.77a	6.66±0.07 ab	5.79±0.03 a	97±2.70 ab	2±0.58 b	7.48±0.03 a	6.61±0.04 a
B3	85±2.71 a-c	0±0.00	5.61±0.04a	4.76±0.06 ab	90±2.52 a-d	1±0.52 ab	6.58±0.05 ab	5.59±0.06 a-c	96±1.62 ab	1±0.50 c	7.39±0.04 a	6.56±0.03 a
B4	86±2.66 a-c	0±0.00	5.36±0.07b-d	4.70±0.04 ab	92±241 a-c	1±0.00 ab	6.71±0.09 ab	5.52±0.04 bc	96±2.79 ab	3±0.00 a	7.47±0.03 a	6.67±0.06 a
B5	87±1.78 ab	0±0.00	5.60±0.06ab	5.16±0.06a	93±2.02 ab	0±0.00 b	6.77±0.04 ab	5.87±0.05 a	98±2.92 a	0±0.81 d	7.39±0.04 a	6.82±0.05 a
B6	88±2.56 a	0±0.00	5.61±0.08a	4.81±0.05 ab	89±2.65 b-e	0±0.50 b	6.69±0.07 ab	5.76±0.06 a-c	93±2.65 ab	0±0.00 d	7.32±0.05 a	6.71±0.07 a
B7	80±2.66 c	0±0.00	5.03±0.05e	4.60±0.04 b	88±2.56 c-e	1±0.52 ab	6.47±0.06 b	5.55±0.04 c	94±3.21 ab	2±0.00 b	7.51±0.03 a	6.51±0.04 a
B8	81±2.71 bc	0±0.00	5.66±0.06a	4.99±0.06 ab	90±2.41 a-d	0±0.00 b	6.55±0.07 ab	5.88±0.07 ab	94±2.12 ab	1±0.00 c	7.32±0.04 a	6.52±0.05 a
B9	85 ±2.88a-c	0±0.00	5.68±0.08a	4.83±0.04 ab	88±2.57 c-e	0±0.00 b	6.49±0.05 cd	5.71±0.05 a-c	96±2.88 ab	0±0.58 d	7.33±0.04 a	6.69±0.06 a
CV%	3.56	0.00	4.20	4.13	2.43	130	1.86	2.48	1.74	94.87	0.97	1.61
$\overline{X}$	84.4	0.00	5.48	4.87	89.7	0.56	6.59	5.69	95.3	1.11	7.40	6.62
B10	88±2.72 a	0±0.00	5.67±0.09a	5.09±0.05 ab	94±1.50 a	0±0.52 b	6.81±0.07 a	5.98±0.03 a-c	98±2.79 a	0±0.50 d	7.44±0.05 a	6.78±0.08 a
B11	86±2.51 a-c	0±0.00	5.66±0.04a	4.99±0.07 ab	93±2.99 ab	0±0.00b	6.76±0.09 ab	5.92±0.05 a	97±2.23 ab	0±0.81 d	7.47±0.03 a	6.63±0.04 a
B12	82±2.19 a-c	0±0.50	5.54±0.08 a-b	4.88±0.06 ab	86±2.20 de	0±0.00 b	6.55±0.10 ab	5.88±0.06 a-c	92±2.52 b	0±0.00 d	7.38±0.04 a	6.63±0.03 a
B13	81±2.09 bc	0±0.00	5.31±0.07c-d	4.72±0.04 ab	85±2.38 e	0±0.00 b	6.42±0.06 b	5.63±0.05 bc	92±2.90 b	0±0.00 d	7.37±0.03 a	6.59±0.05 a
CV%	3.92	0.00	3.02	3.22	5.20	0.00	2.75	2.63	3.38	0.00	0.65	1.26
$\overline{X}$	84.3	0.00	5.55	4.92	89.5	0.00	6.64	5.85	94.8	0.00	7.42	6.66
$\overline{X}$ g.	84	0.0	5.50	4.88	90	0.4	6.57	5.79	95	0.8	7.41	6.63

Tukey multiplex test (P≥0.05)

Table 4- Coefficients of simple correlations (r) between germination and growth of seedling (stem cm, root cm) of oilseed rape varieties (n= 36)

Variety	*Stem (cm)*	*Root (cm)*
B1	*	**
B2	***	***
B3	**	*
B4	*	**
B5	***	*
B6	**	**
B7	*	*
B8	***	**
B9	*	*
B10	***	*
B11	*	**
B12	***	**
B13	**	*

Indicates *, a significant trend at P≥0.05; **, a significant trend at P≥0.01; ***, a significant trend at ≥0.001

Table 5- Coefficients of simple correlations (r) between germination and growthof seedlings in laboratory conditions and in the field of the varieties of oilseedrape for germination, and growth of seedlings (n= 36)

Variety	*Germinability (%)*	*Stem (cm)*	*Root (cm)*
B1	***	**	***
B2	**	***	***
B3	***	*	**
B4	***	***	**
B5	***	***	*
B6	**	**	**
B7	*	*	*
B8	***	***	***
B9	***	*	**
B10	***	***	***
B11	***	**	*
B12	***	***	***
B13	**	**	***

Indicates *, a significant trend at P≥0.05; **, a significant trend at P≥0.01; ***, a significant trend at P≥0.001

of seedling that can withstand unfavorable climatic conditions and/or competition with weeds, etc.

The association of the seed size with germinability and the initial growth of seedling is dependent on the plant species (Ambika et al 2014). Increased germination and initial growth of seedlings are mainly associated with greater reserve food in larger seeds (Willenborg et al 2005). In wheat, larger seeds achieved higher germination by 4% compared to small but the greatest influence was on the growth of seedlings (P≥0.002) (Chaffai & Louchichi 2013). And in the seed of oats increase in germination was the same (4%, the average of five varieties) and was achieved from larger seeds, while depending on the variety, the germination varied more considerably (14%) (Mut & Akay 2010). Thus, our results are consistent with it (Table 2).

Seed dormancy is a form of biological adaptation that prevents germination on the plant itself (viviparia), as well as germination of seeds at an inconvenient time of year, when the seedlings would not endure adverse environmental conditions (Bewley 1997).

The basic division of seed dormancy is on embryonic, where dormancy control is performed within the embryo itself, and the on the dormancy caused by a seed coat that is waterproof and impermeable to gases, as well as a division into primary and secondary dormancy or their combinations Baskin & Baskin (2004). Regardless of the form of seed dormancy, it conditions the subsequent germination of the seed whose seedlings even in the already established crop in competition with the already developed plants are with little chance of contributing to the establishment of crops of agricultural cultures, and from the agronomic aspect in general the seed dormancy is undesirable.

Also in the rapeseed according to Rahman & McVetty (2011) on the seed after harvest, dormancy is pronounced, which means that these seeds will not germinate, although they have ideal germinating conditions (Koornneef & Karssen 1994).

The color of the oilseed rape seeds is determined by genetics, where the yellow seed coat is desirable because these seeds are characterized by a higher oil and protein content as well as their better quality (Rahman et al 2010; 2011). According to Neubert et al (2003) between the color of the seed coat and the chemical composition as well as the seed formation of the rapeseed, there is a connection, and the seeds with a darker seed coat contain more flavonoids that condition the seed inactivity.This also may be the reason for the variability of germination oilseed rape varieties after different concentrations of NaCl (Uyanıket al 2014).

Seeds of different groups of plants (Cereals, Legumes, Oil crops, Miscellaneous crops) are highly variable for germination during the storage period (Nagel & Börner 2010). For oily cultures, it is characteristic that, during seed storage, degradation of the lipids leads to degradation of the quality (Graham 2008). According to Debeaujon et al (2000), the anatomical material and color of the rapeseed seed coat affect germination during the storage period. In our tests, crucial for deterioration or even improvement of the seed germinability was the seed coat color (Tables 2 and 5). That can be brought into conjunction with higher oil content in seeds where the seed color is brighter (Rahman et al 2010; 2011).

Table 6- Application of the aging test to the seed of different sizes (C1-C3), colors (D1-D3) and age (E0-A1) of rape seedlings

Variety	*Seed size*			*Seed color*			*Seed age*		
	C1	*C2*	*C3*	*D1*	*D2*	*D3*	*E0*	*E1*	*E2*
B1	45 ^{aA}	43 ^{aA}	44 ^{aA}	39 ^{aA}	44 ^{aA}	52 ^{aB}	62 ^{aA}	45 ^{aB}	39 ^{aC}
B2	43 ^{aA}	44 ^{aA}	45 ^{aA}	40 ^{aA}	46 ^{aAB}	56 ^{aB}	58 ^{aA}	48 ^{aB}	36 ^{aC}
B3	46 ^{aA}	46 ^{aA}	45 ^{aA}	42 ^{aA}	47 ^{aA}	53 ^{aB}	58 ^{aA}	46 ^{aB}	39 ^{aC}
B4	45 ^{aA}	42 ^{aA}	43 ^{aA}	38 ^{aA}	44 ^{aAB}	55 ^{aB}	60 ^{aA}	47 ^{aB}	39 ^{aC}
B5	44 ^{aA}	44 ^{aA}	42 ^{aA}	41 ^{aA}	47 ^{aAB}	56 ^{aB}	61 ^{aA}	45 ^{aB}	37 ^{aC}
B6	43 ^{aA}	45 ^{aA}	44 ^{aA}	42 ^{aA}	48 ^{aAB}	55 ^{aB}	58 ^{aA}	48 ^{aB}	39 ^{aC}
B7	44 ^{aA}	43 ^{aA}	44 ^{aA}	40 ^{aA}	46 ^{aAB}	54 ^{aB}	59 ^{aA}	46 ^{aB}	36 ^{aC}
B8	45 ^{aA}	46 ^{aA}	44 ^{aA}	38 ^{aA}	47 ^{aB}	56 ^{aC}	62 ^{aA}	46 ^{aB}	38 ^{aC}
B9	46 ^{aA}	46 ^{aA}	44 ^{aA}	39 ^{aA}	45 ^{aAB}	52 ^{aB}	61 ^{aA}	48 ^{aB}	36 ^{aC}
B10	45 ^{aA}	44 ^{aA}	43 ^{aA}	42 ^{aA}	45 ^{aA}	54 ^{aB}	59 ^{aA}	46 ^{aB}	38 ^{aC}
B11	43 ^{aA}	45 ^{aA}	42 ^{aA}	38 ^{aA}	44 ^{aAB}	56 ^{aB}	60 ^{aA}	45 ^{aB}	36 ^{aC}
B12	42 ^{aA}	44 ^{aA}	43 ^{aA}	40 ^{aA}	46 ^{aAB}	52 ^{aB}	61 ^{aA}	46 ^{aB}	37 ^{aC}
B13	45 ^{aA}	43 ^{aA}	44 ^{aA}	41 ^{aA}	44 ^{aA}	56 ^{aB}	60 ^{aA}	48 ^{aB}	38 ^{aC}
$\overline{X}$	44.3	44.2	43.6	40.0	45.6	54.4	59.9	46.5	37.5

Tukey multiplex test (P ≥0.05), small letters show the difference a, b...x for the column, capital letters show the difference A, B...x, for the line

4. Conclusions

Higher seed germination of up to 3.6% is achieved from larger seed. Brighter seeds give higher germination after three months of seed preservation by 4%. In contrast, the darker seed yields higher germination after fifteen months of storage for 11% and has a greater potential for preserving germinability after a long period of storage. The use of seeds with higher germinability leads to a higher growth in root and stem, as indicated by their correlative interdependence (r) which is the most pronounced in the case of the following varieties: KVS Gordon, Jasna, Zlatna.

No clear difference was found between hybrid varieties (B1 to B9) and domestic varieties (B10-B13) for seed germinability and dormancy as

well as the growth of seedlings. The obtained results can usefully serve farmers when choosing varieties for the establishment of rapeseed and/or growers when creating new varieties.

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Production of Multiple Hydrolytic Enzymes by Black Aspergilli Isolated from Date and Grape

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ABSTRACT

Black aspergilli newly isolated from local grape and date were investigated for production of hydrolytic enzymes including cellulase, tannase and pectinase. Isolates were morphologically and molecularly identified as *Aspergillus niger, Aspergillus tubingensis, Aspergillus japonicus* and *Aspergillus aculeatus*. Isolates were screened for enzyme production ability on solid and in liquid media. Enzymatic activity was determined in the culture filtrate of liquid medium. A total of six isolates were found to produce multiple hydrolytic enzymes. The highest activity of cellulase was produced by *A. japonicus* ZGM4 and *A. aculeatus* ZGM6 as 40 and 35 U g^{-1} dry biomass, respectively. All the isolates exhibited high level of tannase activity in the range of 150-343 U g^{-1} dry biomass after 24 h of incubation. *A. tubingensis* ZGM5 and *A. aculeatus* ZGM6 were found to produce the highest pectinase activity at a level of 130 and 117 U g^{-1} dry biomass, respectively. In the light of these results, isolates can be used for multiple hydrolytic enzyme production in industry.

Keywords: *Aspergillus*; Cellulase; Pectinase; Tannase

1. Introduction

Enzymes are widely used in the production of chemicals, food and beverages, pulp and paper, leather, detergents, textiles, biofuels, animal feed, personal care, and pharmaceuticals. Microbial enzymes are preferred as they are relatively more stable, cheaper and have properties more diverse than those originating from plants and animals.

It is important to find a microorganism which has the capacity for producing an enzyme with sufficient activity and yield. In most cases, the scale up of purification methods leads to high loss of yield and operation cost because of multistep procedures (Ma et al 2015). In this respect, enzyme cocktails could be an attractive option for industrial processes utilizing synergistic effect of different enzymes. *Aspergillus, Rhizopus* and *Trichoderma* species have been used for production of enzyme cocktails. These cocktails have been utilized for hydrolysing tissues and cell walls of biological materials and releasing juice and functional ingredients for use in food and other industries. They are currently being investigated for valorization of food waste for production of bioactive compounds (Martinez-Avila

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et al 2014; Xu et al 2014; Dulf et al 2016; Karray et al 2016; Buenrostro-Figueroa et al 2017).

One of the most important industrial hydrolytic enzymes is cellulase which breaks the glucosidic bonds of cellulose, releasing oligosaccharides, cellobiose and glucose. This enzyme has been used for improvement of starch and protein extraction, clarification of fruit juice and releasing of antioxidants from fruits and vegetables (Kuhad et al 2011). Pectinases are composed of depolymerizing enzymes such as polygalacturonase (EC 3.2.1), pectin lyase (EC 4.2.2) and decomposting enzymes such as pectin esterase (EC 3.1.1). They have been used for extraction and clarification of fruit juices and wines, maceration of tea leaves and increase the juice and oil extraction yields as well as various biotechnological processes (Ortiz et al 2017). Another important enyzme for the food industry is tannase or tannin acyl hydrolase (EC 3.1.1.20) that catalyses the breakdown of hydrolyzable tannins or gallic acid esters to release glucose and gallic acid. Tannase is used in production of gallic acid and manufacturing of instant tea to prevent haze and sediment formation by the industry (Ma et al 2015).

Several *Aspergillus* spp. have been discovered which have capacity to produce enzymes. However, the need for isolation of new species which have the ability to produce multiple enzymes from different habitats continues. Local sources can be a good alternative to isolate microorganisms which can produce novel enzymes. Utilization of local sources enables economical and sustainable production and use of industrial enzymes in a country. The aim of this study was to investigate new strains of black aspergilli from local food sources for production of multiple hydrolytic enzymes including cellulase, pectinase and tannase.

2. Material and Methods

2.1. Materials

Dichloran Glycerol agar (DG18), Dichloran Rose Bengal Chloramphenicol (DRBC) agar, Czapek Yeast Agar with 20% Sucrose (CY20S), Czapek Dox Agar (CZ), Czapek Yeast Agar (CYA), and Malt Extract Agar (MEA) were supplied from Merck Chemicals (Darmstadt, Germany). Carboxymethylcellulose sodium salt (CMC), tannic acid, pectin, 3,5-dinitrosalicylic acid (DNS), rhodanine (2-thioxo-4-thiazolidinone), polygalacturonic acid, D-galacturonic acid and gallic acid were purchased from Sigma Chemicals (Taufkirchen, Germany). Yeast extract, agar agar, mycological peptone were purchased from Oxoid (Hampshire, UK). Congo red (Aldrich Chemicals, Taufkirchen, Germany) and Tween 80 (Acros Organic, Geel, Belgium) were also used.

2.2. Microorganisms

Aspergillus oryzae MUCL 14492 was supplied from Mycothèque de l'Université Catholique de Louvain (Louvain-la-Neuve, Belgium) and used as reference culture for qualitative screening of enzyme production.

2.3. Isolation and identification of fungal isolates

Rotten parts of grape (*Vitis vinifera* L.) and date (*Phoenix dactylifera* L.) collected from local markets were transferred onto plates containing MEA, DG18 and DRBC agar after aseptic homogenization in peptone water or directly. Cultures were purified by sub-culturing the spores from black colonies on fresh MEA medium.

Isolated molds were firstly identified according to their macroscopic and microscopic features then molecular identification analysis was performed. Isolated black colonies were grown on CY20S, CZ and MEA media at 25 °C and CYA medium at 25 and 37 °C. Each colony on all media was examined by determination of colony characteristics by naked eye and micromorphological features (MEA medium) under light microscope for species identification (Raper & Fennell 1965; Klich 2002).

DNA of molds grown on MEA medium was extracted with Biospeedy Fungal DNA kit (Bioeksen, Istanbul, Turkey). Molecular identification was performed by amplifying internal transcribed spacer region (ITS) using ITS1-5.8S rRNA and ITS2 (5'TCCTCCGCTTATTGATATGC3') as forward

and (5'GGAAGTAAAAGTCGTAACAAGG3') as reverse primers for real-time quantitative polymerase chain reaction (QPCR) (Schoch et al 2012). QPCR products were purified using PCR Purification Kit (Bio-rad Laboratories, Hercules, CA, USA). DNA sequences were analyzed with Sanger Dideoxy Sequence Termination method using ABI Prism 377 DNA Sequencing Analyser (Applied Biosystems, Foster City, CA, USA). Sequences for 18S and ITS regions were compared with the sequences available in National Center for Biotechnology Information (NCBI) database using online BLAST tool (GenBank 2016).

2.3. Screening of enzymes from solid and liquid media

Mold suspensions were prepared from 3-day old cultures on MEA slant by adding 5 mL sterile distilled water with 0.05% Tween 80. Disloged mold spores were transferred to a tube aseptically and the tube was centrifuged at 2000xg at 10 °C for 20 min. Mold spores were washed two times using peptone water. Molds were enumerated after growth on Sabouraud agar at 30 °C for 72 h.

Mold suspension (app. 10^7 spores mL^{-1}) was inoculated to the middle of the plates containing enzyme specific solid media. Media with CMC (Jayani et al 2005), tannic acid (Bradoo et al 1996) and pectin (Zheng et al 2011) were used for determination of cellulase, tannase and pectinase activity, respectively. Plates were incubated at 30 °C for 72, 48 and 72 h for cellulase, tannase and pectinase, respectively. Enzyme producing isolates were detected by observation of the clear zone around the margins of the colony. Congo red (1%, w/v) was used to dye the medium for cellulase and pectinase. The media prepared with tannic acid had a purple color which became colorless after hydrolysis by tannase.

Mold suspension with 10^7 spores mL^{-1} was added to a liquid medium (25 mL) with the same composition as the solid medium without agar and incubation took place at 30 °C in a shaking incubator at 200 rpm. Culture filtrate from Whatman filter paper (pore size 5-13 µm) was used as the crude extracellular enzyme source. Mycelial biomass was dried to a constant weight at 105 °C for 24 h to determine the biomass yield. pH of the filtrate was measured using pH meter to follow pH changes. Cellulase and pectinase enzymes were assayed according to the DNS method (Debing et al 2006; Sridevi & Charya 2011). One unit of enzyme is defined as the amount of enzyme that liberates 1 µmol of glucose and galacturonic acid by the enzyme per minute under the assay conditions for cellulase and pectinase, respectively. Tannase activity was determined by method described by Lagemaat & Pyle (2001). Tannase activity was expressed as µmol of gallic acid released per minute under the assay conditions.

2.4. Statistical analysis

Experiments and analyses were carried out in triplicate. Data were subjected to one-way analysis of variance (ANOVA). Means were compared by Tukey's test at a significance level of 0.05 (Minitab 16, Minitab Inc, Coventry, UK).

3. Results and Discussion

3.1. Isolation and identification of black aspergilli from local sources

Eight of the black colonies that grew on date and grape with different morphology were isolated for morphological and molecular identification. All isolated molds produced pigmentation with modest color yields such as brownish-black to black in front and cream to yellow in reverse of agar media. The isolates were found to exhibit features of *Aspergillus* species under the section of *Nigri* in micromorphological analysis. The strains belonging to *Aspergillus* section *Nigri* characteristically present dark-brown to black conidia, uniseriate or biseriate conidiophores, spherical vesicles and hyaline or lightly pigmented hyphae.

The molecular analysis showed that isolates belonged to genus *Aspergillus* (Table 1). The isolates ZDM1 and ZGM5 belong to the species *A. tubingensis*, ZDM2 and ZDM3 were classified as *A. niger*, ZGM4 and ZGM6 were *A. japonicus*

and *A. aculeatus*, respectively. Morphological and molecular identification findings were in agreement. Two of the colonies were found to be similar according to molecular and morphological identifications and therefore six molds were selected for further studies.

3.2. Screening of isolates for enzyme production on solid medium

Qualitative screening was performed by the hydrolysis of substrate on solid agar medium. All isolates had the ability to produce cellulase, tannase and pectinase enzymes (Table 2). The activity was detected around the colonies by the appearance of a clear zone revealed by hydrolysis of substrate. In the case of molds which did not produce any enzyme, growth was restricted and no clear zone observed.

Aspergillus oryzae MUCL 14492 was employed in screening on solid medium as a reference culture. This culture has been extensively used before particularly for synthesis of cellulase, tannase and pectinolytic enzymes (Heerd et al 2012; Pirota et al 2016; Koseki et al 2018). There were no significant differences between levels of cellulase and pectinase displayed on solid medium by the isolates and the reference culture. *A. japonicus* ZGM4 and *A. aculeatus* ZGM6 showed the highest tannase activity compared to those of the other isolates and the reference culture exhibited the lowest tannase activity. Taskin et al (2008) and Murugan et al (2007) also reported that *Aspergillus* spp. can produce these enzymes on solid media.

Some researchers found a high correlation between the levels of enzyme production determined by screening on solid and in liquid media (Ten et al 2004; Murugan et al 2007). However, in another study, a mold identified as an enzyme producer by plate screening method did not produce any enzymes in liquid medium (Tseng et al 2000). Therefore, enzyme production by a mold needs to be confirmed in liquid medium.

Table 1- Identification of isolates from grape and date on sequences

Isolate code	*Species*	*Gen bank code*	*Identities (%)*	*Source*
ZDM1	*Aspergillus tubingensis*	KP131626	589/594 (99)	Date
ZDM2	*Aspergillus niger*	KT852982	566/568 (99)	Date
ZDM3	*Aspergillus niger*	KT898789	557/558 (99)	Date
ZGM4	*Aspergillus japonicus*	KC128815	560/568 (99)	Grape
ZGM5	*Aspergillus tubingensis*	GU595290	574/576 (99)	Grape
ZGM6	*Aspergillus aculeatus*	JF439460	546/550 (99)	Grape

Table 2- Clear zone diameter of *Aspergillus* spp. measured by plate screening method

Isolates	*Cellulase*	*Tannase*	*Pectinase*
		*Clear zone (mm)**	
Aspergillus oryzae MUCL 14492	49.8±4.9[a]	15.5±0.4[c]	38.3±1.4[a]
Aspergillus tubingensis ZDM1	51.8±7.1[a]	22.7±2.3[b]	29.6±8.1[a]
Aspergillus niger ZDM2	53.9±7.7[a]	22.9±2.0[b]	36.4±10.4[a]
Aspergillus niger ZDM3	57.1±6.2[a]	22.0±2.3[b]	30.5±6.7[a]
Aspergillus japonicus ZGM4	49.3±5.9[a]	30.3±1.0[a]	28.4±5.5[a]
Aspergillus tubingensis ZGM5	54.4±8.0[a]	22.2±2.5[b]	37.8±7.3[a]
Aspergillus aculeatus ZGM6	51.0±7.4[a]	28.9±1.9[a]	30.7±9.2[a]

*, Mean±Standard deviation (n= 3); Means marked with different letters in the same column are significantly different (P<0.05)

3.3. Screening of isolates for enzyme production in liquid medium

Biomass of all isolates did not change significantly after the first day of incubation in medium used for cellulase (5.36±1.22 mg mL^{-1}) and pectinase (5.71±0.15 mg mL^{-1}) production. Biomass increased at the beginning of incubation (3.76±0.51 mg mL^{-1}) and then remained constant until the end of incubation in the case of tannase.

3.3.1. Cellulase

The pH value of the medium did not change during incubation. Cellulase activity of all isolates increased during the incubation period and peak activity was achieved after 96 h (Figure 1). The highest cellulase activity was produced by *A. japonicus* ZGM4 with a peak activity of 40±4.5 U g^{-1} dry biomass (3.8±0.5 U mL^{-1}). Peak enzyme activity of isolates of *Aspergillus* spp. expressed per volume of medium was ranged between 2-3.8 U mL^{-1}, except *A. tubingensis* ZGM5 which produced an activity of 0.56 U mL^{-1}. Gautam et al (2011) reported the peak cellulase activity for *A. niger* after 96 h of incubation as 1.8 U mL^{-1}. Imran et al (2017) also reported that cellulase activity of *A. tubingensis* IMMIS2 increased continuously up to 96 h and then decreased. Enzyme activity fluctuated in some isolates which can be explained by catabolite repression from excess product during degradation of cellulose (Ang et al 2013).

3.3.2. Tannase

A. niger ZDM2 and *A. japonicus* ZGM4 exhibited the highest tannase activity at 326±57 U g^{-1} dry biomass and 343±107 U g^{-1} dry biomass, respectively, after 24 h of incubation (Figure 2). All isolates were found to produce higher activity of tannase compared to those reported in the literature for *Aspergillus* spp. by Banerjee et al (2007) and Murugan et al (2007). Lal et al (2012) reported higher tannase activity than those in this study for *A. niger* isolated from bark of *Acacia nilotica* which contained high level of tannin.

There are differences in reported incubation times for maximum tannase activity in the literature. Yadav et al (2008) found that maximum tannase production by *A. fumigatus* was obtained after 96 h of incubation, whereas Banerjee et al (2007) found a maximum tannase production by *A. aculeatus* after 72 h of incubation. In this study, the highest tannase activity was obtained

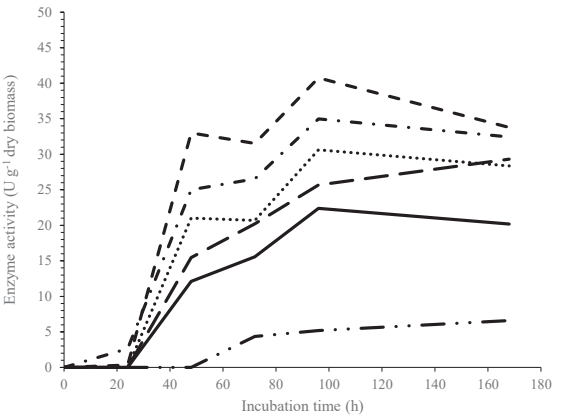

Figure 1- Change in cellulase activity of *Aspergillus* spp. during seven days of incubation (n= 3). *A. tubingensis* ZGM5 (▪ ▪); *A. tubingensis* ZGM1 (▪); *A. niger* ZDM3 (▪); *A. niger* ZDM2 (▪ ▪ ▪); *A. aculeatus* ZGM6 (▪); *A. japonicus* ZGM4 (▬)

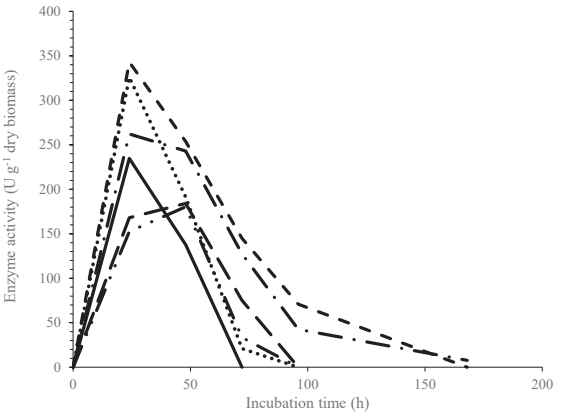

Figure 2- Change of tannase activity of *Aspergillus* spp. during seven days of incubation (n= 3). *A. tubingensis* ZGM5 (▪ ▪); *A. tubingensis* ZGM1 (▪); *A. niger* ZDM3 (▪); *A. niger* ZDM2 (▪ ▪ ▪); *A. aculeatus* ZGM6 (▪); *A. japonicus* ZGM4 (▬)

after 24 h incubation and then there was a decline for all *Aspergillus* spp. The reason for the decline in enzyme production can be explained by the accumulation of end-products like gallic acid and secretion of toxic substances like catechuic acid, benzoic acid and pyrogallol which can cause cell disruption (Kar & Banerjee 2000). Previous studies also reported that pyrogallols, gallic acid and gallaldehyde can inhibit the tannase activity of *A. niger* (Srivastava & Kar 2009).

The pH changes could also affect tannase enyzme production negatively, because tannase enzyme is an acidic protein and its optimum pH is around 5.5 (Banerjee et al 2007). Initial pH of the medium was around 4 and it declined to 3 after 1 day of incubation. It started to increase after 2 days of incubation and it reached to 6-7 after 7 days of incubation. The increase in the pH can be due to the consumption of tannic acids or the production of alkaline compounds during the incubation time (Zeni et al 2011). Lal et al (2012) also reported similar trend for tannase activity of *A. niger* with pH changes where maximum activity was observed at pH 5.0, and tannase activity start to decrease when the pH of medium reached the alkaline range.

3.3.3. Pectinase

All isolates produced pectinase but the incubation time for peak activity was changed according to the isolate (Figure 3). Highest activity of pectinase (130±66.2 U g^{-1} dry biomass; 25±1.5 U mL^{-1}) was obtained from *A. tubingensis* ZGM5. Taskin et al (2008) reported pectinase production by *Aspergillus* spp. isolated from vineyards with activity in the range of 44-122 U mL^{-1}. The difference between these results could be due to the composition of the media and the method used for determination of activity.

For pectinase enzyme, pH of medium showed fluctuations during incubation time depending on the isolate. pH of liquid medium used for all isolates decreased from 4.5 to 3-3.5 after 3 days of incubation. An increase in pH to 5.5 was observed for *A. japonicus* ZGM4 and *A. aculeatus* ZGM6

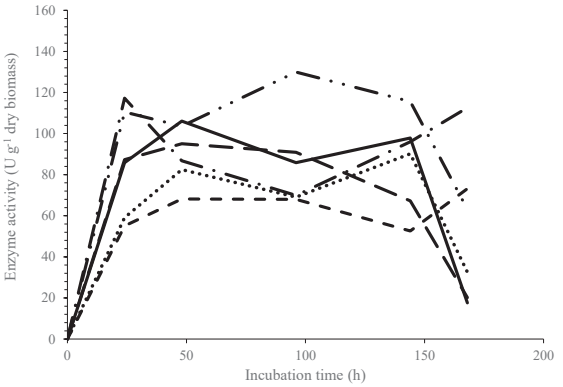

Figure 3- Change of pectinase activity of *Aspergillus* spp. during seven days of incubation (n=3). *A. tubingensis* ZGM5 (▪ ▪); *A. tubingensis* ZGM1 (▪); *A. niger* ZDM3 (▪); *A. niger* ZDM2 (▪ ▪ ▪); *A. aculeatus* ZGM6 (▪); *A. japonicus* ZGM4 (▬)

while the pH remained constant for other isolates after 4 days. Mahesh et al (2016) reported that pH 4 was optimum for pectinase production by *Aspergillus ibericus* and increase in fermentation medium pH from 3 to 4 increased the production of pectinase after that activity started to decrease with increasing pH. The increase in pH can be caused by consumption of organic acids by molds as nutrients because of a lack of carbon source (Botella et al 2005). The decrease in pH can be related to the release of galacturonic acid to the medium due to the action of pectinase enzymes of the molds during the first day of incubation (Zeni et al 2011).

The enzyme production is generally associated with the growth phase of microorganisms. For some isolates, there was a decline in activity of tannase and pectinase after a few days of incubation. This decline might be explained with the hydrolysis of the produced enzymes by isolates due to lack of nutrients in fermentation media (Botella et al 2005). In addition, changes in the fermentation conditions such as pH and production of inhibitory substances compared to the starting conditions could affect the activity of enzymes (Gautam et al 2011; Zeni et al 2011).

4. Conclusions

This study showed that newly isolated black *Aspergillus* spp. have good potential for use as a source of hydrolytic enzymes. All isolates produced tannase with high activity, however *A. japonicus* ZGM4 and *A. aculeatus* ZGM6 were found to be the best isolates for producing tannase. Additionally, *A. tubingensis* ZGM5 can produce tannase and pectinase, while it was not competent to produce cellulase with high activity. Among the isolated molds, *A. aculeatus* ZGM6 had the ability to produce all studied hydrolytic enzymes at a high activity. Isolates from this study can be utilized as a source for multiple enzyme production after further studies on optimization of fermentation conditions and scale-up.

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Effects of Olive Leaf (Oleuropein) Supplementation on Quality of Breast Meat in Broilers

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ABSTRACT

In this study, we investigated the effect of dietary olive leaf (oleuropein) supplementation at different levels on breast meat color and pH_{24} in along with TBA values of breast meats which were stored at +4 °C during the 11-day in broilers. For this purpose 320 one-day-old Ross-308 chicks randomly assigned to four groups (5 replicates per group, 16 chicks per replicates). In trial, dietary treatments consist of corn-soybean meal diet without or with 125, 250 and 500 mg kg^{-1} oleuropein supplementation respectively. At the end of trial, two chicks per replicate were slaughtered and meat samples were collected for lipid oxidation, color and pH_{24} measurement. According to the obtained findings, 250 mg kg^{-1} oleuropein supplementation on broiler diets significantly ($P<0.05$) decreased TBA values (mg MDA kg^{-1} meat) of breast meats compared with other groups. TBA values of breast meats significantly ($P<0.05$) increased during storage time. While breast meat brightness (L*) and yellowness (b*) values and pH_{24} were not significantly ($P>0.05$) affected by oleuropein supplementation at different levels, redness (a*) value significantly ($P<0.05$) increased compared with control group. As a result of the study, it is possible to say that oleuropein demonstrated antioxidant activity linked with supplementation level and it can be used at level of 250 mg kg^{-1} as phytobiotic antioxidant in broiler diets.

Keywords: Olive leaf; Oleuropein; Broiler; Antioxidant; Lipid oxidation

1. Introduction

Lipid oxidation and microbial growth are major problems during the storage of chicken meat. Therefore, the use of antimicrobial and antioxidants in broiler is quite common. Nowadays, the use of natural antioxidants instead of synthetic antioxidants (butyl hydroxyanisole, butyl hydroxy toluene, tertiary butyl hydroxyquinonone and propyl gallates) are on the agenda because of consumers' demand for safety food and natural feed additives (Botsoglou et al 2010a). In this demand, the use of vitamins such as E or C vitamins and some phytobiotics as natural antioxidants are emphasized. Phytobiotics can be defined as extracts or essential oils from various parts of plants. The use of some plants and spices such as carnations, rosemary, sage, green tea, thyme and olive leaf, which are antioxidant, in broiler chickens has been discussed in recent years. These products can be used either directly or processed extract form or essential oil form on broiler feed (Loetscher et al 2013).

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Olive and olive oil production are important and traditional agro-industrial activity in the Mediterranean countries since the ancient times and almost all of the world's olive production and consumption is carried out in Mediterranean countries (Basmacıoğlu-Malayoğlu & Aktaş 2011). It is reported that olive leaf, olive oil processing by products, contains more than 30 phenolic compounds which are generally classified as phenolic acids, phenolic alcohols, flavonoids, secoiridoid and lignans. One of these, oleuropein is the major bioactive compound and may reach concentrations of 90 g kg^{-1} in dried olive leaves (Benavente-Garcia et al 2000). Antimicrobial and antioxidant effects of this compound have been demonstrated with some studies (Bisignano et al 1999; Markin et al 2003; Lee & Lee 2010).

In this study, we aimed the investigate effects of oleuropein supplementation at different levels on meat quality and TBA values of breast meat during the storage time in broilers.

2. Material and Methods

2.1. Animals and experimental treatments

In this trial, 320 one-day-old Ross308 chicks were randomly assigned to 4 groups (5 replicates/group, 16 chicks/replicates). At the beginning of study chicks were weighed, wing banded and distributed into 20 floors. Each 1.2×1.1 m^2 floor pen was furnished with wood shavings litter, a round feeder and a round drinker. Water and feed were offered *ad libitum*. Temperature and relative humidity was maintained within the optimum range. Lighting was 23 h light and one hour darkness. The trial lasted for 6 weeks. The basal diet formulated in granule form for starter (1-10 days of age) and pellet forms for grower (11-24 days of age) and finisher (25-42 days of age) periods. The ingredients and composition of basal diets used at different ages is shown in Table 1. The experimental groups consisted of control group fed the basal diet (no oleuropein supplementation) and three groups fed the basal diet supplemented with oleuropein at levels of 125 (OLE125), 250 (OLE250) and 500 (OLE500) mg kg^{-1} of diet, respectively. For this purpose firstly oleuropein content of olive leaf was analyzed. Olive leaf's chemical composition, oleuropein and total phenol contents is shown in Table 2. Then olive leaf was mixed at different levels to soybean meal used in ration and then mixture was added to the compound feed to provided oleuropein levels. The oleuropein contents of diets supplemented with olive leaf were as follows: 125 mg kg^{-1}, 250 mg kg^{-1} and 500 mg kg^{-1} respectively. Dried olive leaf used in trial was obtained from a commercial company operating in the Edremit/Turkey.

2.2. Determination of breast meat quality

At the end of the trial, 40 broilers (10 chicks in each group) were randomly sampled for determination of breast meat quality. After slaughter carcasses were trimmed for breast meat by removing skin, bones and connective tissue. Following trimming, breast meat from each chick was separated into two sections. Right section was used for lipid oxidation, the other for color and pH_{24} measurements. Samples were placed on the plastic plates and covered with polyethylene film and stored in the refrigerator at +4 °C for lipid oxidation measurement. Thiobarbituric acid value (TBA) was determined on days 1, 5 and 11 as malondialdehyde (MDA) equivalence during 11 days storage. In order to determine the TBA value (mg MDA kg^{-1}), 5 g sample was blended for 2 minute in a homogenizer (AM-7, Nissei Co., Tokyo, Japan) with extracting solution (50 mL 2 M phosphoric acid) which containing 20 percent trichloroacetic acid in. The resulting slurry was diluted to 100 mL with distilled water and homogenized and filtered (Whatman No. 1 filter paper). 5 mL of filtrate was homogenized (5 mL of 2-thiobarbituric acid) and reserved in the dark for 15 h at room temperature. The resulting color was measured with spectrophotometer at 530 nm (Witte et al 1970). For color (L*- lightness, a* - redness and b* - yellowness) and pH at pH_{24} measurement breast meat samples were stored at 4 °C for 24 hours after slaughter. Objective measurement of color was performed at the surface of breast meat using a Minolta CR-300 colorimeter to measure CIE

Table 1- Ingredients and nutrients composition of the basal diets

	Starter (0-10 days)	*Grower (11-24 days)*	*Finisher (25-42 days)*
Ingredients (g kg^{-1})			
Corn	263.60	276.00	224.80
Soybean meal	351.90	305.80	185.00
Wheat	150.00	160.00	200.00
Full fat soybean	64.40	47.30	120.00
Wheat bran	60.00	80.00	140.00
Sunflower meal	20.00	30.00	50.00
Soybean oil	49.80	65.00	47.70
Dicalcium phosphate	15.50	12.50	10.40
Limestone	10.00	9.50	9.50
Common salt	3.50	2.90	2.90
DL-Methionine	3.40	2.80	3.10
L-Lysine	3.00	3.00	2.50
L-Threonine	0.70	0.50	0.50
Sodium bicarbonate	1.10	1.60	1.10
Premix[1,2,3,4]	1.00	1.00	1.00
Enzyme+Phytase	1.00	1.00	1.00
Anticoccidials	0.60	0.60	-
Choline chloride	0.50	0.50	0.50
Analyzed values (g kg^{-1})			
Dry matter	893.50	895.40	896.30
Crude protein	231.07	210.40	190.60
Ether Extract	71.50	92.10	8.40
Crude ash	62.55	50.30	50.40
Crude fiber	34.00	33.70	38.20
Calcium	10.20	9.10	8.40
Total phosphorus	7.37	6.80	7.20
Starch	348.42	372.50	393.70
Sugar	46.47	41.50	40.80
Metabolizable energy*, Kcal kg^{-1}	3025.00	3150.00	3200.00

[1], vitamin premix per kg of starter diet; 11000 IU vitamin A; 5000 IU vitamin D_3; 75 IU vitamin E; 3 mg vitamin K; 3 mg vitamin B_1; 8 mg vitamin B_2; 60 mg niacin; 15 mg Pantothenic acid; 0.15 mg biotin; 2 mg folic acid; 0.16 mg Vit B_{12} [2]vitamin premix per kg of grower diet: 9000 IU vitamin A; 5000 IU vitamin D_3; 50 IU vitamin E; 3 mg vitamin K; 2 mg vitamin B_1; 6 mg vitamin B_2; 60 mg niacin; 15 mg Pantothenic acid; 0.10 mg biotin; 1.75 mg folic acid; 0.16 mg Vit B_{12} [3]Vitamin premix per kg of finisher diet: 9000 IU vitamin A; 4000 IU vitamin D_3; 50 IU vitamin E; 2 mg vitamin K; 2 mg vitamin B_1; 5 mg vitamin B_2; 40 mg niacin; 15 mg Pantothenic acid; 0,10 mg biotin; 1.50 mg folic acid; 0.10 mg Vit B_{12} [4]Mineral premix per kg of diet: 100 mg Zn; 120 mg Mn; 40 mg Fe; 16 mg Cu; 1,25 mg I; 0,30 mg Se. *, Calculated value

Table 2- Chemical composition, oleuropein and total phenol contents of olive leaf

Analyzed values	*(g kg^{-1})*
Dry matter	952.60
Crude protein	91.90
Ether extract	72.80
Crude ash	52.40
Crude fiber	170.21
Oleuropein	25.10
Total phenol (mg GAE g^{-1})	46.47

L*, a*, b* values (Kim et al 2007). The instrument was calibrated with standard white measuring plate before measurements. The pH_{24} value of the meat samples at 3 different locations was determined using a pH meter (Hanna Instruments HI 8314) and measured using a direct electrode by thrusting the probe into the incised breast and probe was cleaned in each measurement.

2.3. Statistical analysis

All experimental data analyzed using general linear model procedure in SPSS 16.0 package program. In addition, the effect of treatment (oleuropein levels) the MDA contents of samples was determined by regression analysis by defining orthogonal polynomial contrast as linear and quadratic. Differences between the treatment groups were assessed according to the Duncan's Multiple Range test and differences were considered significant at P<0.05 (SPSS 2007).

3. Results and Discussion

At the completion of the study, performance parameters did not differ significantly (P>0.05) among groups (data presented in thesis), indicating that the incorporation of olive leaves to the diets had no adverse influence on the growth rate of broilers (Yavaş 2013).

3.1. Lipid oxidation on breast meat

The effect of dietary oleuropein supplementation and storage time on breast meat lipid oxidation was presented in Table 3 and lipid oxidation alteration depending on the storage time was presented in Figure 1. As shown in Table 3, extension of the storage time significantly increased the levels of TBA of breast meat (P<0.05). Moreover, results showed that dietary oleuropein supplementation at level of 250 mg kg^{-1} was significantly decreased lipid oxidation on breast meat compared with other groups (P<0.05).

Our results supported by the findings of studies on Japanese quails (Sarıca & Toptas 2014) mentioned that 150 and 200 mg kg^{-1} oleuropein supplementation on quail diets were effective in delaying lipid oxidation. Similarly, olive leaf supplementation studies on turkeys (Govaris et al

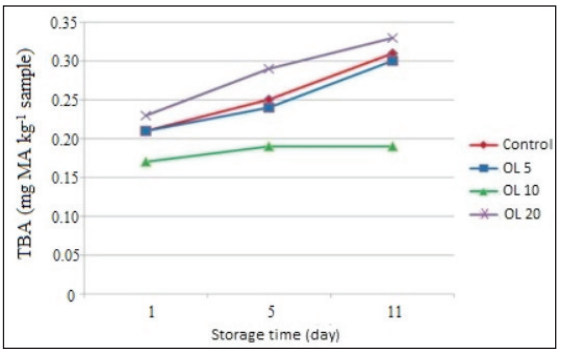

Figure 1- The alteration of TBA value on breast meat samples in experimental groups depends on storage time

Table 3- Effect of treatment and storage time on breast meat lipid oxidation (TBA, mg MDA kg^{-1} sample)

Sources of variation		*TBA (mg MDA kg^{-1} sample)*
Treatments	Control	0.26^{b}
	OLE125	0.25^{b}
	OLE250	0.19^{a}
	OLE500	0.28^{b}
	SEM	0.01
	P	<0.001
	Linear	0.830
	Quadratic	<0.001
Storage time, day	1.	0.21^{a}
	5.	0.24^{b}
	11.	0.28^{c}
	SEM	0.01
	P	<0.001
P values		
Treatment (T)		< 0.001
Storage Time (ST)		< 0.001
T x ST		0.364

$^{a-c}$, means within a column with different superscripts are significantly different (P<0.05); SEM, standard error of means

2010; Botsoglou et al 2010a; Botsoglou et al 2010b) observing that 10 g kg^{-1} olive leaf supplementation on turkey diets was decreased lipid oxidation on breast meat which was stored for 12 days at +4 °C compared to control and summarized that 10 g kg^{-1} olive leaf supplementation more effective to prevent lipid oxidation than 10 g kg^{-1} rosemary and 150 mg kg^{-1} α-tocopherol acetate (Govaris et al 2010) and 10 g kg^{-1} thyme (Botsoglou et al 2010b) supplementation respectively. Study on broilers (Marangoni et al 2017) also reported that 5 g kg^{-1} olive leaf supplementation has antioxidant effect on chicken meat during the frozen storage. But these studies not mentioned olive leaf's oleuropein levels. Other *in vivo* study on laying hens (Aktaş 2012) also reported antioxidant effect of olive leaf extract. In addition to *in vivo* studies, studies conducted on *in vitro* also reported antioxidant effect of olive leaf or its phenolic substances (Briante et al 2002; Lee et al 2009; Kiritsakis et al 2010). The antioxidant effect of oleuropein has been reported in studies that it inhibits the formation of free radicals by binding with metal ions such as iron and copper and under favour of this bond suppresses the activities of many inflammatory enzymes such as lipoxygenase (Andrikopoulos et al 2002; Visioli et al 2002; Botsoglou et al 2010a). Studies also have shown that olive leaf has 15 antioxidant substances besides oleuropein such as hydroxytriosol, luteolin-7-glucoside, verbascoside, tirosol, vanillic acid (Benavente-Garcia et al 2000; Silva et al 2006). Our result of antioxidant effect of oleuropein is consistent with the reported in the literature. However, OLE500 dietary group in our study did not decrease the TBA value in breast meat sample compared to the control group, on the contrary increased as a numerally, in other words its preventive effect on lipid oxidation was not detected. In a study conducted in *in vitro* conditions with turmeric extract (Saefudin et al 2014), reported that the use of turmeric extract in high levels causes prooxidant properties instead of antioxidant activity. It is known that polyphenol compounds can display both antioxidant and prooxidant effects (Decker 1997) depending on several factors (chelating potential, solubility, bioavailability and stability in tissues). In this respect 250 mg kg^{-1} oleuropein leaf supplementation to diet showed the expected antioxidant effect but highest level of supplementation showed prooxidant effect related to usage level.

3.2. Breast meat color and pH_{24}

The effect of dietary oleuropein supplementation on 24 hours stored at 4 °C breast meat color and pH_{24} were presented in Table 4. As shown in Table 4, while there was no difference in breast meat lightness, yellowness and pH_{24} among the treatments ($P>0.05$) dietary oleuropein supplementation at different level significantly increased redness value compared to the control group ($P<0.05$).

Table 4- Effect of treatment on breast meat color and pH_{24} value

Treatments	*L**	*a**	*b**	*pH_{24}*
Control	48.85	3.19[a]	5.16	6.15
OLE125	50.67	5.67[b]	5.47	6.11
OLE250	51.43	6.52[b]	5.62	6.10
OLE500	49.33	7.83[b]	5.66	6.19
SEM	0.874	0.774	0.619	0.054
P	0.156	0.001	0.937	0.600
Linear	0.575	0.000	0.550	0.601
Quadratic	0.031	0.459	0.829	0.232

L*, lightness; a*, redness; b*, yellowness; [a-b], means within a column with different superscripts are significantly different ($P<0.05$); SEM, standard error of means

Our results supported by the findings of study (Marangoni et al 2017) observing that olive leaf supplementation to broiler feed has no effect on L* and b* values of breast meat but were contrast to findings of a* value of breast meat and pH. In that study, while olive leaf supplementation has no effect on a* value, it decreased pH of the meat during long storage condition (60 days). Studies with different phytobiotics on broiler breast meat color and pH (Hong et al 2012; Kırkpınar et al 2014; Cho et al 2014; Li et al 2015) reported that increase or decrease in meat color might be related to type and rations of carotenoids found in phytobiotic structures, management, breeding type, carcass weight and pH. Olive leaf's carotenoid amount was reported by

Cayan & Erener (2015) as 2 mg β-carotene and 8 mg lutein 100 g^{-1} olive leaf in literature and olive leaf supplementation at any level on layer diet increased yolk yellowness in that study. In our study, amount of carotenoid in olive leaf was not determined and increases in a* value may be related to amount and type of carotenoid in olive leaf.

4. Conclusions

As a result, 250 mg kg^{-1} oleuropein supplementation to broiler diets, decreased lipid oxidation by decreasing TBA value of breast meat sample and it is possible to say that olive leaf demonstrated antioxidant activity linked with oleuropein supplementation level and it can be used at level of 250 mg kg^{-1} as phytobiotic antioxidant in broiler diets.

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Determination of the Main Socio-Economic Factors of the Sustainable Production of Forage Crops: Research of Kayseri Province

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ABSTRACT

Today, about 21% of the Turkey's population lives engage plant production and animal husbandry; the most crucial aspect of livestock breeding is producing forage crops. Since 2000, growers in Turkey have been subsidized and encouraged to increase both their production and quality of forage crops. However, despite all this support and assistance, desired production levels have not yet been achieved. Therefore, it is equally crucial analyze the effects of factors other than the subsidies provided on forage crops production. This study aims to determine the socio-economic factors that affect sustainable forage crops production. The study was carried out in the province of Kayseri, specifically in 11 villages where both plant production and animal husbandry systems are common. The subjects of the study were selected through the Random Sampling Method and data was collected by surveying 310 forage crops growers. To identify dependency relationships between qualitative variables used mainly based on a statistical chi-square statistic. According to the analysis results, 35.5% of growers found forage crops production sustainable, whereas 64.5% of them thought that it was unsustainable. When both socio-economic variables and the answers to survey questions were taken into account, variables such as grower's age, education level, income level, land for fodder crops, livestock count, recent increase in livestock count, subsidies, and the conditions under which sustainability may be maintained were found to be related to forage crops production sustainability.

Keywords: Sustainability; Forage crops; Subsidies; Kayseri-Turkey

1. Introduction

Forage crops production is the backbone of livestock breeding. Forage crops both directly and indirectly improve the productivity of land use (Maç & Yilmaz 2016). The fact that forage plants are rich in minerals and vitamins makes them enhance livestock productivity and produce quality foods from animals (Peters et al 2001). However, the amount of coarse fodder produced in Turkey is far outstripped by the feed demands of the existing livestock population (Yolcu & Tan 2008; Temel & Şahin 2011). In Turkey, forage crops are grown in as much as 2.689,253 hectares. Specifically, forage crops that are grown most often in Turkey are corn (976.698 ha), clover (676.172 ha) and common vetch (669.432 ha). When cultivated lands (15.464 ha) and fallow lands (4.286 ha) are combined, the proportion of the forage crop lands is 13.6% (BUGEM 2017). The first reason for this is that the coarse fodder yield in meadows and rangelands is low. The second reason is that the

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forage crops production is insufficient (Kusvuran et al 2011). Ecologically, Turkey has the necessary resources to meet the coarse fodder demand and feed its livestock population.

Turkey has a great potential for forage production, but this potential can be accomplished with active and more specific policy measures (Yilmaz & Mac 2013). Therefore, there are plans to ameliorate Turkey's coarse fodder deficit. For this, efforts to improve meadows and rangelands have been intensified and forage crops growers are subsidized. Forage crop growers have been supported since 2000 to foster, promote, and keep track of livestock breeding in Turkey. One objective of the forage crops subsidy policies in place is sustainable forage crops production. It is well known that government-provided subsidies positively impact the amount of forage crops produced (Cevher et al 2012). However, the increase in the amount of production and productivity depend on both the subsidies and the forage crop growers' socio-economic features (Ward et al 2016). For instance, a study on developing livestock breeding found that socio-economic features such as breeders' age, education level, non-agricultural activities, and production objectives affect livestock breeding (Demir & Yavuz 2010). In another study (Karadavut et al 2011), proved that the growers' socio-economic features help their success in producing forage crops.

This study determines the socio-economic factors that affect growers' forage crops production and its sustainability. In addition, the socio-economic variables that impact the sustainability of forage crops production and the subsidies given by the government have been studied. It is crucial to determine these variables to reinforce the government support for producing forage crops.

2. Material and Methods

2.1. Data

This study's subjects are registered growers who grow forage crops in Kayseri province, of whom there are 1.600. A random sampling method is used to select subjects among them (Yamane 2001) Equation 1 is used to determine the sample size.

$$n = \frac{Npqz_{\alpha/2}^2}{Nd^2 + pqz_{\alpha/2}^2} \qquad \text{Equation 1} \qquad (1)$$

In this equation, n is the sampling size, p is the estimated percentage of the subjects saying that forage crops production is sustainable (0.5), q is the estimated percentage of subjects saying that forage crops production is unsustainable (0.5), N is the population size, d is the sampling error (0.05), α is the first type error level (0.05), and z is the standard normal distribution value (1.96). When all these values are placed into Equation 1, we have:

$$n = \frac{1600(0.5)(0.5)(1.96)^2}{1600(0.05)^2 + (0.5)(0.5)(1.96)^2} = 309.78 \qquad (2)$$

Based on this, the sample size should be at least 310 subjects. Accordingly, 310 registered growers were randomly selected for this study. In addition, 30 growers, roughly 10% of the sample size, were picked as substitute subjects, in case some primary ones chose not to answer the questionnaire or could not be reached. Thus, 310 growers were surveyed through face-to-face interviews.

2.2. Analysis

In this study, since the state of sustainability and socioeconomic variables are categorical, the relationship between variables was analyzed via chi-squared test. c was the number of columns and r was the number of rows; the expected frequencies for each cell in a $c \times r$ frequency table were calculated as follows:

$$E_{ij} = \frac{1}{n}\left(\sum_{k=1}^{r} O_{kj}\right)\left(\sum_{k=1}^{c} O_{ik}\right) \qquad \text{Equation 2} \qquad (3)$$

The test statistics showing the differences between the observed and expected frequencies are defined as follows:

$$\sum_{j=1}^{c}\sum_{i=1}^{r}\left(\frac{(O_{ij} - E_{ij})^2}{E_{ij}}\right) \qquad \text{Equation 3} \qquad (4)$$

While the variables were independent, this statistic had a chi-squared distribution with an approximate degree of freedom of (r - 1) (c - 1) (Ozkan et al 2016).

$$\sum_{j=1}^{c}\sum_{i=1}^{r}\frac{(O_{ij}-E_{ij})^2}{E_{ij}} \sim \chi^2_{(r-1)(c-1)} \qquad \text{Equation 4} \qquad (5)$$

The Cramer v correlation coefficient was calculated for the relationships that were found in the chi squared analysis.

$$v=\sqrt{\frac{\chi^2}{n\times\min\{(r-1),(c-1)\}}} \qquad \text{Equation 5} \qquad (6)$$

This coefficient has a value between [0.1] and as it approaches 1, the correlation between the variables increases. As a measure of association, making the proviso that this indicator should not be considered as absolute support to guarantee or not the association between variables; however, it serves to clarify the type and magnitude of a possible relationship between the variables of interest. Additionally, a correlation graph is provided to make it easier to compare correlation coefficients and the data based on the analysis are evaluated by the significance level of P<0.05.

In this study, the concept of sustainability in fodder crop production indicates whether the producer will continue to produce fodder crops when the supports (subsidizations) are removed.

3. Results and Discussion

3.1. The demographic features

The demographic features of subjects in this study are given in Table 1. Analyzing these features revealed that more than half of them were aged 46 or older. Another similar study found that the average age of the forage crop farmers was 46.2 years and they have an average education level of the farmers was nine years (Maç & Yilmaz 2016). The percentage of growers who were elementary school graduates was 67.4%, and 88.7% of growers live in rural areas. The percentage of growers who said that they had a low income was 10%, while 17.1% said that they had a high income. The sustainability rate was 35.5 (Figure 1).

Table 1- Growers' demographic features (n= 310)

Variables	*Value*	*f*	*%*
Age	≤ 30	30	9.7
	31-45	117	37.7
	≥ 46 +	163	52.6
Education	Primary school	209	67.4
	Middle school	44	14.2
	High school and higher	57	18.4
Living place	Rural	275	88.7
	Urban	35	11.3
Income level	Low	31	10.0
	Medium	226	72.9
	High	53	17.1

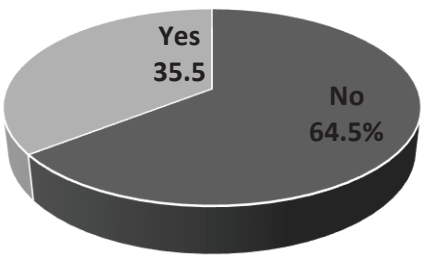

Figure 1- The distribution on the state of sustainability

3.2. The chi square analysis results

As shown in Table 2, the area in which growers reside and whether they had non-agricultural income had no significant effect on forage crop production sustainability (P>0.05). But, it is statistically significant for P<0.10. In addition, growers' age, education level, income level, whether they owned forage crop land, livestock count, whether they received subsidies, the conditions under which sustainability was ensured and the effects of subsidies on livestock count had a significant relationship with sustainability (P<0.05).

Although (Cevher & Tatlidil 2001; Çukur & Işın 2008) found no significant relationship between

Table 2- The Correlation of socio-economic variables with sustainability (n= 310)

Variables		*Sustainable*					χ^2	*P*
		No		*Yes*				
	Value	*f*	*%*	*f*	*%*	*Total*		
Age	≤ 30	13	43.3	17	56.7	30	10.47	0.005*
	31-45	86	73.5	31	26.5	117		
	≥ 46 +	101	62.0	62	38.0	163		
Education	Primary	128	61.2	81	38.8	209	8.58	0.014**
	Middle	37	84.1	7	15.9	44		
	High	35	61.4	22	38.6	57		
Living place	Rural	183	66.5	92	33.5	275	3.11	0.078***
	Urban	18	51.4	17	48.6	35		
Having off farm job	No	174	63.3	101	36.7	275	1.65	0.200
	Yes	26	74.3	9	25.7	35		
Income level	Low	27	87.1	4	12.9	31	30.64	0.000*
	Medium	156	69.0	70	31.0	226		
	High	18	34.0	35	66.0	53		
Fodder crops area	No	36	90.0	4	10.0	40	16.58	0.000*
	Yes	152	56.3	118	43.7	270		
Animal unit	1-15	121	93.1	9	6.9	130	101.41	0.000*
	16-30	68	56.2	53	43.8	121		
	31 or more	11	19.3	46	80.7	59		
Taking forage crop incentives	No	154	93.3	11	6.7	165	127.96	0.000*
	Yes	46	31.7	99	68.3	145		
Factors that ensure sustainability	Feed support	88	46.6	101	53.4	189	70.32	0.000*
	Animal prices	61	87.1	9	12.9	70		
	Number of animals	51	100.0	0	0.0	51		
The effect of incentives to increase animal numbers	No	200	79.7	51	20.3	251	132.49	0.000*
	Yes	0	0.0	59	100.0	59		

Significance level: 0.01*, 0.05**, 0.10***

sustainability and age, we found that the two were significantly related. The rate of growers, who were 30 years old or below and considered forage crops production sustainable was higher than that among growers in other age groups. Hence, sustainability is possible as it is easy to make young growers adopt innovations in agriculture. The percentage of growers whose education level was either primary or high and who considered forage crops production sustainable was greater than the percentage among middle school graduate growers. Several studies on Turkey's different crops found that education level and sustainability were related (Çukur & Işın 2008; Kılıç & Kıymaz 2014; Yildiz & Boyacı 2017), indicating that growers with a higher level of education had a high level of sustainability. This could stem from the increased awareness of sustainability associated with an increased level of education.

Regarding income, the higher the income level, the higher the rate of growers who consider forage crops production sustainable; 66% of growers who have a high income maintain that forage crops production is sustainable, whereas only 12.9% of those with a low income consider it sustainable. Similarly (Topcu 2008), found that as the level of income increased, the willingness to benefit from agricultural subsidies also increased. Using logistic

regression analysis (Kaya & Atsan 2013) concluded that there was a significant relationship between income and the adoption of agricultural innovations. Out of the growers who owned forage crop lands, 68.3% stated that forage crops production was sustainable while only 6.7% of growers without any lands were positive about the sustainability of forage crops production. In a study conducted (Kaya & Atsan 2013) concluded that there was a significant relationship between owning land area and subsidies. The subsidies for sustainable agriculture increased in proportion to land ownership.

In addition, the rate of sustainability visibly increased in accordance with the livestock count; 80.7% of growers who owned 31 or more livestock saw forage crops production as sustainable. This rate was only approximately 6.9% among growers with 1-15 livestock. In a study on this subject (Aksu & Dellal 2016) concluded that increasing the number of the livestock positively influenced sustainability.

Whereas 68.3% of the growers who benefit from subsidies for producing forage crops saw it as sustainable, the rate of those without any subsidies who consider it sustainable was only 6.7%. When the distribution between the suggestions for ensuring sustainability and sustainability itself were analyzed, the most valuable suggestion was to "increase the subsidies for forage crops"; 53.4% of the growers confirmed that forage crops production would be sustainable if subsidies increased. All growers who had increased their livestock count thanks to subsidies found forage crops production sustainable.

Figure 2 shows the Cramer *v* correlation coefficients between the significant variables and the state of sustainability; the thicker the line, the more significant the correlation. The increase in the livestock count was the most significant variable (*v*= 0.654), followed by the variable for whether the grower received any subsidies (*v*= 0.642). The third most significant variable that had an effect on sustainability was the livestock count (*v*= 0.574).

3.3. Discussion

To benefit from support for feed plants in the study area, manufacturers with sensible fields should be provided with convenience. Because 41% of the tapestries in the research area are sensible territories

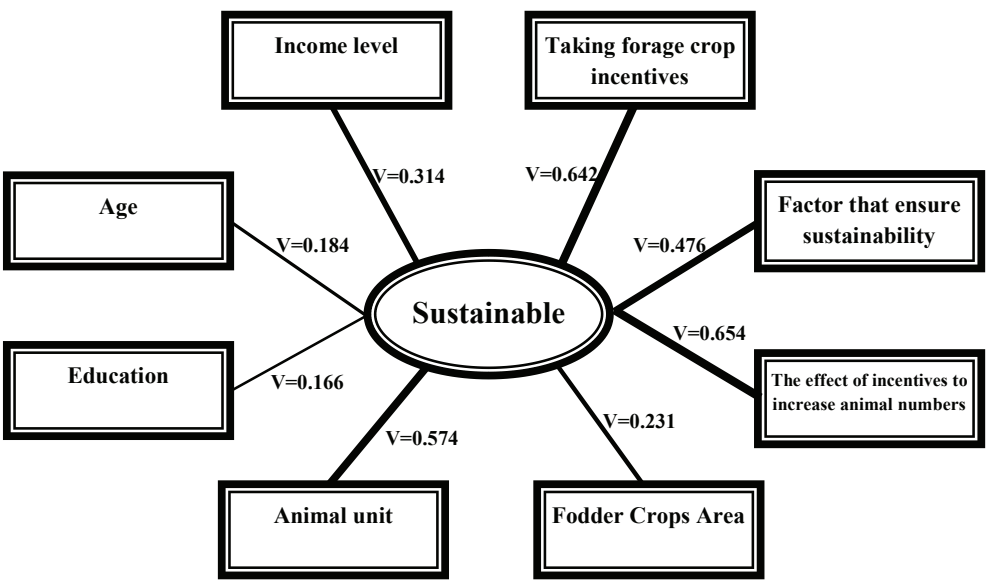

Figure 2- Correlation graph (the thicker the line is, the more significant the correlation is)

and producers can't benefit from the incentive. This is an obstacle to increased production. Manufacturers who have leased treasury and neighboring land have stated that they do not benefit from support. This is an obstacle to the increase in production of feed plants. If this situation is done in favor of the producer, it will contribute to the increase of production. In addition to support for increased production of feed crops, agricultural publication studies and increased need for coarse feeds have also been effective. In this context, emphasis on agricultural publishing studies will increase production. 50% of perennial feed plants should be given in breeding. This will lead to an increase in production. Feed plant supports have put production of feed plants in an advantageous position in production pattern (according to other products). This has contributed to the increase in production of feed plants. Maintaining this support will contribute to the production of feed plants, the improvement of soil structure and the increase in the profit of operation in operation.

4. Conclusions

This study reveals that forage crops production closely depends on growers' income level, whether they own forage crop lands, livestock count, whether they receive subsidies, the conditions under which sustainability is maintained, and the effects of subsidies on livestock count. In particular, livestock count significantly correlates with sustainability. This study indicates that the increase in livestock count due to subsidies given is the most important factor for ensuring sustainability. The rate of growers who have increased their livestock count thanks to subsidies is 19% (59 out of 310 growers). Moreover, all these growers find forage crops production sustainable. Thus, forage crops production subsidies are insufficient and should be increased. In addition, stockbreeding should be turned into a more lucrative line of business by stabilizing the prices of animal-based products in commodity markets, which would encourage increased livestock counts. It is necessary to boost breeders' purchasing power. Apart from enhancing the livestock, receiving subsidies is confirmed as directly impacting sustainability. One reason for this could be that forage crop lands and the amount of production soar after subsidies are reimbursed. Another reason could be that subsidies greatly encourage forage crops production compared to regular crop production. It is seen that growers' financial situations really matter with regard to sustainability. This paper found that the rate of sustainability among growers with a larger income was high, which was why extending additional subsidies and supplying equipment and labor support to the growers with low income in particular could play an important role in ensuring sustainability. Since whether a person owns forage crop lands affects the sustainable production of such crops, a hike in the amount of forage crops would mean a hike in the sustainability. When growers were interviewed on the conditions under which forage crops production could be sustainable, it was concluded that increasing subsidies and livestock prices would encourage sustainability. In addition, the following course of action should be taken to reinforce coarse fodder production in Turkey: it is particularly necessary to enhance the quality of fodder, add forage crops to growers' growing cycle, make use of publications on forage crops, and collaborate with universities. Promotion of young population through support (subsidizations) and training activities, the effectiveness of producer organizations to benefit from support (subsidizations) will contribute to sustainable forage production.

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Drip Fertigation In Apple Orchards: Impact on Soil Chemical Properties and Nutrient Distribution In Relation to Soil Texture

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ABSTRACT

The aim of this research was to determine the influence of drip fertigation on soil chemical properties and vertical distribution of nutrient in the root zone of three apple orchards planted on soils with different textures (loam, clay loam and sandy loam). The fertigation led to a significant decrease in soil pH, concentration of calcium carbonate ($CaCO_3$) and organic carbon (C). Changes of these parameters were more pronounced in sandy loam soil than in loam and clay loam. Fine textured soils (loam and clay loam) had higher accumulation of available forms of P, K, Fe, Zn, Mn and Cu in the surface layer at 0-10 cm depth, compared to sandy loam soil. The results showed that, despite numerous advantages, drip system of fertigation can lead to negative changes of soil properties and that the sandy loam soils are more exposed, but in the same time more suitable for fertigation due to better vertical distribution of nutrients in a soil profile compared to loam and clay loam.

Keywords: Soil fertility; Irrigation; Nitrogen; Phosphorus; Potassium

1. Introduction

Irrigation can affect soil quality in different ways. Consequently, monitoring is necessary to avoid negative consequences of irrigation on soil quality in the form of reduced fertility, and crop productivity (Sun et al 2018). The influence of irrigation on physical and chemical soil properties in arid and semi-arid conditions is well documented, while there is very little data on effects of irrigation in humid and sub-humid conditions where crops can be grown without irrigation. In Serbia where the total amount of precipitation and its distribution during the vegetative growth period is highly variable, irrigation is used as an additional tool for supplying plants with water during critical crop growth. One of the most commonly used irrigation system in modern apple orchards is drip irrigation. The main advantage of this system compared to others is that irrigation timing and quantity can be very accurately adjusted to the needs of plants in accordance with plantation age, phenophase and weather conditions (Haynes 1985). The drip system is more efficient than other irrigation systems because the dripping water is added to a relatively small soil volume around the root of the cultivated plants, but at the same time the effect on physical and chemical soil properties in wetted zone

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is more pronounced compared to other systems (Alva & Obreza 1993). Drip irrigation can have a significant impact on soil fertility due to a change in pH values of the soil which largely influences the availability of certain soil nutrients and thus their absorption by the plants and the crop productivity (Neilsen et al 1993; Treder et al 1997).

Groundwater (Artesian aquifers) is most commonly used for drip irrigation. Groundwater can contain high concentrations of calcium and magnesium bicarbonate (Čuvardić et al 2004), which lead to an increase in soil pH value and a lower availability of nutrients. On the other hand, irrigation and fertilizer application through irrigation system (fertigation) can cause soil acidification due to the process of washing out of base cations (Neilsen & Stevenson 1983), or the application of physiologically acidic fertilizers which lower the soil pH value (Belton & Goh 1992; Komosa et al 1999). Apart from changes in soil pH value, irrigation can have a negative effect on soil organic matter content. Increased soil moisture has a positive effect on microbiological activity and decomposition of organic matter which can lead to a decrease of soil organic carbon and indirectly to a reduction in soil fertility (Condron et al 2014).

It is well know that soil texture has an important role in nutrient management because it influences nutrient retention (Gaines & Gaines 1994), water infiltration rate (Mamedov et al 2001), cation exchange capacity (Hepper et al 2006), content of organic matter (Bechtold & Naiman 2006), and other characteristics that define soil fertility.

The aim of this research was to determine the influence of drip fertigation on soil chemical properties and vertical distribution of nutrients in the root zone of three apple orchards planted on soils with different textures.

2. Material and Methods

2.1. Research site

Research was conducted on three localities in the northern part of Serbia (Vojvodina province) during September 2014. The analyzed samples were taken from three farming households with modern apple orchards of the Golden Delicious cultivar. The trees were on M9 T337 rootstock, planted at a 3.2×0.8 m distance. The orchards were planted in 2007 on soil of different textures (Table 1). The basic chemical properties of the experiment soils are presented in Table 2. In the last seven years, during the vegetation period (from April to September), all three orchards have been watered using drip irrigation with annual irrigation norm from 50 to 150 mm of water. The chemical composition of the irrigation water is shown in Table 3. In each orchard, the experiment was set up using completely randomized design with four repetitions, where one row of apple trees represented one repetition. During the seven-year period the fertigation has been conducted in all three orchards using same types and doses of fertilizers: urea (100 kg ha^{-1}), ammonium nitrate (100-150 kg ha^{-1}), monoammonium phosphate (60-80 kg ha^{-1}), and potassium sulfate (160-200 kg ha^{-1}). The total amounts of nitrogen, phosphorus and potassium applied trough fertigation system during each vegetation were: 80-100 kg N ha^{-1}, 30-40 kg P_2O_5 ha^{-1} and 80-100 kg K_2O ha^{-1}. The application of fertilizers with microelements has been done each year using foliar spraying. The microelements were applied as iron sulphate (4.5 kg ha^{-1}), zinc sulphate (1.5 kg ha^{-1}) and manganese sulphate (1.5 kg ha^{-1}) in the first half of the vegetation every year. Copper was not used as a fertilizer, but copper hydroxide is used every year as a fungicide.

2.2. Soil sampling and analysis

During September 2014, soil samples were taken from all three orchards from soil layers at 0-10, 10-20, 20-30 and 30-50 cm depth. Samples were taken from soil that was under irrigation emitter tubing and unirrigated soil of the inter-row spaces at a distance of 1.5 m from the irrigation tubing. In each site soil sample were taken from four repetitions, and in total 120 samples were analyzed. The soil pH was determined in soil suspension with 1 M KCl, potentiometrically using a pH meter (Mettler-Toledo, Switzerland) (ISO 10390:2007). The content

Table 1- Soil textures

Site	*GPS coordinates*	*Coarse sand (%)*	*Fine sand (%)*	*Silt (%)*	*Clay (%)*	*Texture*
Remeta	N 45º 06' 03.05'' E 19º 44' 20.08''	1.0	41.0	34.0	24.0	Loam
Rumenka	N 45º 17' 42.25'' E 19º 45' 28.83''	0.0	31.0	33.0	36.0	Clay loam
Ljutovo	N 45º 17' 42.25'' E 19º 45' 28.83''	2.0	60.4	27.8	9.8	Sandy loam

Table 2- Basic chemical characteristics of the soils

Site	*Ph (H_2O)*	*pH (KCl)*	*$CaCO_3$ (%)*	*Total N (%)*	*Organic C (%)*	*AL-P_2O_5 (mg $100g^{-1}$)*	*AL-K_2O (mg $100g^{-1}$)*
Remeta	7.92	7.16	19.33	0.11	1.30	10.70	34.10
Rumenka	7.54	6.56	4.15	0.13	1.55	12.10	31.80
Ljutovo	8.36	7.79	14.34	0.09	1.09	31.80	26.80

Table 3- Chemical properties of irrigation water

Parameters		*Sites*		
		Remeta	*Rumenka*	*Ljutovo*
Dry residue (mg L^{-1})		375.00	472.20	340.00
pH		7.42	7.35	7.31
SAR*		0.76	3.17	0.93
EC dS m^{-1}		0.78	0.95	0.45
Anions (mmol L^{-1})	CO_3^{2-}	0.20	2.14	0.20
	HCO_3^-	3.60	3.70	4.40
	Cl^-	2.50	1.90	0.50
	SO_4^-	0.68	1.13	0.31
Cations (mmol L^{-1})	Na^{2+}	1.11	4.10	1.37
	K^+	0.16	0.11	0.05
	Ca^{2+}	2.04	0.99	1.87
	Mg^{2+}	2.21	2.38	2.46

*SAR, sodium adsorption ratio

of calcium-carbonate ($CaCO_3$) was determined volumetrically using the Scheibler calcimeter (ISO 10693:2005). The content of organic carbon (C) was determined using the Tyurins method (Tyurin 1940). Mineral N concentration under field conditions during the vegetation was determined by the Wehrmann & Scharpf (1979) method. The content of easily available phosphorus and potassium was determined using the AL method (Enger et al 1960). For the determination of plant-available

fractions of Fe, Mn, Cu and Zn in the soil, samples were extracted with diethylenetriaminepentaacetic acid-triethanolamine (DTPA-TEA) buffer solution (0.005 M DTPA+0.01 M $CaC1_2$+0.1 M TEA) (ISO 14870:2001). For each sample, 20 mL of DTPA-TEA solution was added to 10.0 g of soil, shaken for 2 h on an orbital shaker, gravity filtered through filter paper, and analyzed by atomic absorption spectrometer with flame technique (Shimadzu 6300, Japan). The soil texture was determined based on triangle classification of the International Soil Science Society (Verheye & Ameryckx 1984). The results were subjected to analysis of variance (ANOVA) and treatment means were compared using the Tukey test ($P<0.05$) with STATISTICA 9 (StatSoft Inc, USA).

3. Results and Discussion

The influence of fertigation on the soil pH, concentration of $CaCO_3$ and organic C is shown in Figure 1. The fertigation led to a decrease in the soil pH value in the layers of the wetted zone at 0-10 cm and 10-20 cm, while the soil acidification intensity differed between the observed sites/soils. The biggest change in soil pH value was measured in sandy loam soil where there was a decrease of 0.84 pH units due to fertigation, that is, the soil turned from slightly alkaline class to neutral. Belton & Goh (1992) and Neilson et al (1993), also reported a considerable decrease in soil pH value under the drip irrigation system due to the application of physiologically acidic fertilizers such as urea and monoammonium phosphate. The fact that the type of fertilizer greatly influences acidification intensity is also confirmed by Treder (2005). Also, the decrease in soil pH value can happen as a result of base cation leaching K, Ca and Mg, and their replacement in the soil adsorption complex with hydrogen ions (Neilsen & Stevenson 1983).

In our research, the fertigation led to a significant decrease in concentration of soil $CaCO_3$ in loam and sandy loam soils (Figure 1). On other hand, this effect did not occur in the soil with finer texture (clay loam) (Tables 1 and 2). Considering that the content of organic C and the share of clay fraction are in correlation with the cation exchange capacity (CEC) (Caravaca et al 1999; Hepper et al 2006), and infiltration rate (Wakindiki & Ben-Hur 2002), it is possible that due to the higher CEC, and clay fraction the fertigation in clay loam soil did not lead to a significant decrease of $CaCO_3$ content in soil profile under the drip.

Fertigation can have a positive effect on organic C content in arable land due to a higher production of biomass (root and post-harvest residue of cultivated plants) (Entry et al 2002). On the other hand, due to increased soil moisture and higher microbiological activity, fertigation can lead to a decrease in organic C content caused by a more intense mineralization (Kumar & Goh 2000). In our research, measurements of the three experiment soils showed a lower concentration of organic C in all four layers of the fertigated area of the soil in comparison with the unfertigated area. However, there were statistically significant differences only in the surface soil layer of clay loam, and in all four soil layers of sandy loam soil (Figure 1). The lower concentration of organic C in the soil under the drip compared to that of the inter-row space is probably the result of more intense mineralization of organic matter caused by more favorable conditions. Other than that, it is possible that a part of the organic C labile fractions was washed down into deeper soil layers (Kalbitz et al 2000). Supporting this is the fact that the biggest decrease in organic C content was measured in the sandy loam soil (Ljutovo site) with lighter mechanical composition, higher infiltration rate and deeper wetting layer.

On all three sites, soil samples from the fertigated area (space inside the rows) had higher concentration of mineral N, available P and K compared to soil samples from unfertigated area. The concentration of mineral forms of N did not significantly differ in soil layers, except in loam soil, where surface layer had significantly higher concentration than layer at 30-50 cm depth. The application of P fertilizers through fertigation led to an accumulation of phosphorus in surface soil layers of the soils with finer texture (loam and clay loam). In these two soils, an almost double concentration

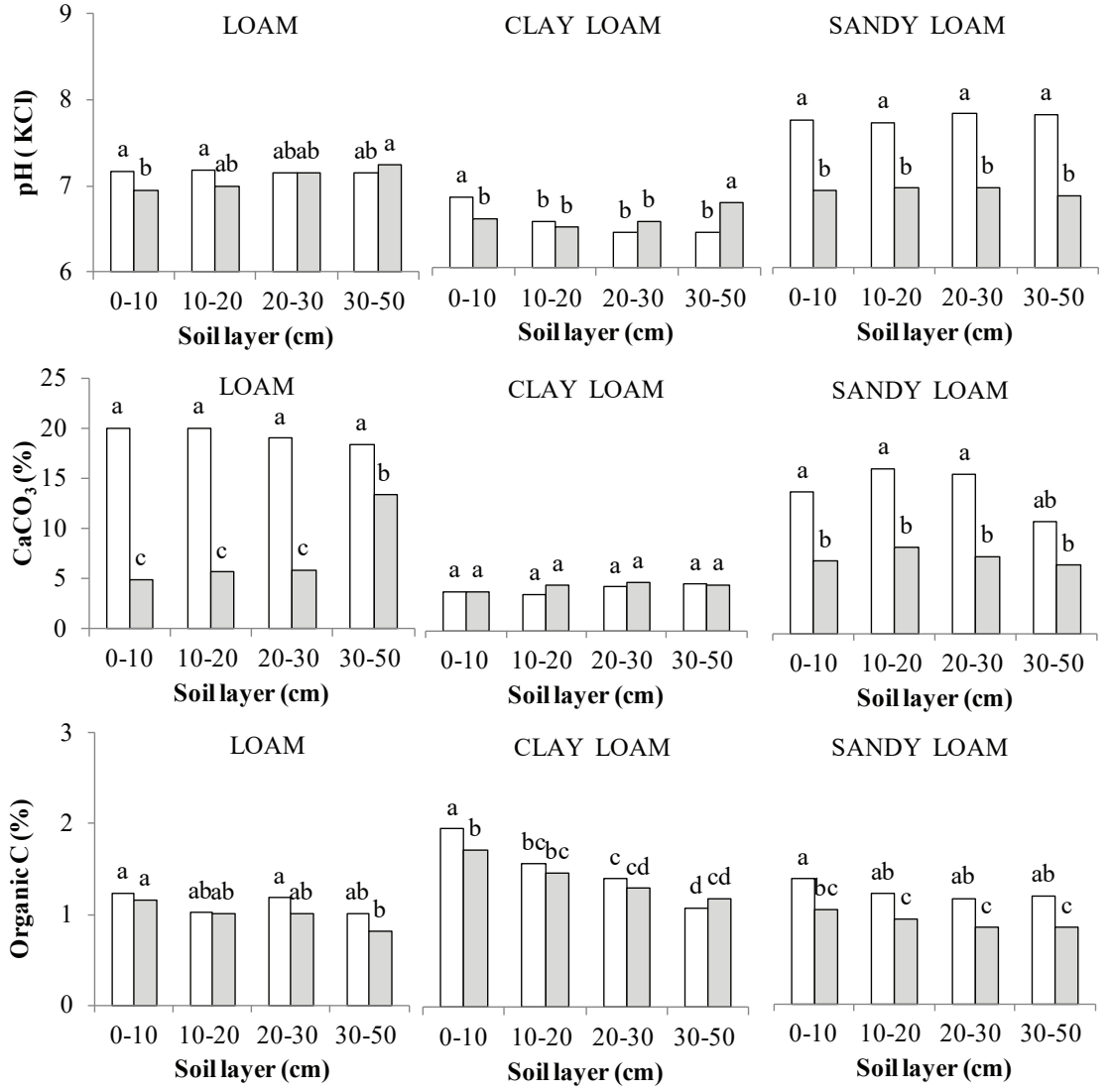

Figure 1- Impact of fertigation on soil pH, $CaCO_3$ and concentration of organic C in soils with different texture (gray columns-fertigated soil; white columns-unfertigated soil). Columns followed by different letters indicate LSD at P≤0.05

of available P and K was measured in the surface soil layer at 0-10 cm, as opposed to the layer at 10-20 cm depth or deeper. On the other hand, at site Ljutovo (sandy loam), a higher concentration of available P was measured in all four layers on the section that was fertigated, compared to the soil that was not under the direct influence of fertigation. In sandy loam soil was not recorded surface layer accumulation, as was the case in the other two soil textures, where the 0-10 cm layer contained more than 50% of the total P content in the root system zone (soil layer 0-50 cm) (Table 4).

Table 4- Distribution of available N, P and K in fertigated soil (% of total amount in soil profile 0-50 cm). Values followed by different letters are significantly different at P<0.05

Site/soil texture	*Depth (cm)*	*N*	*P_2O_5*	*K_2O*
Remeta/loam	0-10	28.9 a	52.3 a	44.3 a
	10-20	25.9 ab	22.0 bc	23.2 b
	20-30	24.5 ab	17.4 c	19.6 bc
	30-50	20.5 b	8.20 d	12.6 c
Rumenka/clay loam	0-10	25.5 a	50.9 a	44.3 a
	10-20	21.9 a	19.7 b	19.8 b
	20-30	24.7 a	13.1 b	18.3 b
	30-50	27.7 a	16.1 b	17.4 b
Ljutovo/sandy loam	0-10	27.0 a	26.3 a	31.2 a
	10-20	24.5 a	30.5 a	24.8 b
	20-30	22.2 a	20.9 b	24.4 b
	30-50	26.2 a	22.4 b	19.4 c

It is also well known that soil $CaCO_3$ have a negative effect on phosphorus availability and its movement toward deeper soil layers, due to process of P precipitation (Tunesi et al 1999). However, in our research P accumulation (in the surface layer) in the clay loam soil with lower $CaCO_3$ content and finer texture was higher than in the sandy loam soil with higher $CaCO_3$ content and coarser texture. These results indicate that P movement under the irrigation emitter into the deeper soil layers was far more conditioned by soil texture than $CaCO_3$ content and soil pH value. The studies of Zheng et al (2003), Salas et al (2003) and Hanson et al (2006) also indicate a great influence of soil texture on phosphorus shift and accumulation.

The available K distribution in the apple root zone was identical to that of phosphorus. In the soil with coarser texture (sandy loam), the concentration of K in the deepest soil layer (40-50 cm) at fertigated area was significantly higher compared to corresponding layer at unfertigated area. Simultaneously, in loam and clay loam soils, significant differences in K concentration were found only in the surface soil layers (Figure 2). Better vertical distribution of K in sandy loam soil, compared to the other two soil textures is probably the result of coarser soil texture and lower clay content, as it was previously noticed by Rosolema et al (2010), who found that K movement into deeper soil profile was significant in the lighter textured soil but was not apparent on the heavier textured soil.

The concentrations of available forms of microelements in all the soil samples were above critical values where deficiency symptoms can be expected (Lindsay & Norvell 1978). The fertigation led to a higher concentration of all the analyzed microelements (Fe, Cu, Zn, Mn), especially in the surface soil layers, although fertilizers containing microelements were not used in the fertigation (Figure 3). The higher concentration of microelements in the soil samples can be explained by the fact that the samples were taken directly under the apple trees (inside the row). Therefore, it is possible that microelements were washed down from the apple tree trunks and leaves and then accumulated in the soil surface within the sampling zone. On the other hand, the use of acidic fertilizers in the fertigation led to a decrease in soil pH value under the emitter, which could have had a positive effect on the concentration of available forms of microelements (Haynes & Swift 1987).

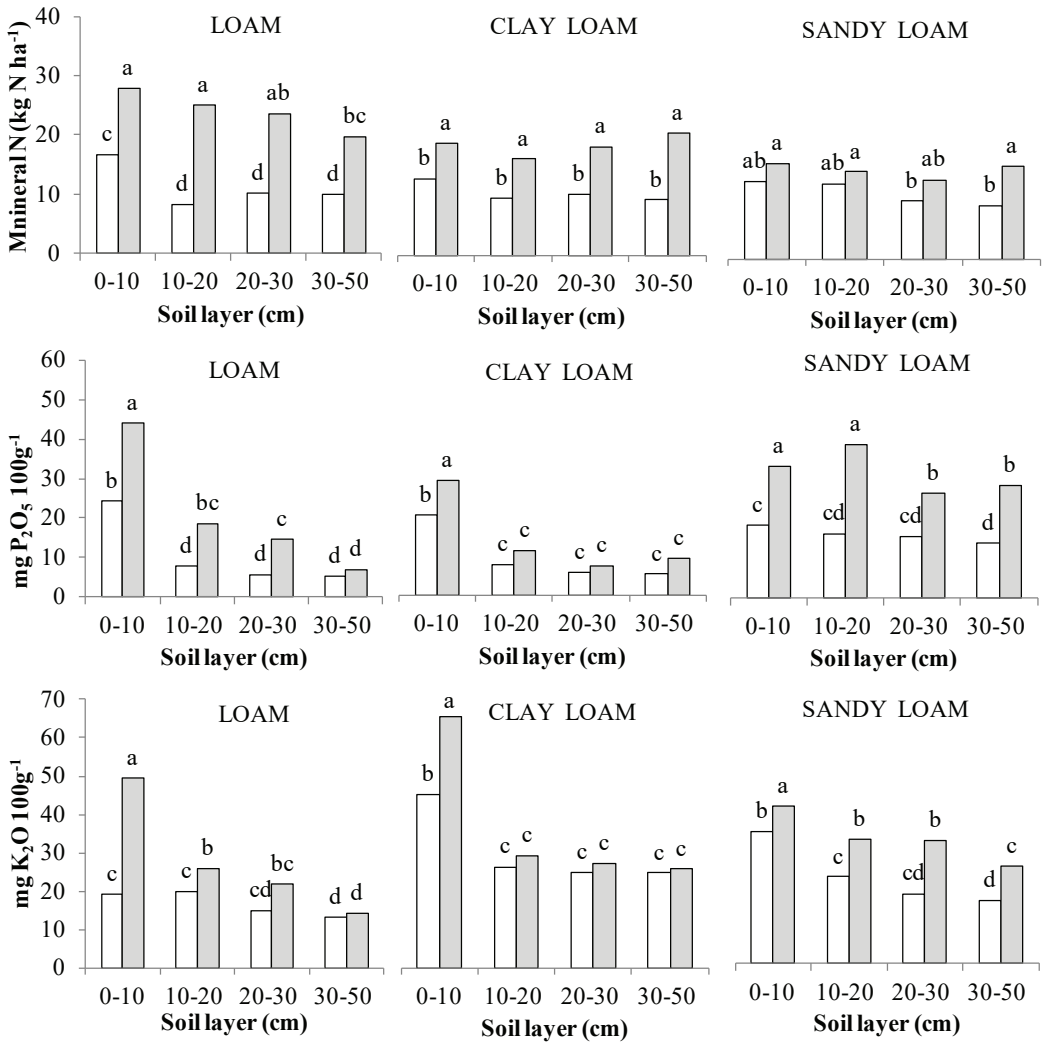

Figure 2- Impact of fertigation on concentration of mineral N, available P and K in soils with different texture (gray columns-fertigated soil; white columns-unfertigated soil). Columns followed by different letters indicate LSD at P≤0.05

4. Conclusions

Drip fertigation led to a significant decrease in soil pH values, as well as a lower concentration of $CaCO_3$ and soil organic C. Changes of these fertility parameters in soils with finer texture (loam and clay loam) were recorded in the surface layers (0-10 cm and 10-20 cm), while in soil with coarser texture (sandy loam), fertigation had significant effect on all of the analyzed layers down to 50 cm depth. Fine textured soils (loam and clay loam) had a significant accumulation of available forms of P, K, Fe, Zn, Mn and Cu in the surface layer at 0-10 cm depth, while sandy loam soil had a better distribution these nutrients in soil profile (root system zone 0-50 cm) than loam and clay loam.

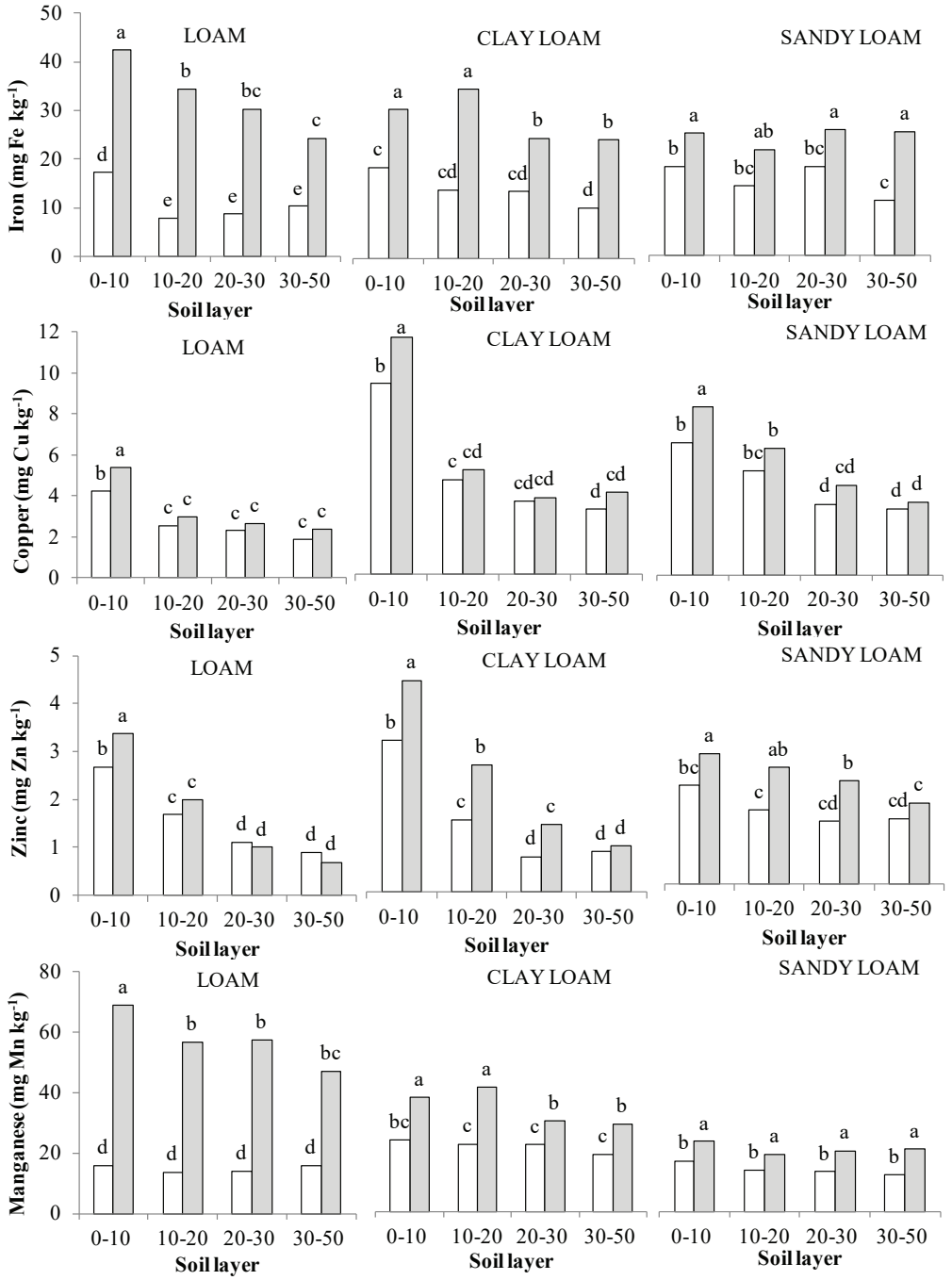

Figure 3- Impact of fertigation on concentration of available microelements (DTPA-extraction) in soils with different texture (gray columns-fertigated soil; white columns-unfertigated soil). Columns followed by different letters indicate LSD at P≤0.05

The results showed that, despite numerous advantages, drip system of fertigation can lead to negative changes of soil properties and that the sandy loam soils are more exposed, but in the same time it is more suitable for fertigation due to better nutrients distribution in a soil profile compared to loam and clay loam. However, more detailed research covering a different soil types and orchards with longer period of fertigation is necessary in order to determine changes of soil properties and to take measures to prevent eventual soil degradation.

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Effects of Biofloc Technology (BFT) on Growth of Speckled Shrimp *(Metapenaeus monoceros)*

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ABSTRACT

The effects of biofloc technology (BFT) on growth and hepatopancreas histology of speckled shrimp *Metapenaeus monoceros* (initial weight: 8.32±0.69 g) were investigated for 30 days. The trial was conducted in fiberglass tanks (45 L) with biofloc (zero-water exchange) and without biofloc (water exchange 50% day^{-1}). Different carbon sources (corn starch: CS and glycerine: G) and mannan oligosaccharides (MOS) supplementation (with and without BFT) were tested in triplicate groups. At the end of the experiment, better growth performances, feed conversion ratio, and specific growth rates were detected in shrimps reared in BFT (CS, G, CS+MOS, G+MOS) groups. Measured water quality parameters (dissolved oxygen, pH, temperature, salinity, ammonium-NH_4, and nitrate-NO_3) did not differ between BFT and control groups. The total number of bacteria count of shrimp reared in the biofloc groups were higher ($4.9x10^{-6}$±$8.5x10^{-4}$ CFU mL^{-1}) than that of in the without biofloc ($3.7x10^{-6}$±$5.4x10^{-4}$ CFU mL^{-1}) groups. In this study BFT had no negative effects on hepatopancreatic tissue by histological assessment. It is suggested that corn starch as a carbon source in BFT with 3 g kg^{-1} MOS supplementation could be applied as healthy growth enhancer in speckled shrimp culture.

Keywords: Corn starch; Glycerol; Growth; Histology; Mannan oligosaccharides; *Metapenaeus monoceros*

1. Introduction

Aquaculture plays a crucial role for supporting the world's needs of protein (Jackson 2007; Kuhn et al 2010). According to FAO and World Bank 2030-2050 projections the production capacity of aquaculture should be doubled because of the population increases and global warming. The report also proposes that agriculture will be practiced in marine environment. In order to achieve this successful and secure food production, the industry will obviously need to develop new technologies that will increase sustainable economic profitability and environmental concerns. Intensive aquaculture applications work with great amounts of clean water exchange ratios to sustain acceptable water quality (Wang 1990; Hopkins

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et al 1993; Moss et al 1999; Mishra et al 2008). However, due to environmental concerns like conservation of water sources, the requirement for more ecologically sound management and aquaculture practices is also growing steadily (Emerenciano et al 2012).

It is known that microorganisms have an important influence on nutrition cycle, water quality, and nourishment of cultured species (Emerenciano et al 2011). Biofloc technology is a technique utilized to increase water quality and create extra protein resources by adding an external carbon source into environmentally friendly aquaculture systems. The sustainable and recyclable food production approach based on such a system is dependent on the growth of microorganisms. Biofloc technology today has been implemented with minimum or zero water exchange in culture systems. Moreover, it is possible to reduce feed consumption by recycling all the waste for the edible food sources by BFT for cultured species (Kaya & Genc 2018).

Studies investigating alternative/new species for aquaculture are vitally important to gain insight regarding market diversification and minimization of food shortage. Speckled shrimp, *Metapenaeus monoceros*, is the one of highly demanded penaeid shrimp in fish market of Mediterranean and Indo-Pacific region and is considered as an alternative species in aquaculture. The main objective of the current study was to determine the growth parameters, survival, and hepatopancreas histology of speckled shrimp with BFT (in zero water exchange) and without BFT application (50% day^{-1} water exchange).

2. Material and Methods

2.1. Experimental design and culture conditions

The study was conducted at the Fisheries Research Unit (Faculty of Agriculture, Ankara University), Ankara, Turkey. The trial was conducted in fiberglass tanks (45 L) with biofloc (zero-water exchange) and without biofloc (water exchange 50% day^{-1}). Shrimps used in the study were obtained from the Faculty of Marine Sciences and Technology of Iskenderun University and transported to the Ankara University.

Effects of biofloc treated with two different carbon sources (corn starch: CS and glycerine: G) and mannan oligosaccharide (MOS) as a prebiotic on growth of speckled shrimp were investigated. In the experiment, 90 shrimps (8.32±0.69 g) were used in four different biofloc groups (CS, G, CS+MOS, G+MOS) and two groups without biofloc (MOS and Control) for 30 days. Each treatment was randomized with three replicates (5 shrimps/tank). Each biofloc was produced with different carbon sources (by starting before the 15 days of experiment and maintained with daily carbon addition, at 28.82-29.33 ppt salinity and 25.10-25.45 °C water temperature). Biofloc volume was controlled with imhoff to ensure that it remains at the level of 10-20 mL L^{-1} in biofloc groups. Shrimp were fed with 45% crude protein and 20% lipid marine fish commercial diet (Sibal LTD. Sinop, Turkey) at 3% of the estimated biomass (three times a day; 08.00, 13.00, 18.00) throughout the experiment. In the trial, a 12-h photoperiod was sustained by using fluorescent lighting. Corn starch and glycerine were added daily after feeding to maintain C:N as 15 during the study to ensure heterotrophic bacterial growth.

2.2. Water quality parameters

Throughout the experimental period, pH, temperature, salinity, and dissolved oxygen were measured daily using a multi-parameter instrument (YSI® 556, YSI Inc., Yellow Springs, OH, USA) at 08:30 hours in all tanks. Ammonium (NH_4) and nitrate (NO_3) were monitored once a week according to standard methods (APHA 1998).

2.3. Growth parameters

At the end of the study, the final weight, daily weight gain, feed conversion ratio (FCR), specific growth rate (SGR), and survival rate of six treatments were calculated as indicated below.

Weight gain (g/shrimp)= Final weight (g) - Initial weight (g) (1)

Feed conversion ratio (FCR)= Total feed given (g) / Weight gain (g) (2)

Specific growth rate (SGR; %/day)= ((*ln* (Final weight) - *ln* (Initial weight))/days) x 100 (3)

Survival rate (%)= (Final number of shrimp) / (Initial number of shrimp) x 100 (4)

2.4. Histology

Three shrimp samples from each triplicate group were anaesthetized with 5 mg L^{-1} quinaldine (Sigma Chemical Company, St. Louis, Missouri, USA) (Genc et al 2007), the carapax were opened, and hepatopancreas tissue were removed by dorso-ventral incision. The hepatopancreas samples were fixed for 24 h in 10% buffered formaline solution. After dehydration by passing tissues through a series of ethanol solutions (70, 85 and 98%) and clarification by two series of xylene, the samples were vacuum embedded in paraffin at 59±1 °C. The microtome sections (4-5 µm, Thermo Shandon, Germany) were stained for morphological purposes with haematoxylin and eosin (H & E) and then were analysed on microphotographs (Leica CM40) (Roberts & Smail 2004).

2.5. Total bacterial counts

Water samples from the culture tanks were stored in sterile glass bottles (50 mL) and analysed for total heterotrophic bacteria (THB). The preparation of the sample dilutions and bacteriological assays of the water were conducted separately and their averages were computed using the method described by APHA (2005). Total viable bacterial count from tank water was determined by the spread-plate technique on Plate Count Agar (PCA) with 2% NaCl. In each sample, serial dilutions were prepared in macro dilution tubes with concentrations ranging between 10^{-1} to 10^{-8} and were incubated at 37 °C for 48 hours. After the incubation period, the colonies were counted by eye and CFU values (CFU mL^{-1}) were calculated. Cultures containing less than 30 colonies and more than 300 colonies were not considered in the analyses.

The CFU mL^{-1} values were calculated using the formula;

CFU mL^{-1}= (no. of colonies x dilution factor) / volume of culture plate (5)

2.6. Statistical analysis

Statistical analysis was performed using SPSS, IBM, Statistics 23.0 for Windows. Analysis of variance (ANOVA) was used on shrimp performance and water quality parameters data to analyse the effect of BFT. The α level of 5% was used for all tests.

3. Results and Discussion

Water quality values are listed in Table 1. There were significant differences ($P<0.05$) among groups with regards to dissolved oxygen and pH parameters. Levels of dissolved oxygen and pH ranged from 6.02 to 6.29 mg L^{-1} and 7.73 to 7.96, respectively. The concentrations of ammonium (NH_4) and nitrate (NO_3) were found similar for all groups ($P>0.05$). The total number of bacteria count of shrimp reared in the biofloc groups were higher ($4.9x10^{-6}$±$8.5x10^{-4}$ CFU mL^{-1}) than that of in the no-biofloc ($3.7x10^{-6}$ ±$5.4x10^{-4}$ CFU mL^{-1}) groups ($P<0.05$) (Table 2).

Shrimp growth performance is presented in Table 3. Final weights of shrimp reared in CS+MOS group were significantly higher ($P<0.05$) than that of shrimp reared in MOS and C groups. There was no significant difference among the biofloc treated groups in terms of final weights ($P>0.05$). The highest weight gain (g) was obtained in group CS+MOS and the lowest value was observed in the control group ($P<0.05$). The best feed conversion ratio (FCR) of shrimp was observed in group CS+MOS and the highest FCR was calculated from the control group ($P<0.05$). The specific growth rate (%/day) and survival rate (%) of shrimp were higher in the biofloc treatments compared to the MOS and C, but no significant differences were detected among all groups ($P>0.05$).

Table 1- Water quality parameters of speckled shrimp culture environments

	Biofloc groups				*Without biofloc groups*	
Water parameters	*CS*	*G*	*CS+MOS*	*G+MOS*	*MOS*	*Control*
DO (mg L^{-1})	6.29±0.42^{a}	6.06±0.26^{ab}	6.02±0.68^{b}	6.07±0.35^{ab}	6.06±0.23^{ab}	6.15±0.41^{ab}
pH	7.95±0.18^{b}	7.73±0.45^{a}	7.88±0.19^{b}	7.96±0.14^{b}	7.95±0.10^{b}	7.94±0.10^{b}
T (°C)	25.15±0.50	25.30±0.55	25.10±0.70	25.45±0.50	25.20±0.64	25.29±0.72
Salinity ppt	29.33±0.76	29.07±0.87	29.03±0.85	28.93±0.90	28.82±0.86	29.13±1.22
NH_4 (mg L^{-1})	0.18±0.01	0.23±0.05	0.14±0.01	0.15±0.01	0.15±0.01	0.14±0.01
NO_3 (mg L^{-1})	0.21±0.10	0.21±0.10	0.21±0.10	0.25±0.09	0.23±0.09	0.25±0.09

CS, Corn starch; G, Glycerol; MOS, Mannan oligosaccharides. Different superscripts in the same line indicate significant differences (P<0.05)

Table 2- Results of total aerobic bacteria counts

	Biofloc groups				*Without Biofloc groups*	
Total aerobic bacteria	*CS*	*G*	*CS+MOS*	*G+MOS*	*MOS*	*Control*
TBC CFU (mL^{-1})	$4.8x10^{-6}$± $5.8x10^{-4b}$	$4.8x10^{-6}$± $5.8x10^{-4b}$	$5.0x10^{6}$± $5.8x10^{-4c}$	$5.0x10^{-6}$ ± $5.8x10^{-4c}$	$4.1x10^{-5}$± $3.3x10^{-4a}$	$3.3x10^{-5}$± $1.5x10^{-4a}$
Mean CFU (mL^{-1})	$4.9x10^{-6}$±$8.5x10^{-4}$				$3.7x10^{-6}$±$5.4x10^{-4}$	

CS, Corn starch; G, Glycerol; MOS, Mannan oligosaccharides. Different superscripts in the same line indicate significant differences (P<0.05)

Table 3- Growth parameters

	Biofloc groups				*Without Biofloc groups*	
Growth parameters	*CS*	*G*	*CS+MOS*	*G+MOS*	*MOS*	*Control*
IW (g)	8.33±0.69^{a}	8.30±0.79^{a}	8.36±0.83^{a}	8.36±0.81^{a}	8.32±0.74^{a}	8.35±0.75^{a}
FW (g)	10.99±0.9^{ab}	10.89±0.77^{ab}	11.51±0.75^{b}	10.93±0.93^{ab}	10.65±1.06^{a}	10.44±0.83^{a}
WG (g)	2.64±0.62^{ab}	2.63±0.32^{ab}	3.15±0.28^{b}	2.56±0.36^{ab}	2.36±0.69^{ab}	2.08±0.36^{a}
FCR	2.81±0.63^{ab}	2.89±0.32^{ab}	2.38±0.26^{a}	2.92±0.49^{ab}	3.24±0.90^{ab}	3.59±0.73^{b}
SGR (%/day)	0.92±0.21^{a}	0.92±0.09^{a}	1.07±0.11^{a}	0.89±0.13^{a}	0.83±0.24^{a}	0.75±0.16^{a}
SR (%)	93.33^{a}	86.67^{a}	93.33^{a}	93.33^{a}	86.67^{a}	86.67^{a}

CS, Corn starch; G, Glycerol; MOS, Mannan oligosaccharides. Different superscripts in the same row indicate significant differences (P<0.05)
IW: Initial weight, FW: Final weight, WG: Weight gain, FCR: Feed conversion ratio, SGR: Specific growth rate, SR: Survival rate

Hepatopancreas morphology was normal and did not differ among the groups (Figure 1). Different cell types were recognized but the structures were normal.

There are limited studies on growth of speckled shrimp, *M. monoceros*. In this study, water quality parameters (DO, pH, temperature, and salinity) were measured in the recommended range of

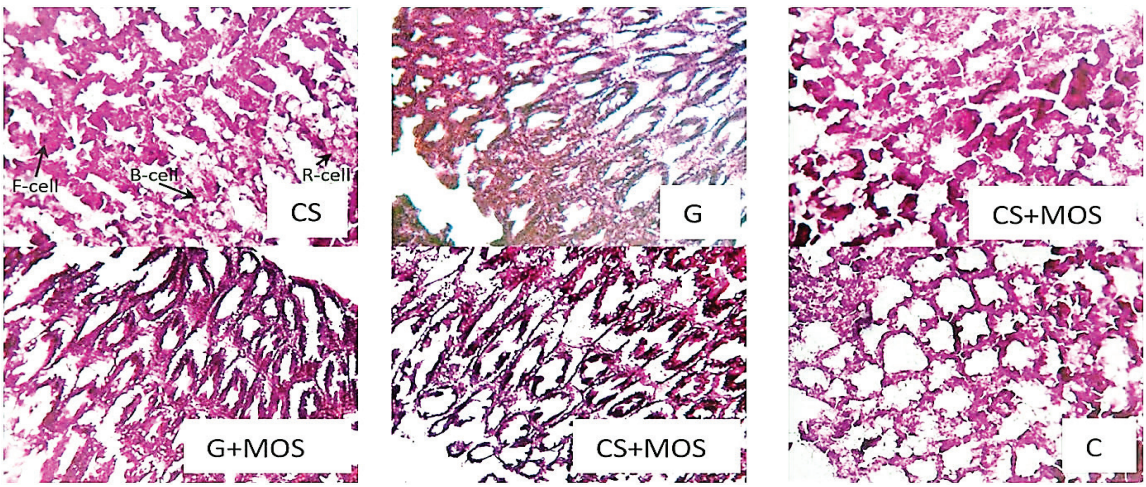

Figure 1- Speckled shrimp hepatopancreas tissue sections (10x, H & E)

marine shrimp culture with BFT. Water quality parameters observed in the current study were found to be similar to the study by Rahman et al (2010) for speckled shrimp. In BFT, where water exchange is limited, the inhibition of accumulation of nitrogenous toxic compounds may be possible by maintaining high C:N ratio and the consumption of ammonia by the microbial community (Avnimelech 1999; Kuhn et al 2010; Emerenciano et al 2012). In this study, ammonium and nitrate levels did not differ statistically between BFT and without BFT groups. Results reveal that the use of corn starch and glycerol as the carbon source and the maintenance of the optimum C:N ratio can sustain the low level of toxic nitrogen compounds in the culture environment. The mean total number of bacteria counts in the BFT groups were observed in similar ranges compared to the ranges reported in previous study ($4.23\text{-}4.55 \times 10^{-6}$ CFU mL^{-1}) by de Paiva Maia et al (2016). However, bacterial count ranges not only in BFT groups but also in without BFT groups of the present study were higher (245.67×10^{-8} CFU mL^{-1}) compared to the study by Anand et al (2014). This discrepancy may be explained by the low salinity (13.67-14.29 ppt) conditions used in the previously mentioned study.

Digestive glands consist of diverticula of the intestine and the main functions of the hepatopancreas are absorbing nutrients, storing lipids, and producing digestive enzymes (Johnson 1980; Genc et al 2007). According to the literature on physiology of digestion, absorption plays an important role in lipoprotein metabolism and could be used to monitor nutritional value of shrimp diets. In this study, the speckled shrimp reared with BFT and without BFT conditions fed on commercial marine fish diet with or without MOS supplementation showed similar and normal morphology.

It is argued that it can be possible to obtain better yield from a unit of area by using BFT in aquaculture. In our experiment, the growth parameters (the final weight, daily weight gain, feed conversion ratio, specific growth rate, and survival rate) were higher in BFT groups than the shrimp in the without BFT groups. The final weight of shrimp treated with CS+MOS was statistically higher than that of shrimp reared in the control group ($P<0.05$). These results revealed that BFT increased growth parameters in shrimp culture in parallel with previous studies (Krummenauer et al 2011; Emerenciano et al 2011; Xu & Pan 2012; Emerenciano et al 2012; Kumar et al 2017; Lara et al 2017).

4. Conclusions

Although previous studies focused on the effects of different carbon sources in BFT on growth parameters (Crab et al 2012; Emerenciano et al 2013), the current study investigated the effect of prebiotic supplementation with two different carbon sources. In conclusion, the combined use of corn starch and mannan oligosaccharide in biofloc technology could be considered as a productive application for shrimp culture. In addition, BFT application on speckled shrimp larvae is recommended for future studies. This study is the first record on speckled shrimp *M. monoceros* culture with BFT treatment.

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Characterization of Wild Apricot (*Prunus armeniaca* L.) Genotypes Selected from Cappadocia Region (Nevşehir-Turkey) by SSR Markers

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ABSTRACT

Cappadocia region of Anatolia hosts the third largest wild apricot population in Turkey. The objective of the study was to characterize 44 wild apricot genotypes selected from Cappadocia Region (Nevşehir-Turkey) as prominent with their late flowering, resistance to spring late frosts, large fruit sizes and/or late fruit ripening characteristics and 5 reference apricot cultivars ('Hacıhaliloğlu', 'Kabaaşı', 'Hasanbey', 'Aprikoz' and 'Levent') with SSR (simple sequence repeats) markers. A total of 16 SSR primers were used and 13 of them were successfully amplified. Total number of alleles was 107, average number of alleles was 8.23; average *He* and *Ho* values were 0.722 and 0.669, respectively. Polymorphism information content (PIC) values varied between 0.471 and 0.845. There was a quite high genetic diversity among wild apricot genotypes that genetic similarity values varied between 12 and 96%. Homonymous and synonymous genotypes were not encountered.

Keywords: Wild apricot; *Prunus armeniaca;* Genetic diversity; Genetic relationship; Molecular characterization; SSRs

1. Introduction

Vavilov (1951) indicated the origin centers of culture apricots (*Prunus armeniaca* L.) as China, Central Asia and defined Near-East centers extending from Northeastern Iran to Caucasus and Central Anatolia as the secondary origin center of cultured forms. Kostina (1969) divided *P. armeniaca* species into 4 large eco-geographical groups and 13 regional sub-groups and placed Turkey into Iran-Caucasus eco-geographic group (Layne et al 1996; Zhebentyayeva et al 2012). Anatolia (Turkey) is located within the secondary origin center of apricots, thus has a great genetic diversity (Ercisli 2004). Nevşehir province is located right at the center of Cappadocia region of Anatolia, and the province hosts the 3rd largest wild apricot population with about 145000 trees (TUIK 2017). This population is characterized with late flowering, resistance to spring late frosts, large fruits and late ripening. Thus the population exhibits a large variation in fruit physical and quality attributes. Such a diverse population was evaluated for the first time by Dumanoğlu et al (2018) within

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the scope of a scientific research project, and superior genotypes were identified. These genotypes were then put under protection in a collection orchard. The genotypes constitute significant materials for apricot breeding studies and genetic relationships among these genotypes should be identified with further molecular techniques. Microsatellites or simple sequence repeats (SSRs) are short repeat sequences (1-6 base length) and have co-dominant characteristics, greater polymorphism ratios, are abundant in genome and have quite high repeatability. Therefore, they have a significant place among DNA markers (Litt & Luty 1989; Gupta et al 1996). These markers are commonly used in identification of species, preservation of genetic materials, population genetics, quantitative trait loci mapping, marker assisted selection and similar studies. SSR markers are also used in genetic characterization of *Prunus* species, including apricots. However, SSR markers were not developed at the same rates for each one of the significant species (apricot, peach, plum, and almond), thus potential use of SSR markers of a species in other *Prunus* species (cross-transferability) have become a significant issue (Hormaza 2002; Romero et al 2003; Zhebentyayeva et al 2003; Hagen et al 2004; Messina et al 2004; Mnejja et al 2005; Sanchez-Perez et al 2005; Ruthner et al 2006; Bouhadida et al 2009; Wünsch 2009; Akpınar et al 2010; Bourguiba et al 2010; Liu et al 2013; Wang et al 2014; Eroglu & Cakir 2015; Gürcan et al 2015; Murathan et al 2017).

In this study, genetic relationships between wild apricot genotypes selected from the wild apricot gene sources of Nevşehir province with regard to late flowering, resistance to spring late frosts, large fruits and or late fruit ripening characteristics were identified with SSR markers developed from *P. armeniaca* and *P. persica*.

2. Material and Methods

2.1. Plant material and DNA isolation

In this study, 44 wild apricot genotypes selected from Nevşehir (Cappadocia Region-Turkey) locality and the reference apricot cultivars of 'Aprikoz', 'Kabaaşı', 'Hasanbey', 'Hacıhaliloğlu' and 'Levent' were used as the plant material. DNA isolations were performed from fresh shoot tips and young leaf samples collected from the genotypes (Lefort et al 1998). DNA purity and concentrations were determined in ND-1000 spectrophotometer and isolated DNA was visually controlled in 1% agarose gel.

2.2. SSR reactions

A total of 16 SSR loci were selected as of 10 *P. armeniaca* (apricot), 4 *P. persica* (peach) and 2 *P. armeniaca* EST-SSR loci (Table 1). Selected SSR loci were tested and the polymorphic ones were used in genetic identifications. PCR amplifications were performed by using M13-tailed primer according to the methods described by Schuelke (2000) in *Prunus* genotypes. A tail (M13 universal sequence (-21), TGTAAAACGACGGCCAGT) was added to the 5' end of each forward primers. PCR amplifications were performed in 15 µL reaction mixture containing 90 ng genomic DNA, 0.1 µM of each SSR primer, 0.1 µM labelled M13 (-21) universal primer, 0.2 mM of each dNTPs, 1X DreamTaq Green Buffer (includes $MgCl_2$ at a concentration of 2 mM) (Thermo Scientific) and 0.5 U DreamTaq DNA Polymerase (Thermo Scientific). The amplification program consisted of an initial step of 3 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 50-66 °C, 2 min at 72 °C, followed by 8 cycles of 1 min at 94 °C, 1 min at 53 °C, 2 min at 72 °C, and a final extension at 72 °C for 10 min. The M13 (-21) primer was 5'-fluorescently tagged with HEX, 6-FAM or ROX to facilitate multiplexing. A set of three PCR products (0.5 µL each) was mixed with 0.5 µL GeneScan-600 LIZ size standards (Applied Biosystems, USA) and 9.5 µL Hi-Di™ formamide (Applied Biosystems) and denatured at 95 °C for 5 min, chilled on ice and electrophoresed on the Applied Biosystems Prism 3500 Genetic Analyzer System (Applied Biosystems, USA). GENEMAPPER software v5.0 (Applied Biosystems, USA) was used to determine fragment size.

Table 1- SSR markers used for characterization of wild apricot genotypes selected from Nevşehir locality

No	*Locus*	*Primer sequences (5'→3')*	*Tm (ºC)*	*Species*	*Reference*
1	UDAp-407F*	ttctgctacttacaatcgtgttctc	56	*Prunus armeniaca*	Messina et al (2004)
	UDAp-407R	agagcaccaggtctttctgg			
2	UDAp-410F*	ttgttgacaagaagaaaacaaagc	56	*Prunus armeniaca*	Messina et al (2004)
	UDAp-410R	caacgggttggtttcagaag			
3	UDAp-411F*	tcggtggagaaagagactgg	56	*Prunus armeniaca*	Messina et al (2004)
	UDAp-411R	gtcccccaccctttacaatg			
4	UDAp-414F*	caagcacaagcgaacaaaat	56	*Prunus armeniaca*	Messina et al (2004)
	UDAp-414R	ggtggtttcttatccgatgc			
5	UDAp-415F*	aactgatgagaaggggcttg	56	*Prunus armeniaca*	Messina et al (2004)
	UDAp-415R	actcccgacatttgtgcttc			
6	UDAp-418F*	cagaaatagccccagcacat	56	*Prunus armeniaca*	Messina et al (2004)
	UDAp-418R	ttcttgcgccaaaaacaact			
7	UDAp-420F*	ttccttgcttcccttcattg	56	*Prunus armeniaca*	Messina et al (2004)
	UDAp-420R	cccagaacttgattctgacca			
8	UDAp-423F*	ccatgtagaaactggctgagg	56	*Prunus armeniaca*	Messina et al (2004)
	UDAp-423R	cactcgactctctcgcctct			
9	AMPA105F*	ctgctctcactcaactcaatgc	55	*Prunus armeniaca*	Hagen et al (2004)
	AMPA105R	ctcccctacccctctgtatctc			
10	AMPA096F*	ttttgtgccaaagtagcatcag	55	*Prunus armeniaca*	Hagen et al (2004)
	AMPA096R	tcaactaaccaaaaggagtggc			
11	UDP96-010F*	cccatgtgtgtccacatctc	55	*Prunus persica*	Cipriani et al (1999)
	UDP96-010R	ttgatgattccatgcgtctc			
12	UDP96-019F*	ttggtcatgagctaagaaaaca	55	*Prunus persica*	Cipriani et al (1999)
	UDP96-019R	tagtggcacagagcaacacc			
13	UDP98-406F *	tcggaaactggtagtatgaacaga	55	*Prunus persica*	Cipriani et al (1999)
	UDP98-406R	atgggtcgtatgcacagtca			
14	Ma040aF*	agaaattggagtgacgtaac	55	*Prunus persica*	Yamamoto et al (2002)
	Ma040aR	acgtgatgagaagtagggag			
	EST-SSR Primers	tcggaaactggtagtatgaacaga	55	*Prunus persica*	Cipriani et al (1999)
15	AMPA116F*	attgaaggccccttatgtgag	55	*Prunus armeniaca-EST*	Hagen et al (2004)
	AMPA116R	caaaaaggcgttacagatgatg			
16	AMPA119F*	gtgcccacttacctgttttagg	55	*Prunus armeniaca-EST*	Hagen et al (2004)
	AMPA119R	tcgacgatcagacttgctacag			

*, Fluorescent labeled primer

2.3. Statistical analysis

For each locus, the expected heterozygosity (*He*), observed heterozygosity (*Ho*) and polymorphism information content (PIC) (Nei 1973) were calculated with PowerMarker V3.025 software (Liu & Muse 2005). The neighbor-joining (NJ) and unweighted pair-group method using arithmetic average (UPGMA) were used to construct and draw a dendrogram from the genetic similarity matrix by using the MEGA6 (Tamura et al 2007) and PowerMarker software programs. Bootstrap analyses with 100 replicates were performed and a consensus tree was obtained to measure the confidence levels for the clusters.

3. Results

Allele sizes (bp, base-pair) for 13 SSR loci of wild apricot types selected from Nevşehir locality and reference apricot cultivars are provided in Table 2. Considering the success ratios of 16 SSR loci selected for genetic characterization of wild apricot types, 10-15 of them were thought to be used in genetic analyses. Thirteen SSR loci were successfully amplified. Of the remaining 3 SSR loci, UDP98-406 locus yielded successful PCR reactions, but was not able to be assessed well because of mixed peaks in capillary electrophoresis system. While PCR reactions of AMPA096 locus were unsuccessful, UDP96-019 locus was identified as monomorphic. Therefore, these 3 loci were not used in genetic analyses. The highest number of alleles was observed in UDAp-418 locus with 14 alleles (Table 3). It was followed by both UDP96-010 and AMPA105 loci with 10 alleles and the lowest number of alleles was observed in Ma040 locus with 5 alleles. Total number of alleles was 107 and average number of alleles was 8.23. Average *He* and *Ho* values were calculated 0.722 and 0.669, respectively. *He* values varied between 0.501 and 0.860 and *Ho* values varied between 0.449 and 0.816. Polymorphism information content (PIC) value varied between 0.471 (AMPA119) and 0.845 (UDP96-010) (Table 3).

Genetic similarity dendrogram indicated that genotypes were basically separated into 3 groups (Figure 1). When the dendrogram was evaluated based on the places from where the samples were collected, it was observed that grouping was independent from the sampling locations. Considering the place of reference apricot cultivars in the dendrogram, 'Hasanbey' was placed in the second group, and the other cultivars were placed in 3rd group. Reference cultivars were not placed in the first group.

Genetic similarity index values varied between 12 and 96% with the greatest similarity (96%) between wild apricot genotypes of #6 and #61. These genotypes were followed by the #32-#34 and #39-#68 with a similarity ratio of 92%. The lowest genetic similarity (12%) was observed between the genotypes #13 and #38, #38 and #50, #38 and #64, and #45 and #47. Degree of genetic similarity was independent from the sampling locations. Genotypes #6 and #61, 45 km away from each other (Çavuşin and Gümüşkent, respectively) had the highest genetic similarity (96%) in the study. However, more distantly located (>65 km) genotypes #39 (Gümüşkent) and #68 (Çakıllı), or genotypes #32 and #34 at the same location (Yeşilöz) had the same genetic similarity level of 92%.

4. Discussion

Hormaza (2002) used 37 SSR loci developed from *Prunus* species to identify the genetic relationships among 48 apricot genotypes collected from different geographical regions. Of these loci, 31 were successfully amplified, 20 had repeatable polymorphic characteristics and a total of 82 alleles were identified in 48 genotypes. The common primer UDP96-100 was also found to be polymorphic in this study. Polymorphic UDP98-406 primer was not able to be assessed because of complex peak profile in our study. In another study, Romero et al (2003) used 16 SSR loci developed from peach genome to identify the relationships among 40 apricot genotypes collected from different eco-geographical regions. Of these loci, 11 presented polymorphism in apricot genotypes and allowed clear identification of each genotype. The common primer UDP96-010 similarly yielded the greatest separation power in this study. Zhebentyayeva et al (2003) tested 30 SSR loci developed through enriched library method from peach genome for 74 apricot genotypes. Of 30 SSR loci tested, 20 were amplified in apricot and 14 were reported to be used in separation of apricot genotypes and apricot germplasm diversity studies. Messina et al (2004) isolated 99 SSR loci from apricots and tested 20 of them in 16 apricot genotypes to determine polymorphism ratios. Of 20 SSR loci tested, 9 (UDAp-401, UDAp-404, UDAp-407, UDAp-410, UDAp-411, UDAp-414, UDAp-415, UDAp-418 and UDAp-420) were recommended to be used in apricot fingerprinting studies. Researchers also implied that 20% of

Table 2- Allelic data (bp) for wild apricot genotypes of Nevşehir locality and standard apricot cultivars (A, 'Aprikoz'; L, 'Levent'; HB, 'Hasanbey'; HH, 'Hacıhaliloğlu'; KA, 'Kabaaşı')

Genotype #	*UDAP411*		*UDAP415*		*Ma040*		*AMPA116*		*UDAP414*		*UDAP423*	
3	84	110	163	175	225	225	135	165	166	178	184	198
4	88	110	163	175	233	233	135	135	166	186	184	206
6	110	110	169	179	223	241	135	137	166	186	184	184
7	88	110	169	179	223	223	135	137	178	190	184	204
13	88	88	169	169	223	223	135	165	166	166	184	198
14	84	110	175	179	223	233	135	135	178	186	184	204
15	88	110	169	177	223	241	135	165	166	186	184	204
16	84	84	169	169	223	223	135	135	178	186	184	204
17	110	110	163	175	223	223	135	135	178	178	190	206
18	110	110	163	169	223	233	135	135	166	178	184	206
19	88	88	169	175	233	241	135	159	178	186	184	190
20	110	110	169	179	223	241	135	137	166	186	184	184
21	110	110	163	163	233	233	135	153	166	180	180	204
22	110	110	169	169	233	241	135	135	186	186	184	204
23	110	122	163	163	223	225	165	165	166	178	184	206
24	110	122	167	167	223	233	135	153	178	178	198	204
26	84	109	163	163	223	241	135	153	178	186	198	206
27	110	110	163	169	223	223	135	159	178	186	184	206
28	104	104	167	179	223	233	135	137	166	178	184	204
29	110	122	169	175	223	223	135	137	178	190	184	184
31	88	122	163	167	223	223	135	165	166	166	184	198
32	110	110	169	175	223	223	135	153	179	179	184	198
33	110	122	179	179	223	233	135	159	166	178	184	200
34	110	110	169	175	223	223	135	153	178	178	184	198
35	110	110	169	179	223	233	135	135	178	186	184	184
38	110	110	175	181	233	233	145	145	178	178	184	184
39	84	84	169	175	233	233	135	135	178	178	184	184
41	104	110	175	175	223	223	135	135	166	178	184	204
42	88	88	167	175	223	223	135	135	178	186	184	200
43	110	110	167	175	223	233	135	159	166	186	184	204
45	110	110	163	179	223	233	135	135	166	178	184	200
46	84	110	163	179	233	241	135	165	166	178	184	204
47	88	88	175	175	223	223	135	166	166	166	198	204
48	104	104	169	175	223	223	135	137	186	186	186	199
49	88	110	163	167	223	223	135	153	178	178	184	204
50	84	109	163	163	223	233	135	135	166	186	184	206
53	84	104	169	175	223	233	135	137	179	186	186	206
54	88	110	169	175	225	241	135	165	166	186	190	204
59	110	122	163	169	223	223	159	165	166	186	184	204
60	88	110	175	175	223	233	135	135	178	186	184	204
61	110	110	169	179	223	241	135	137	166	186	184	184
64	88	110	169	169	223	223	129	135	184	184	184	200
68	84	84	169	175	233	233	135	135	178	178	184	184
76	88	88	163	175	233	233	135	165	166	186	184	206
A	110	110	163	169	223	225	129	159	184	184	184	198
HB	84	84	175	181	223	233	135	143	166	180	180	198
HH	84	84	167	175	223	223	135	153	180	180	180	204
KA	84	110	163	167	223	223	135	153	180	186	184	204
L	84	110	167	167	223	241	135	153	180	186	204	204

Table 2 (Continue)- Allelic data (bp) for wild apricot genotypes of Nevşehir locality and standard apricot cultivars (A, 'Aprikoz'; L, 'Levent'; HB, 'Hasanbey'; HH, 'Hacıhaliloğlu'; KA, 'Kabaaşı')

Genotype #	*UDAP96-010*		*UDAP418*		*AMPA105*		*AMP119*		*UDAP420*		*UDP407*		*UDP410*	
3	93	117	176	180	203	203	114	122	195	195	204	206	142	162
4	93	99	180	180	195	195	120	122	195	195	186	206	140	168
6	113	117	176	182	203	203	116	120	179	195	204	204	140	142
7	99	105	154	170	205	205	116	120	195	195	186	204	166	166
13	117	117	170	170	205	231	120	124	179	179	186	204	142	142
14	105	117	176	180	227	227	120	130	179	195	182	204	136	166
15	93	99	176	180	203	205	116	120	169	195	186	204	142	142
16	113	113	170	180	231	231	116	122	195	195	186	208	162	166
17	99	103	180	180	227	231	120	120	169	195	186	204	136	168
18	93	103	150	180	195	231	120	120	169	171	186	206	142	168
19	97	117	180	180	195	205	116	122	179	195	182	186	140	140
20	114	117	176	184	203	203	120	120	179	195	204	204	140	142
21	99	113	150	152	203	227	120	120	169	195	186	186	136	136
22	103	105	176	180	195	217	120	120	179	195	186	204	140	162
23	113	117	150	180	195	227	116	120	169	195	186	204	140	168
24	105	117	170	180	195	227	116	120	179	195	204	209	140	162
26	93	117	152	176	217	231	120	120	169	179	186	206	140	168
27	99	103	176	176	205	217	120	120	189	195	206	206	140	166
28	93	113	174	180	195	195	120	120	171	195	182	186	136	142
29	91	117	176	180	205	227	120	122	195	195	186	208	162	162
31	103	117	180	180	205	227	120	120	171	195	204	204	142	168
32	103	117	176	180	195	195	120	120	169	195	182	206	140	162
33	99	105	182	182	195	205	120	120	169	195	182	206	136	142
34	103	117	176	180	195	195	120	120	169	195	182	206	140	162
35	97	105	176	180	205	227	116	120	187	195	186	186	162	168
38	97	97	170	180	205	205	129	129	195	195	209	209	144	144
39	105	105	154	164	195	195	120	120	195	195	186	209	140	162
41	93	93	176	180	227	231	120	130	183	195	186	186	142	166
42	105	117	176	180	195	227	120	120	171	179	186	204	142	162
43	97	99	176	176	195	205	116	120	179	195	182	186	142	166
45	97	97	150	150	226	226	120	130	171	195	182	182	140	168
46	93	97	154	180	195	195	120	120	169	195	186	186	140	166
47	103	117	164	176	205	231	116	122	169	169	186	208	142	166
48	91	117	150	180	227	231	120	122	169	171	186	206	166	166
49	91	105	152	176	203	227	120	120	169	185	186	186	166	166
50	97	117	176	181	195	231	120	122	169	195	184	186	140	162
53*	105	117	150	170 180	205	217 227	120	120	169	195	186	206	136	162
54	97	99	154	176	195	205	120	120	169	195	204	204	142	168
59	97	105	176	176	195	205	120	120	169	169	182	186	136	168
60	105	113	154	184	195	205	116	120	183	195	186	204	140	140
61	113	117	176	182	203	231	116	120	179	195	204	204	140	142
64	113	113	170	170	191	191	120	120	169	171	186	204	136	140
68	105	105	154	154	195	195	120	122	195	195	186	209	140	162
76	93	99	164	182	195	195	120	122	195	195	186	206	140	168
A	105	113	169	176	227	227	120	122	169	195	186	205	162	162
HB	97	105	164	182	195	205	116	120	169	195	186	206	142	168
HH	99	105	164	180	205	205	120	120	185	195	204	206	140	168
KA	105	105	162	164	205	227	120	120	171	195	204	206	140	142
L	99	103	148	180	227	227	120	120	169	185	186	204	142	168

*, Genotype #53 has 3 alleles and shows triploidy for the loci of UDAP418 and AMPA105

Table 3- Genetic parameters for wild apricot genotypes of Nevşehir Locality (number of alleles (n), expected (*He*) and observed (*Ho*) heterozygosity, polymorphism information content, PIC)

Locus	*n*	*He*	*Ho*	*PIC*
UDAp-411	6	0.675	0.449	0.634
UDAp-415	7	0.787	0.714	0.755
Ma040	5	0.601	0.469	0.546
AMPA116	9	0.607	0.673	0.585
UDAp-414	7	0.751	0.673	0.710
UDAp-423	9	0.715	0.816	0.684
UDP96-010	10	0.860	0.796	0.845
UDAp-418	14	0.821	0.755	0.801
AMPA105	10	0.812	0.571	0.788
AMPA119	7	0.501	0.510	0.471
UDAp-420	8	0.682	0.755	0.641
UDAp-407	8	0.747	0.735	0.710
UDAp-410	7	0.825	0.776	0.801
Total	107	9.385	8.694	8.971
Average	8.23	0.722	0.669	0.690

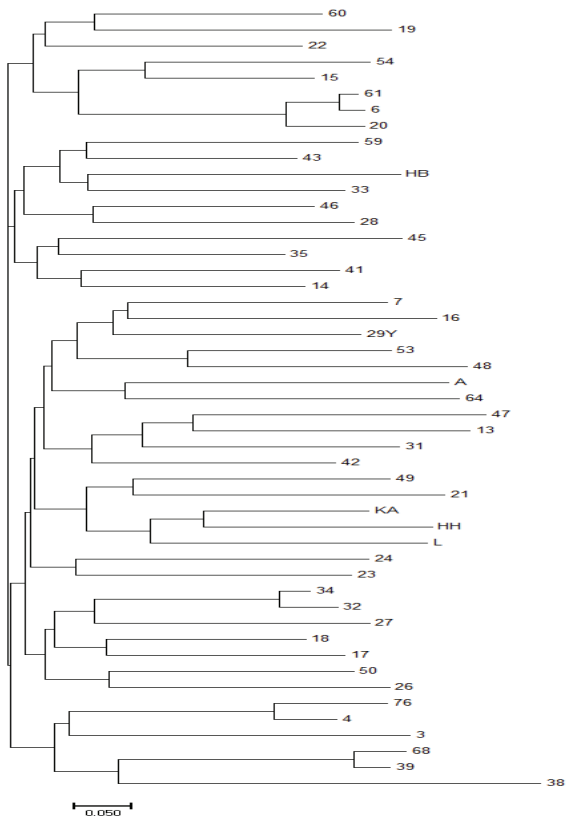

Figure 1- UPGMA (Unweighted pair group method with arithmetic average) based dendrogram showing genetic similarity between 44 wild apricot genotypes of Nevşehir locality and 5 reference apricot cultivars (A, Aprikoz; L, Levent; HB, Hasanbey; HH, Hacıhaliloğlu; KA, Kabaaşı) based on 13 SSR loci

SSR loci were successfully amplified in different *Prunus* species including peaches, nectarine, almond, European plum, Japanese plum, cherry and sour cherry. Of the relevant primers, UDAp-401 and UDAp-404 were also used in the present study and successfully amplified and had quite high polymorphism ratios. Akpınar et al (2010) used 10 SSR loci of which 2 were developed from apricots and 8 from peaches for genetic identification of 25 local apricot genotypes and 4 foreign reference cultivars. In the present study, the primer UDP96-010 developed from the peaches had also similar polymorphisim. Ullah et al (2017) employed 20 SSR loci for genetic characterization of 12 apricot genotypes significant for the economy of Pakistan and reported polymorphic bands for 18 of them.

Zhebentyayeva et al (2003) characterized 74 apricot genotypes with 30 SSR markers and reported the number of alleles between 2 (Pchgms 17) and 16 (UPD 96-001) and total number of alleles as 107 with an average number of alleles as 7.64. Messina et al (2004) tested 20 of 99 SSR loci isolated from apricots in 16 apricot genotypes to determine polymorphism ratios and reported the number of alleles between 2 and 9, expected heterozygosity between 0.26 and 0.82. Liu et al (2013) developed 19 microsatellite loci for marker assisted selection (MAS) of *P. sibirica* L. with regard to late flowering and characterized them in 40 genotypes. Researchers reported the number of alleles between 3 and 11, expected and observed heterozygosity ratios between 0.063 and 0.917, and between 0.295 and 0.876, respectively. In another study carried out with *P. sibirica* L. species, 31 SSR loci were used to assess genetic diversity and population structure. The number of alleles were reported between 5 and 33 with an average value of 19.323, and average expected and observed heterozygosity ratios were reported 0.639 and 0.774, respectively (Wang et al 2014). Gürcan et al (2015) assessed 278 apricot genotypes with 20 SSR loci and reported the number of alleles between 5 and 25 with an average number of alleles as 12.78, expected and observed heterozygosity values of 0.75 and 0.63, respectively. Study, 49 *P. armeniaca* genotypes including wild apricot genotypes and reference cultivars were screened through 13 SSR markers and total number of alleles was identified as 107, average number of alleles per locus as 8.23, polymorphism information content as 0.69.

SSR markers are used for various purposes (genetic mapping and etc.), especially for genetic characterization of *Prunus* species, including apricots. However, SSR markers were not able to be developed at the same rates for each one of the significant species (apricot, peach, plum, almond), thus potential use of SSR markers of a species in other *Prunus* species (cross-transferability) have become a significant issue. In this study, two different sources were preferred in selection of SSR loci for genetic assessment of wild apricot genotypes. Of these loci, while only one of 10 SSR loci (AMPA096) was unsuccessful, peak quality was poor in UDP98-406 locus developed from *P. persica*, and the UDP96-019 locus developed from the same source was monomorphic. EST-SSR primers developed from *P. armeniaca* were successfully amplified and identified as polymorphic.

5. Conclusions

In this study, 16 SSR primers developed from apricot and peaches were used. Thirteen SSR primers were successfully amplified, and they had quite high polymorphism ratios. These 13 SSR loci were found sufficient and successful for characterization and identification of selected wild apricot genotypes. There was a quite high genetic diversity among the genotypes. Genetic similarity varied between 12 and 96%, and homonymous and synonymous genotypes were not encountered.

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Effects of Sowing Date, Cultivar and Chitosan on Quality and Quantity of Rapeseed (*Brassica napus* L.) Oil

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ABSTRACT

The effects of cultivars and sowing date along with chitosan application on oil yield, yield components and fatty acids of rapeseed (*Brassica napus* L.), were investigated. Five cultivars (RGS003, Sarigol, Zafar, Dalgan, and Julius) were sown in three sowing dates (October 7, 17, and 27), for two years (2014-2015 and 2015-2016). A factorial split-plot experiment was conducted in a complete randomized blocks design with three replications, where the sowing dates and the two levels of chitosan (0 {control} and 0.2% concentrations) were allotted to main plots and the cultivars were allotted to subplots. ANOVA revealed a significant ($P<0.01$) effects of the three studied factors on studied characters. Sarigol cultivar had the highest amount of seed yield (4447 kg ha^{-1}), seed oil (45.51%) and biological yield (15672 kg ha^{-1}). These characters had the highest values in the first sowing date. Application of chitosan solution increased the amount of seed yield from 3916 to 4233 (kg ha^{-1}), seed oil from 44.83 to 45.24% and biological yield from 13628 to 14797 (kg ha^{-1}). Delayed sowing dates, increased the linolenic and erucic acids and decreased the palmitic, oleic, and linoleic acids. The results of the present study indicated that early sowing date and chitosan application had positive effects on the quantity and quality of rapeseed oil. Cluster analysis divided the cultivars into two main clusters. The PCA revealed that the three first PC confirmed about 96% of the total variance among the studied cultivars.

Keywords: Rapeseed (*Brassica napus* L.); Seed oil; Biological yield; Year

1. Introduction

Rapeseed or oilseed rape (*Brassica napus* L.) is one of the most important oilseeds with 40-44% oil and it is the world's third annual edible oil after soybean (*Glycine max* Merril.) and oil palm (*Elaeis guineensis* Jacq.) (Enjalbert et al 2013). Also, the oil of the plant is currently considered as the best oil in human nutrition with 61% oleic acid, 20% linoleic acid (Spasibionek et al 2003).

Oil yield and glucosinolate accumulation in canola are inheritable, however, they are affects by environmental factors, too (Fieldsend et al 1991; Azizi et al 2006). Early and late sowing dates causes the plant to be exposed to undesirable environmental conditions. The determination of the best sowing

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date plays an important role in the confirmation of plant growth stages to desirable environmental conditions, resulting in maximum quality and quantity yield (Siadat & Hemayati 2009). The sowing date is the most effective treatment on the physiological and physiological characteristics of the crop (Khayat 2007). Optimum sowing date cause in the better seedling establishment and improved cold tolerance (Sultan & Angadi 2016). However delay sowing date cause decreasing in plant height and seed yield (Alisial et al 2005). The early and late sowing date of this plant causes the plant to face a variety of stresses during its growth. The chitosan is a useful natural polymer that produced by alkaline N-deacetylation of chitin. It is a component of the cell walls of some algae, fungi, and insects. The chitosan can be sprayed on plant aerial parts to induce the accumulation of bioactive secondary metabolites (Lei et al 2011; Yin et al 2011). Spraying plants with chitosan compensated to some extent for the negative impact of drought stress on some main characters. Reduction of the negative impact of drought on some characters by foliar application of chitosan has been reported in different plants (Dzung et al 2011; Mahdavi et al 2011). Bittelli et al (2001) reported that it reduced plant transpiration in pepper (*Capsicum* sp. L.), resulting in 26-43% reduction in water use without a reduction in dry matter yield. With increased levels of chitosan and drought stress, the amount of oil of Thymus (*Thymus daenensis* Celak.) increased (Bistgani et al 2017). The chitosan is used to cover seeds, leaves, and fruits (Devlieghere et al 2004). Studies on the effect of sowing time and application of the chitosan on quality and quantity of rapeseed oil using different cultivars are limited in number. Therefore, the aim of this study was to determine the effects of three sowing times and chitosan on some traits in rapeseed cultivars.

2. Material and Methods

2.1. Experiment conditions

The study was conducted at the Karaj Seed and Plant Improvement Research Institute, Karaj, Iran (35°49′N, 50°59′E; altitude of 1321 m asl) during the 2014-2015 and 2015-2016. This area has average annual rainfall and temperature of 354 mm and 14.2 °C, respectively. The soil of the experimental was a loam clay, with montmorllionite clay mineral, low in nitrogen (0.06-0.09%), with a Ec= 0.66 dS m^{-1} and pH of 7.2-7.9 (Table 1). The experiment was organized in a randomized complete block design, with factorial split plot arrangement, with three replications. There were three factors including (5) cultivars of rapeseed (RGS003, Sarigol, Zafar, Dalgan and Julius), (3) sowing dates (October 7, 17

Table 1- The result of soil analysis

Parameter	*2014- 2015*		*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	7.90	7.20	7.80	7.40
Total neutralizing value (%)	8.56	6.68	8.25	8.46
Moisture content (%)	36.00	38.00	35.00	37.00
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})	14.70	15.80	14.20	15.30
Available K (mg kh^{-1})	197.00	155.00	165.00	148.00
Clay (%)	28.00	25.00	29.00	27.00
Silt (%)	47.00	49.00	45.00	46.00
Sand (%)	25.00	26.00	26.00	27.00
Soil texture	Clay loam	Clay loam	Clay loam	Clay loam

and 27) and (2) levels of chitosan (0: control and 0.2%). The sowing dates and chitosan were allotted to main plots. However the cultivars were allotted to subplots. A solution of chitosan (0.2%) was sprayed at budding time. Soil samples were collected at the depth of 0-30 cm and 30-60 cm, before seed sowing. The soil physicochemical properties were presented in Table 1. According to the soil analysis, 150 kg ha^{-1} of ammonium phosphate and potassium sulphate before sowing date and 350 kg ha^{-1} urea at (3) different times (100, 150 and 100 kg ha^{-1} at sowing time, stem elongation and flowering stage, respectively), were applied.

Each experiment plot consisted of 6 lines 6 meters with lines of 60 cm and plant spacing on the 4 cm line, as well as two lateral lines as margins and its four middle lines were used to determine all phonological stages of the plant and various traits. Irrigation and disease control measures were done as per requirement. At full maturity ten randomly selected sample plants were collected separately from each plot.

2.2. The measured characters

In the maturity stage the following characters were measured, seed yield, number of pods per plant, number of seeds per pod, 1000-seed weight, biological yield, and seed oil yield. The oil content was extracted by Soxhlet method (Joshi et al 1998). Also, seed glucosinolate content and fatty acids in the oilseeds were measured using with a high-performance liquid chromatography device (HPLC; Unicam 4600, England) (Yang et al 2009).

2.3. Data analysis

In order to verify the homogeneity of error variance of combined analysis, Bartlett's χ^2 test was used. Since the data of the two years had homogeneous variances, the combined analysis was performed on the data. The data were analyzed using Statistical Analysis Software (V. 9.1; SAS Institute, Cary, NC). In addition, the mean values were compared by using the LSD test (Steel &Torrie 1980). Cluster analysis was conducted to distinguish among the five cultivars based on the arithmetic mean (UPGMA) method. The cluster analysis was performed by SPSS software on Windows 20.0 (SPSS Inc., Chicago, IL).The principal component analysis (PCA) was conducted with MetaboAnalyst Software (v. 3.0) (Xia & Wishart 2016).

3. Results and Discussion

3.1. Analysis of variance

The result of Bartlett's χ^2 test for all characters showed that the data of the two years had homogeneous variances, therefore the combined analysis was performed on the data. Analysis of variance indicated that the effects of the cultivar, year, sowing date, and the chitosan were significant ($P<0.01$) in all studied characters (Table 2). Rad et al (2015) reported that all assessed traits in canola were significant by the different sowing dates. Siadat & Hemayati (2009) described the variety factor had a significant effect on all rapeseed characters (except for single seed weight). The result revealed that interaction between sowing date and cultivar was significant ($P<0.01$) on all studied traits (except for palmitic and oleic acids). The factors interaction means that the sowing dates produced a differential effect on the response of cultivars for many traits. The similar significant interaction was found between sowing date and canola hybrid for seed yield and oil content (Lima et al 2017). The interaction between sowing date and chitosan was significant ($P<0.05$) only for a number of pods per plant. There was no any other significant interactions between the factors studied (Table 2).

3.2. Quantity characters

The highest number of pods per plant was observed on the first sowing date (October 7; Table 3). The delay in the sowing date from the first to the third had a 25.22% decrease in the number of pods per but 0.2% of chitosan increased the trait about 10% compared to the control. Sarigol and Julius cultivars had the highest (169.44) and lowest (140.18) mean values for this character, respectively. The delay in the cultivation time from September 12 to October 12 decreased the number of pods per plant and

Table 2- Variance analysis results of the measured agronomic traits in the cultivars

S.O.V	*df*	*Number of pods per plant*	*Number of seeds per pod*	*1000-seeds weight (g)*	*Seed yield (kg ha^{-1})*	*Biological yield (kg ha^{-1})*	*Oil (%)*	*Glucosinolate (µmole g^{-1})*	*Palmitic acid (%)*	*Oleic acid (%)*	*Linoleic acid (%)*	*Linolenic acid (%)*	*Erucic acid (%)*
Y	1	**	**	**	**	**	**	**	**	**	**	**	**
E	4	332.40	1.50	0.57	859383	1209269	0.49	11.35	0.14	0.73	3.27	1.58	0.01
S	2	**	**	**	**	**	**	**	**	**	**	**	**
Y×S	2	ns	*	**	ns	ns	ns	ns	ns	**	**	*	**
CH	1	**	**	**	**	**	**	**	**	**	**	**	**
Y×CH	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
S×CH	2	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Y×S×CH	2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
E	20	137.78	1.88	0.20	309898	677004	0.23	1.08	0.14	1.44	1.08	0.11	0.002
C	4	**	**	**	**	**	**	**	**	**	**	**	**
Y×C	4	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
S×C	8	**	**	**	**	**	**	**	ns	ns	**	**	**
Y×S×C	8	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CH×C	4	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Y×CH×C	4	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
S×CH×C	8	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Y×S×CH×C	8	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
E	96	99.44	1.29	0.15	243283	784397	0.17	0.46	0.16	0.56	0.39	0.06	0.001
C.V (%)		6.58	6.37	9.20	12.10	6.23	0.91	5.24	8.17	1.17	3.28	4.38	9.39

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

seed yield (Pasban-Easlam 2009). The interaction between the sowing date and the chitosan was significant (P<0.05) for this trait. The application of chitosan in 7th October had the highest mean value for this character (221.2; Figure 1).

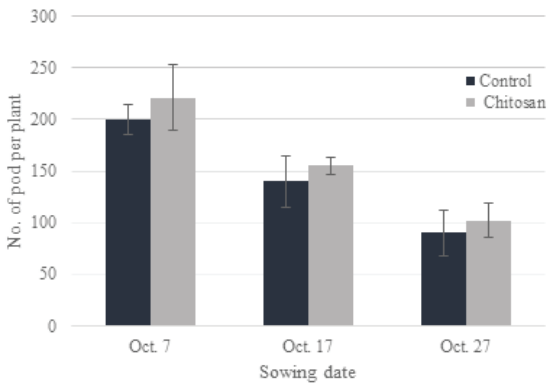

Figure 1- The interaction between the sowing date and the chitosan on a number of the pod^{-1} plant

Delay in sowing date from first to third reduced the number of seeds per pod about 33%, but the 0.2% chitosan solution increased to 4.76% compared to the control (Table 3). The reduction of this character due to a delay in sowing date was reported in some studies (Fagheh 2000; Rahnama & Bakhshandeh 2005). The interaction between cultivar and sowing date, and the interaction between Sarigol with the first sowing date and Julius with the third sowing date had the highest (22.88) and the lowest (13.45) mean value of a number of seed per pods (Table 4). The 1000-grain weight decreased from 5.26 g at the first sowing date to 3.18 g on the third sowing date (Table 3). In delay sowing date, the seed-filling period is associated with high temperature and the heat prevents grain seed filling (Robertson et al 2004; Fallah et al 2011). The application of 0.2% chitosan solution increased the character to 6.8% more than the control (Table 3). In the interaction between sowing date × cultivars, Sarigol in the first sowing date had the highest average (5.71 g) of this trait (Table 4).

The highest (5283 kg ha^{-1}) and the lowest (2844 kg ha^{-1}) grain yield were recorded on the first and third sowing dates, respectively. The chitosan solution 7.5% increased the seed yield than the control. RGS003 and Sarigol cultivars had more average for this character than the others cultivars (Table 3). When canola seeds were sown on 7th and 27th October, Sarigol and Julius cultivars showed the maximum and minimum mean value of seed yield, respectively (Table 4). In many studies, delay in sowing date caused seed yield reduction (Taylor & Smith 1992; Johnson et al 2006; Siadata & Hemayati 2009; Turhan et al 2011; Delkhosh et al 2012). Delay sowing date causes the maturity period to be exposed to high temperatures, which lead to reduced photosynthetic quantities and seed weight and ultimately reduced seed yield (Gan et al 2004; Rafiei et al 2011). Also, the delay sowing date cause reduces vegetative and reproductive growth times (Adamsem & Coffelt 2005). As seen in Table 3, delay in sowing date from the first (18321 kg ha^{-1}) to third (10260 kg ha^{-1}) reduced the biological yield about 44%. The biological yield was increased by chitosan solution than the control treatment. Sarigol cultivar had the highest biological yield (15672 kg ha^{-1}). In the interaction between cultivar and sowing date, the maximum (15533 kg ha^{-1}) and minimum (12912 kg ha^{-1}) biological yield were observed in the interaction between Sarigoa with first sowing date and Julius with the third sowing date, respectively (Table 4). The weather and soil temperatures in delay sowing time are colder than early sowing as date, it causes reduce in the growth and development of dry matter in leaves and stems (Karakaya & Altinok 2002). Sharif & Keshta (2002) obtained the highest biological yield and dry matter of the plant in November than December.

The highest amount of seed oil (46.43%) was observed at the first sowing date. Also, Sarigol cultivar had the maximum amount of the character (45.51%) than other cultivars (Table 3). The interaction between sowing date and cultivar showed that the highest and lowest seed oil were in Sarigol with first sowing date and Julius with third sowing date interactions, respectively (Table 4). Delay

Table 3- Main effects of the sowing date, chitosan and cultivar in agronomic traits

Factor	*Levels*	*Number of pods per plant*	*Number of seeds per pod*	*1000-seeds weight (g)*	*Seed yield (kg ha^{-1})*	*Biological yield (kg ha^{-1})*	*Oil (%)*	*Glucosinolate (µmole g^{-1})*	*Palmitic acid (%)*	*Oleic acid (%)*	*Linoleic acid (%)*	*Linolenic acid (%)*	*Erucic acid (%)*
Sowing date	October 7th	210.50a	21.27a	5.26a	5283a	18321a	46.43a	9.68c	5.46a	65.23a	20.87a	4.57c	0.21c
	October 17th	147.69b	17.84b	4.25b	4098b	14056b	45.04b	12.97b	4.96b	63.87b	19.06b	5.68b	0.32b
	October 27th	96.37c	14.42c	3.18c	2844c	10260c	43.63c	16.13a	4.10c	62.30c	17.40c	6.78c	0.47a
Chitosan	-	143.53b	17.41b	4.09b	3916b	13628b	44.83b	13.35a	4.75b	63.63a	18.89b	5.82a	0.35a
	+	159.51a	18.27a	4.37a	4233a	14797a	45.24a	12.50b	4.93a	63.97a	19.33a	5.53b	0.32b
Cultivar	RGS003	159.14b	18.46a	4.40a	4287a	14740a	45.30b	12.41b	4.99a	64.08a	19.41a	5.49b	0.32b
	Sarigol	169.44a	18.93a	4.55a	4447a	15672a	45.51a	11.95c	5.03a	64.27a	19.62a	5.31c	0.29c
	Zafar	143.81cd	17.35b	4.10b	3913b	13598b	44.83c	13.38a	4.77b	63.62b	18.88b	5.83a	0.35a
	Dalgan	145.04c	17.38b	4.10b	3914b	13722b	44.83c	13.33a	4.74b	63.59b	18.89b	5.83a	0.35a
	Julius	140.18d	17.11b	4.01b	3814b	13328b	44.70c	13.57a	4.67b	63.43b	18.76b	5.92a	0.36a

In each column, the same letters show that there are no significant

Table 4- The interaction between the sowing date and cultivar in agronomic traits

Cultivar	*Number of pods per plant*			*Number of seeds per pod*			*1000-seed weight (g)*			*Seed yield (kg ha^{-1})*			*Biological yield (kg ha^{-1})*		
	7th	*17th*	*27th*	*7th*	*17th*	*27th*	*7th*	*17th*	*27th*	*7th*	*17th*	*27th*	*7th*	*17th*	*27th*
RGS003	194.24d	169.67a	113.50a	20.47b	19.22a	15.67a	5.00b	4.59a	3.62a	4996b	4546a	3317a	17030b	15533a	11656a
Sarigol	239.58a	159.62b	109.10a	22.88a	18.54a	15.35a	5.71a	4.43a	3.52a	5791a	4374a	3176a	20769a	14974a	11273a
Zafar	200.10cd	140.84c	90.48b	20.72b	17.40b	13.95b	5.12b	4.13b	3.04b	5109b	3952b	2678b	17495dc	13576b	9725b
Dalgan	213.01b	135.91cd	86.19bc	21.27b	17.16b	13.70b	5.28b	4.10b	2.92b	5296b	3866b	2581b	18391b	13284bc	9491b
Julius	205.55bc	132.42d	82.57c	21.02b	16.86b	13.45b	5.19b	4.01b	2.83b	5225b	3752b	2466b	17918bc	12912c	9155b
	Oil (%)			*Glucosinolate (µmole g^{-1})*			*Linoleic acid (%)*			*Linolenic acid (%)*			*Erucic acid (%)*		
	7th	*17th*	*27th*	*7th*	*17th*	*27th*	*7th*	*17th*	*27th*	*7th*	*17th*	*27th*	*7th*	*17th*	*27th*
RGS003	46.10c	45.58a	44.23a	10.50a	11.73c	15.00b	20.48b	19.77a	17.99a	4.86a	5.24c	6.37b	0.24a	0.28b	0.42b
Sarigol	47.11a	45.33a	44.09a	8.26d	12.24c	15.35b	21.56a	19.46a	17.83a	4.07d	5.42c	6.45b	0.14d	0.30b	0.43b
Zafar	46.21bc	44.87b	43.42b	10.17ab	13.35b	16.61a	20.63b	18.84b	17.17b	4.75ab	5.81b	6.94a	0.23ab	0.34a	0.49a
Dalgan	46.42b	44.78b	43.27b	9.60c	13.64ab	16.77a	20.89b	18.69b	17.08b	4.56c	5.92ab	7.02a	0.21c	0.35a	0.50a
Julius	46.32bc	44.65b	43.15b	9.89bc	13.88a	16.93a	20.78b	18.55b	16.95b	4.64bc	6.00a	7.13a	0.215bc	0.36a	0.51a

In each column, the same letters show that there are no significant

sowing date typically reduces the amount of canola seed oil (Hocking & Stapper 2001). Temperature change during the filling stage can reduce the oil yield. The delay on sowing date causes flowering period falls in May-June when evaporation and transpiration reach high values caused the crop to confront water stress (Yau 2007). During the flowering period, rapeseed is susceptible to drought stress, however, the cultivars were found to possess varying sensitivity. Water stress decreased the seed yield of the crop, during the flowering stage (Bitarafan & Shirani-Rad 2012).

3.3. Fatty acid compositions and glucosinolate

As seen in Table 3, the highest range of fatty acid value in the cultivars, was oleic acid (63.43-64.27%), and it was followed by linoleic acid (18.76-19.62%), Linolenic acid (5.31-5.92%), palmitic acid (4.67-5.03%) and erucic acid (0.29-0.36%). According to review literature, the amount of fatty acid compositions in canola oil is as follows: palmitic acid 2-6%, oleic acid, 55-75%, linoleic acid 10-24%, linolenic acid 8-15%, and erucic acid <1% (Naseri 1991; Shariati & Ghazi Shahinzadeh 2000; Azizi et al 2006). The effects of sowing date and chitosan were significant for all fatty acids measured. Delay in sowing dates causes to increase of linolenic and erucic acids and a decrease of oleic, linoleic, and palmitic acids (Table 3). Sowing time significantly affected on oleic and linoleic acids and the mean values of them decreased as sowing times were delayed (Turhan et al 2011). The application of 0.2% of chitosan solution cause to increase of oleic, linoleic, and palmitic acids and a decrease of linolenic and erucic acids. The highest (19.62%) and lowest (18.76%) oleic and linoleic acids were found in Sarigol and Julius, respectively (Table 3). As known as oleic and linoleic acids are the most important components of unsaturated fatty acids, which are important in terms of nutrition (Weber et al 2008). On the other hand, the quality of seed oil is mainly determined by oleic and linoleic acids contents (Ul-Hassan et al 2005). However linoleic acid is not synthesized in the body and must be supplied by diet (Naseri 1991; Dastpak 2001). Sarigol and Julius cultivars had the highest (5.03%) and lowest (4.67%) average of palmitic acid (Table 3). Gecgel et al (2007) showed that the level of palmitic and oleic acids decreased when oil synthesis happened in hot weather. The result indicated that delayed sowing from 7th to 27th October increased the percentage of linoleic and erucic acids. The chitosan solution reduced both of these two harmful fatty acids than control treatment. Sarigol and Julius cultivars had the lowest and highest percentages of the two acids, respectively (Table 3). The interaction of the treatments showed that the lowest percentage of linolenic and erucic acids were recorded in Sarigol cultivar in the first sowing date (Table 4). These two fatty acids are very harmful to human health, and cultivars without these fatty acids have a high nutritional value (Dastpak 2001).

The result of this study showed delayed sowing date had a negative effect on the oil quality. It should be noted that the increase in temperature during seed formation changes the amount of fatty acids in seeds and affects the quantity and quality of seed oil (Ul-Hassan et al 2005). The three studied factors had effects on glucosinolate content. Sarigol and Julius cultivars had the lowest and highest glucosinolate content, respectively (Table 3). The interaction between sowing date and cultivar showed that Sarigol and Julius cultivars had the lowest and highest glucosinolate content at the first and third sowing dates, respectively (Table 4). Fieldsend et al (1991) showed that glucosinolate accumulation in canola seed is inherited, but also affects by environmental factors (Jan et al 2002; Grant et al 2003). Glucosinolate component is considered toxic to human and unfavorable for animal feed and egg production, however, its play an important role in plant's defense mechanism against pests (Kozlowska et al 1990). Early sowing date and application of the chitosan had a positive affected to reduce of this component. Sulisbury et al (1987) reported that an increase in glucosinolate reduces the quality and nutritional value of canola meal. The result of the present study showed that early sowing date and application of chitosan were useful to obtained high seed oil quality.

3.4. Cluster and PC analyses

All measured characters were used to dendrogram generated, and the cluster analysis classified for the five rapeseed cultivars into two main clusters (Figure 2). Three cultivars including Zafar, Dalgan, and Julius were in the first cluster. The cultivars of this group had high values of linolenic acid, erucic acid, and glucosinolate than the second group. However, for the valuable fatty acids, the first group had lower values than the second group. The result indicated that the quality and quantity of the cultivars in the first cluster were more than the second cluster. The second group consist of two other cultivars (RGS003 and Sarigol cultivars). These cultivars had high levels of grain and oil yields and oleic and linoleic acids, indicated that the cultivars are suitable to cultivate in the area. The PCA revealed that the three first PC confirmed about 96% of the total variance among the five cultivars (Figure 3). The measured characters correlated substantially with the first component (PC1); therefore, it was named "Genotype". About 78% of the total variance among the cultivars was affected by different genotype, because the cultivars were cultivated in the same condition, so their variation due to genetic factors. The second and third components confirmed about 12.2 and 5.52% of the total variance among the cultivars, respectively. The three plots revealed that the results of the cultivars were in agreement with the results of the cluster analysis (Figure 3). In both of them, the cultivars were divided in to main groups.

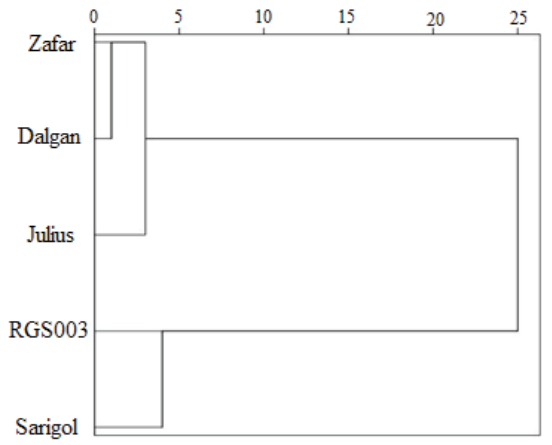

Figure 2- Dendrogram generated based on traits measured using the UPGMA method

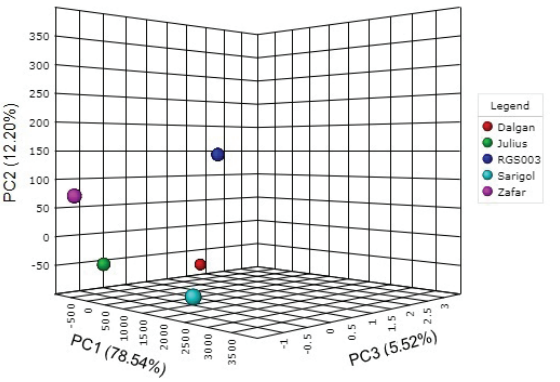

Figure 3- Three plots derived from the principal component analysis

4. Conclusions

The results of the study showed that the rapeseed cultivars responded differently to the sowing dates. Therefore, choosing a suitable sowing date is essential depending upon growing conditions and cultivar. The amount of three beneficial fatty acids including palmitic, linoleic, and oleic acids decreased due to delayed sowing date. Application of 0.2% of chitosan solution increased these three useful fatty acid and decreased harmful fatty acids including erucic and linolenic acids. The result of the present study showed that early sowing date and application of chitosan had a positive effect on the quality and quantity oil yield of the plant. According to the result, it can be suggested that the most appropriate sowing time to obtain a high quantity and quality of rapeseed oil, is early of October and application of chitosan and Sarigol cultivar as a way to increase canola yield. In the end, the determination of sowing date and choosing a suitable cultivar for each region are very important to obtain high quality and quantity of rapeseed oil.

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Numerical Investigation of Multiphase Transport Model for Hot-Air Drying of Food

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ABSTRACT

Drying is widely used to prevent microbial spoilage by evaporating the determined amount of liquid in the food sample. In order to reduce energy consumption and increase food flavor quality, modeling the drying process is crucial. In the literature, different approaches are used for investigation of drying characteristic. Among these approaches, the porous media approach have complex phenomena. Molecular diffusion for gases (water vapor and air), capillary diffusion for liquid (water), and convection mechanisms (Darcy flow) were used in drying model in porous media. In this study, firstly, the effect of shrinkage on drying of porous media was investigated. Non-linear partial differential equations for air and food material in the drying problem were solved numerically for non-steady state condition. The shrinkage effect in the drying process was studied by using the ALE (Arbitrary Lagrangian Eulerian) method. In this study, air velocities of 0.5, 0.8 and 1 m s^{-1}, air temperatures of 40, 50 and 60 °C and the geometric forms of rectangular, cylindrical and square were selected for hot air drying process. The fastest drying was obtained at square shape food at the air temperature of 60 °C and the air velocity of 0.5 m s^{-1}. The analysis result showed that the air velocity and temperature have effect on the drying.

Keywords: Heat and mass transfer; Drying; Numerical modeling; Shrinkage

1. Introduction

The porous medium is a material containing pores filled with liquid and/or gas. Most of these pores are connected and so, the heat and mass transfer occur through these pores. A faster transport process occurs in pores, compared to the solid structure, in general. Many materials such as food products, soil, paper, stored grains can be defined as porous media. Heat and mass transfer processes in porous materials play an important role in the industry because of its product flavor quality, energy intensity, and process control requirements. There are different approaches in this field, which was investigated by numerous researchers either numerically or experimentally in the literature (Machado et al 1998; Sanjuan et al 1999; Datta 2007a). Drying of porous materials is an important process for food, textile, paper and pharmaceutical industry. The drying process assures the formation of simultaneous heat and mass transfer by applying hot air to the food product. Therefore, while the moisture content of the food decreases, its temperature value increases. Thus, it is ensured that food materials will last long without

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spoiling (Curcio et al 2008). The multiphase porous model helps efficiently identify the heat and mass transfer process, especially containing the internal evaporation mechanism, in food materials. During drying, water evaporation occurs inside and outside the material to be dried. The energy required for evaporation is provided by hot air in convective drying. In this case, the vapor and liquid phases formed within the material cannot be neglected. The mass transfer model formed for both phase states should be defined separately. During the drying of agricultural foods such as fruits and vegetables with high moisture content, a significant deformation effect is usually observed. The shrinkage effect, an observable phenomenon, has a strong effect on drying rate and food structure (Lima et al 2002). Defraeye et al (2016) used a neutron X-ray film to scan the moisture distribution in the food when drying fruit slices with forced convection. They examined the effects of air velocity, temperature, and radiation on the internal moisture distribution. Thus, mass transfer in food during the drying process has been visualized with the imaging technique used in their research. Ateeque et al (2014) developed a numerical model for estimating the moisture content of food material. The mass transfer was modeled as considering diffusion of liquid water from inner layer to outer surface of the food product followed by evaporation of water from the surface to the dry air which flows over the moist food product. Nguyen & Price (2007) examined the effect of experimental parameters such as temperature, moisture, and thickness in the drying of bananas. Yan et al (2008) investigated the effect of porosity, deformation and specific volume changes on the drying of banana, pineapple and mango with hot air. Ruhanian & Movagharnejad (2016) experimentally examined the drying of thin potato layers in the infrared convective dryer. They performed their experiments with three different thicknesses and three different drying powers. It was also observed that almost all of the drying process occurred in the falling-rate period. It was found out that increasing the drying power reduced the moisture content and drying time and increased the deformation and effective moisture diffusion coefficient. The drying process is a complex and multivariable thermal process that should be investigated from different perspectives since the underlying physical mechanisms are still not perfectly understood. Therefore, in this study, a mathematical model was developed to calculate the moisture and temperature distribution of the food material inside the dryer. The multiphase transport model was developed for convective drying. Capillary diffusion was used for the liquid phase in the porous food material, and molecular diffusion and convection mechanisms were used for vapor. The effect of shrinkage rate was included in the model of heat and mass transfer for the prediction of moisture and temperature distributions during drying, with and without shrinkage rate cases. The obtained nonlinear unsteady-state partial differential equations were solved by Comsol Multiphysics 5.3. Thus, parameters affecting the drying process such as temperature, air velocity and geometric shape of the food were investigated. The main aim of this study was to predict the temperature and moisture distributions of different geometric shaped including rectangular, cylindrical and square food using multiphase transport model on convective drying process under different drying conditions (Inlet air velocity and air temperature).

2. Material and Methods

2.1. Mathematical model

The transfer mechanisms that occur in both the solid and the flow area were taken into consideration in developing the mathematical model for drying. While the liquid transfer was provided by the pressure and capillary pressure gradient in the solid, the vapor was transferred by the pressure and concentration difference as activation force. The pressure driven flow can be neglected because the amount of inner evaporation in forced convection drying is generally lower than that of microwave drying and vapor molecular diffusion model can be also used as a prevailing mechanism (Datta 2007a). The molecular diffusion for vapor flow and the capillary flow for liquid water flow are preferred. Mass transfer equations determined with liquid

water and vapor for the porous media are given by Equation 1 and 2.

$$\frac{\partial c_w}{\partial t} + \nabla(-D_w \nabla c_w) + u_w \nabla c_w = 0 \quad (1)$$

$$\frac{\partial c_v}{\partial t} + \nabla(-D_v \nabla c_v) + u_v v \nabla c_v = 0 \quad (2)$$

Where D_w is the capillary diffusivity in food, D_v is the diffusion coefficient of vapor in food, u_v and u_w are the velocity of the water vapor and the velocity of liquid water within the solid, respectively. c_w is the water concentration inside food, c_v is the vapor concentration of the food (Bird et al 1960; Welty et al 2001). The equation between the water concentration (c_w) and liquid phase saturation (S_w) can be calculated as follows:

$$S_w = \frac{c_w M_w}{\rho_w \varepsilon} \quad (3)$$

Where; M_w is the moisture content on wet basis, ρ_w is the density of water and ε is the porosity. In order to calculate this liquid phase velocity, the pressure gradient of the gas phase was taken into consideration. The liquid phase velocity is given by:

$$u_w = -\frac{\kappa \kappa_{rl}}{S_w \varepsilon \mu_w} \nabla p_g \quad (4)$$

Where; κ_{rl} is the liquid phase permeability, κ is the porous material permeability, ∇p_g is the pressure gradient, μ_w is the water viscosity (Datta 2007a). The liquid phase permeability is identified in Equation 5.

$$\kappa_{rl} = \begin{cases} \left(\frac{S_w - S_{li}}{1 - S_{li}}\right)^3 & S_w > S_{li} \\ 0 & S_w < S_{li} \end{cases} \quad (5)$$

Where; S_{li} is the irreducible liquid phase saturation; this equation is used to identify the amount of liquid phase evaporation that remain in the porous material (Datta 2007b). The velocity field for the water vapor transfer calculated as follows:

$$u = \frac{u_g}{S_g \varepsilon} - \frac{M_a D_{eff}}{M_{ma} \rho_{ma}} \nabla \rho_{ma} \quad (6)$$

Where; M_{ma} is the molecular weight of the moist air (kg mol^{-1}), ρ_{ma} is the density of the moist air, D_{eff} is the effective diffusion coefficient (m^2 s^{-1}), S_g gas phase saturation, u_g is the flow field of moist air and M_a is the molecular weight of air (kg mol^{-1}). The effective diffusion coefficient is defined as:

$$D_{eff} = D_{va} \varepsilon^{4/3} {S_g}^{10/3} \quad (7)$$

Where; D_{va} is the air-vapor diffusivity (m^2 s^{-1}), the equation of continuity for the laminar flow field is given by Equation 8 and 9

$$\rho \frac{\partial u}{\partial t} + \rho(u\nabla)u = \nabla\left[-pl + \mu(\nabla u + (\nabla u)^T) - \frac{2}{3}\mu(\nabla u)l\right] \quad (8)$$

$$\frac{\partial P}{\partial t} + \nabla(\rho u) = 0 \quad (9)$$

Where; ρ is the air density (kg m^{-3}), ρ is the pressure within drying tunnel (Pa), u is the air velocity vector (m s^{-1}). The energy equation for the drying air is given by Equation 10 (Bird et al 1960; Welty et al 2001; Comsol Multiphysics 5.3 2017).

$$\rho c_p \frac{\partial T}{\partial t} + \rho c_p u \nabla T + \nabla q = 0 \qquad q = -k \nabla T \quad (10)$$

Where; ρ is the air density (kg m^{-3}), c_p is the specific heat (J kg^{-1} K^{-1}), k is the thermal conductivity (W m^{-1} K^{-1}), q is the heat flux (W m^{-2}) and u is the velocity vector (m s^{-1}). The energy equation within the solid according to Fourier's law can be written as Equation 11.

$$\frac{\partial T}{\partial t} + \rho c_p u \nabla T + \nabla q = 0 \qquad q = k_{eff} \nabla T \quad (11)$$

Where; k_{eff} is the food effective thermal conductivity W m^{-1} K^{-1}, ρ is the density of food (kg m^{-3}), c_p is the specific heat (J kg^{-1} K^{-1}) and u is the velocity vector (m s^{-1}).

While the vapor transfer occurs in the air, the liquid water transfer does not occur. Mass transfer equations for the air were provided with convection and diffusion. The mass balance is given by Equation 12 (Bird et al 1960; Welty et al 2001; Comsol Multiphysics 5.3 2017).

$$\frac{\partial c_a}{\partial t} + \nabla(-D_a \nabla c_a) + u\nabla c_a = 0 \quad (12)$$

Where c_a is the water vapor concentration in the air and D_a is the diffusion coefficient of water vapor in air (m^2 s^{-1}) and u is the velocity vector (m s^{-1}). It is difficult to calculate the shrinkage effect experimentally. Therefore, the shrinkage velocity should be estimated, to take into account the shrinkage in this study. The velocity value on the surface is given by Equation 13:

$$u(b) = \frac{b - b(old)}{\Delta t} \quad (13)$$

Where; *b(old)* is the half-thickness of the food at the next time, and *b* is the half-thickness of drying material (Karim & Hawlader 2005). The following expression can be used to calculate the half-thickness of the food at any time (Desmorieux & Moyne 1992).

$$b = b_0 \left[\frac{\rho_w + M\rho_s}{\rho_w + M_0\rho_s}\right] \quad (14)$$

Where ρ_w is the water density (kg m^{-3}), ρ_s is the food density (kg m^{-3}) and M_0 is the initial moisture content (kg kg dry^{-1}), M is the moisture content (kg kg dry^{-1}), b_0 is the initial half-thickness. The fluid flow, heat transfer and transport phenomena should be considered during the drying process. It was assumed that porous media contains water vapor and liquid water. The flow area around the material in the tunnel was assumed laminar (Figure 1). Food material with different geometric shapes was modeled for numerical simulation. Mesh sizes used in the analysis affects the solution. Therefore, it is required to test grid independency (Sabarez 2012). Different mesh structures were applied to rectangle, cylinder and square food and air domains (Figure 2). The solution of grid independency was carried out with accuracy 1%. Finally, for the accurate solution, grid structure of rectangle, cylinder and square which consists of 8868, 8047 and 4745 elements was selected based on preliminary study, respectively. The time-dependent problem was solved by using an implicit time stepping method. The nonlinear PDE (partial differential equations) were solved using Newton's method with relative tolerance 0.001 and absolute tolerance 0.0001 using the commercial Comsol Multiphysics 5.3. Dimensions of the rectangular, the diameter of cylinder and the edge of the square used in the model were considered 1×2.5 cm, 1.2 cm and 1 cm, respectively. The channel dimensions were 15 cm height and 40 cm width. The parameters and thermophysical properties used in the analyses are presented in Table 1. Non-linear partial differential transport equations were solved by the numerical method in order to determine the temperature and moisture content values of the air and food material. The deformed mesh was used to consider the volume change due to the fluid transfer. Therefore, an Arbitrary Lagrange Eulerian (ALE) was applied in the Comsol Multiphysics 5.3. The ALE method is used as an application between the Lagrangian and Eulerian approaches that allow the identification of moving boundaries. The Laplace smoothing type was defined as the deformed mesh.

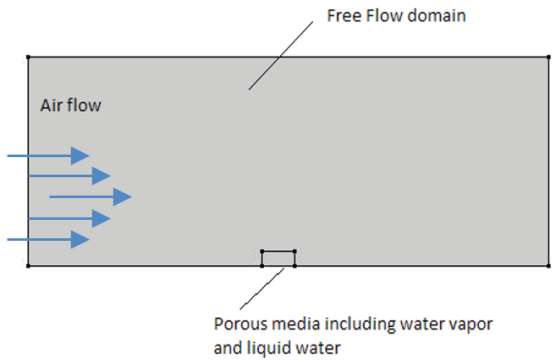

Figure 1- The model and flow field used in analyses

2.2. Predicted models

In order to determine the effect of the shape deformation, models with and without shrinkage were compared with the experimental results. The rectangular shape food was experimentally dried to observe the deformation effect on drying. Therefore, the experimental study should be performed. The numerical results were compared with experimental data in Figure 3.

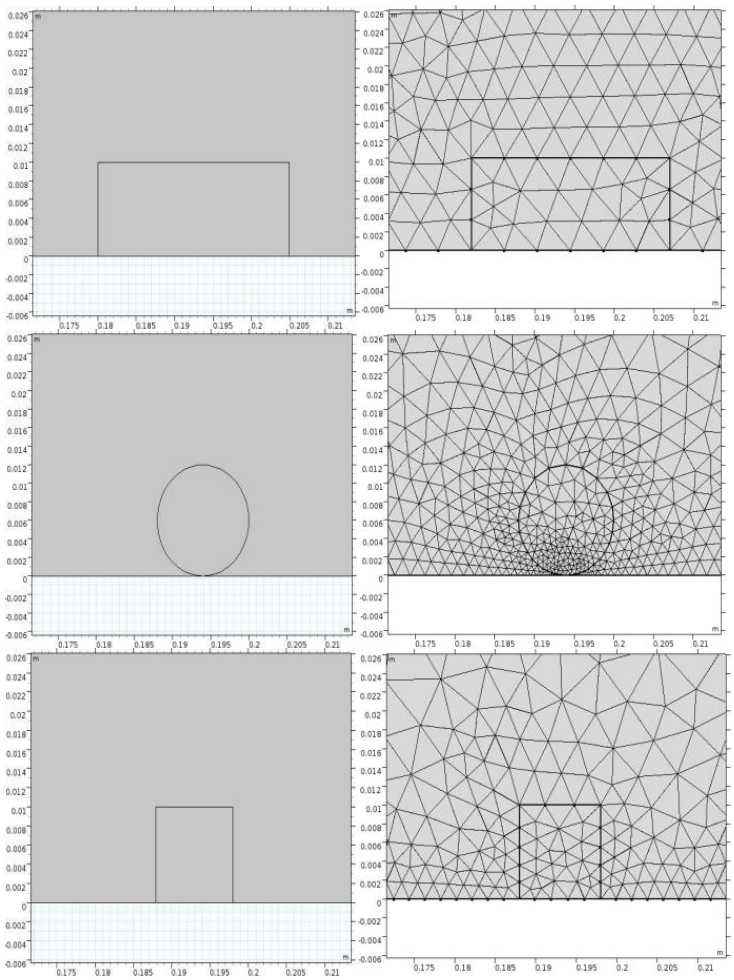

Figure 2- Rectangular, cylindrical and square shaped geometries and mesh structures

Table 1- Drying parameters and thermophysical properties of banana used in the model (Karim & Hawlader 2005; Bart-Plange et al 2012)

Parameter	*Value*
Air velocity (m s^{-1})	0.5-0.8-1
Food temperature (°C)	21
Food density (kg m^{-3})	980
Air temperature (°C)	40-50-60
Food initial moisture content (g g dry^{-1})	2.62
Thermal conductivity of food (W m^{-1} K^{-1})	0.006M+0.120
Specific heat of food (J kg^{-1} K^{-1})	$0.811M^2$-24.75M+1742
Heat of vaporization (kJ kg^{-1})	2383-2407-2359
Water density (kg m^{-3})	0.018

M, moisture content (g g dry^{-1})

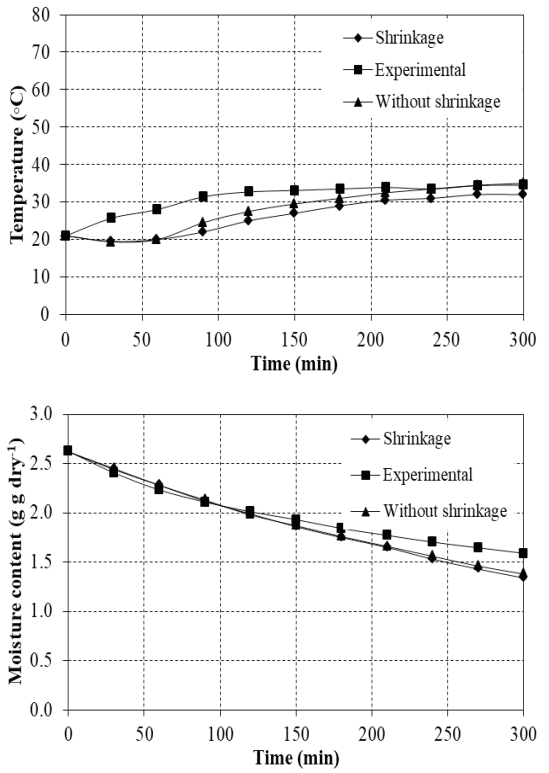

Figure 3- Variation of temperature and moisture content with/shrinkage and experimental study with drying time

2.3. Test condition

The drying characteristics at different air velocities (0.5, 0.8 and 1 m s^{-1}) and temperatures (40, 50 and 60 °C) for rectangular, cylindrical and square shaped food material (banana) were obtained.

3. Results and Discussion

The numerical results which were obtained from these two theoretical models (with and without shrinkage effect) were compared with the obtained experimental results. The numerical results of both models were found to be in good agreement with the obtained experimental results. 0.8% difference for moisture content and 6.2% difference in temperature between with and without shrinkage were found. Therefore, it was used in the analysis the theoretical model which is without shrinkage effect. Difference between the experimental and the analytical results can be explained by the constant thermophysical properties of the food in the calculations and difficulties in the experimental measurement (Liu & Cheng 1991). The dimension and moisture content changes of food due to shrinkage effects are presented in Figure 4. It was observed that the shrinkage effect exists with the evaporation in the food over time. It is difficult to determine the moisture distribution inside the food sample experimentally. Therefore, simulation can be used to calculate moisture distribution inside the material (Hussain & Dincer 2003; Kumar et al 2015).

The center moisture content of the samples was obtained during drying. Figure 5 shows the predicted moisture contents calculated during the drying at different air velocities and geometric forms of food at 40 °C. Air velocity affects the drying process. It is clear that increasing drying air inlet velocity increase drying rate (Karim & Hawlader 2005; Aversa et al 2007; Curcio et al 2008). The square-shaped food material dried in a shorter time than the cylindrical and rectangular-shaped foods. At the end of the fifth hour, for the drying air velocity of 1 m s^{-1}, while the moisture content was 1.06 g g dry^{-1} in the rectangular shape, it was calculated to be 0.33 and 0.1 g g dry^{-1} in cylinder and square, respectively. At the end of the fifth hour, the moisture content of square-shaped food was decreased by 28% for increasing air velocity from 0.5 m s^{-1} to 0.8 m s^{-1} and by 14% for increasing air velocity from 0.8 m s^{-1} to 1 m s^{-1}. Figure 5 shows predicted variation of the temperature of food during drying at the air temperature of 40 °C. The surface temperature values of rectangular, cylindrical and square shape food material were determined to be 38.2, 37.5 and 39.5 °C after the five-hour period at the air velocity of 1 m s^{-1} (Figure 6). As the drying air velocity increased, the surface temperature of the food material increased (Curcio et al 2008). The result showed that the highest surface temperature was determined for the square-shaped food. Figure 7 shows the predicted moisture content of food at air velocity of 0.5 m s^{-1} for different air temperatures.

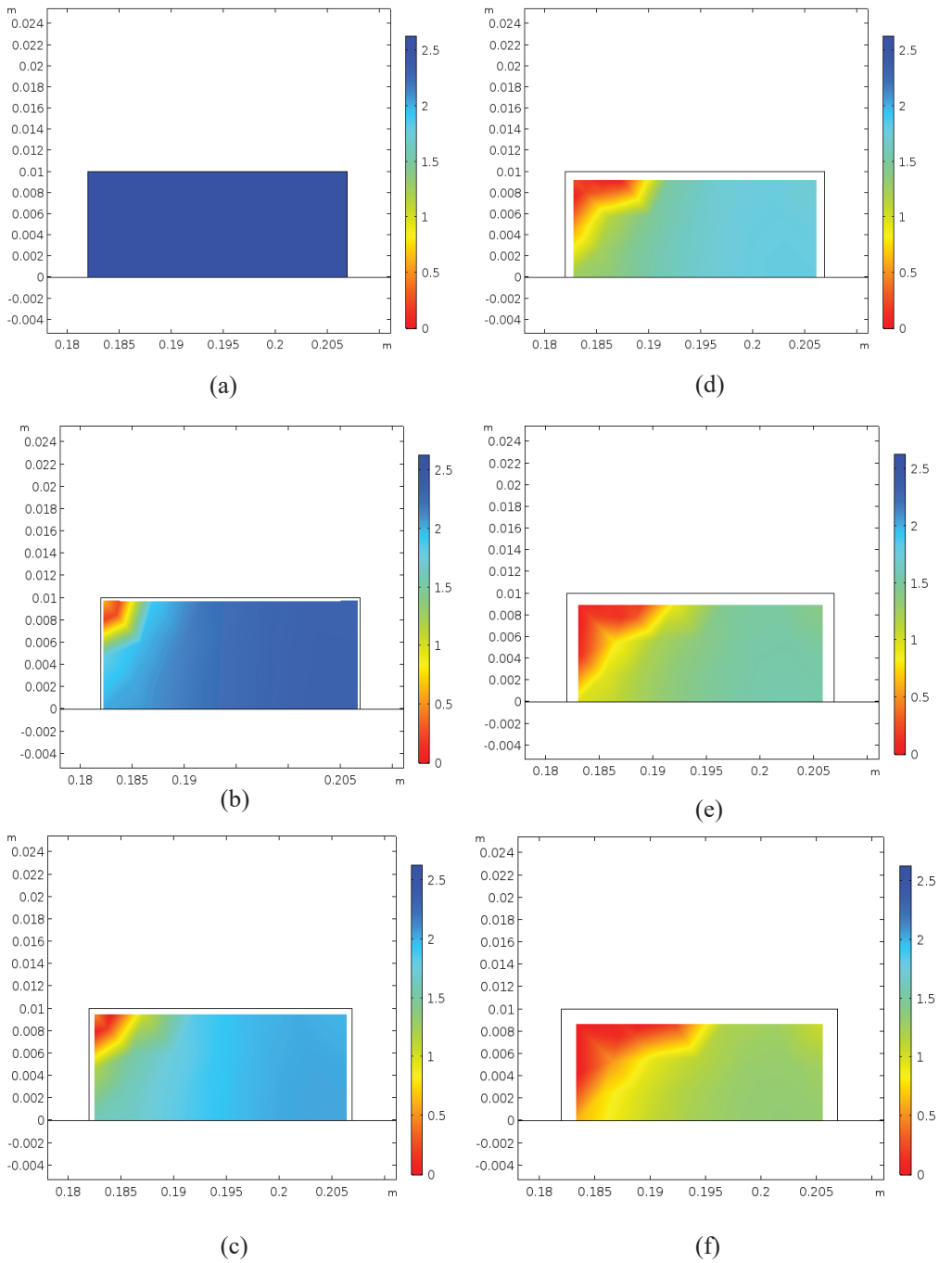

Figure 4- Evolution in the moisture content of the food over time during drying process considering the shrinkage effect (a, start of drying; b, first hour; c, second hour; d, third hour; e, fourth hour; f, fifth hour) (air temperature of 40 °C, air velocity of 0.5 m s^{-1})

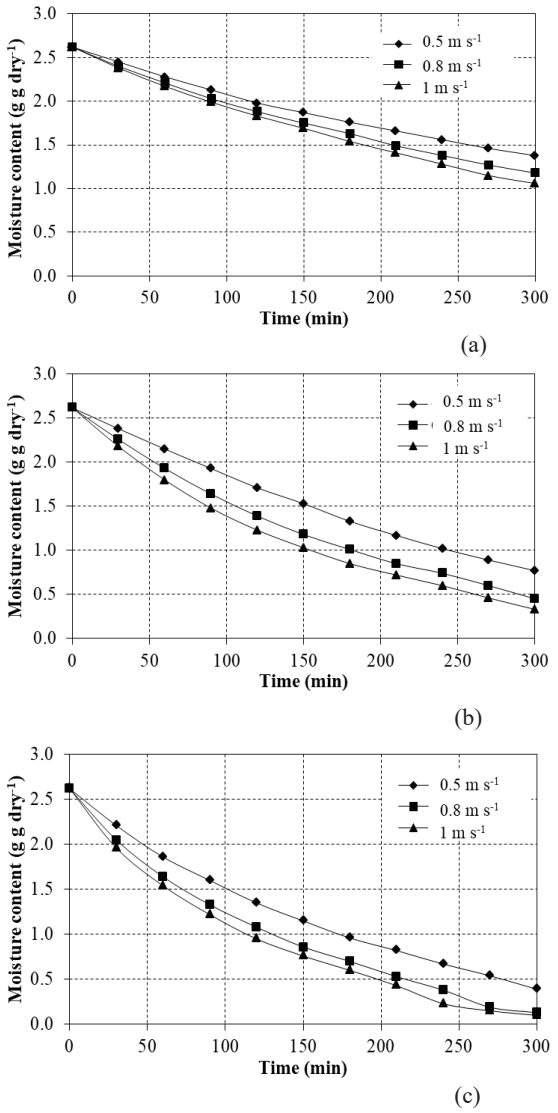

Figure 5- Moisture profiles of foods with different shapes at different air velocities at air temperature 40 °C (a, rectangle; b, cylinder; c, square)

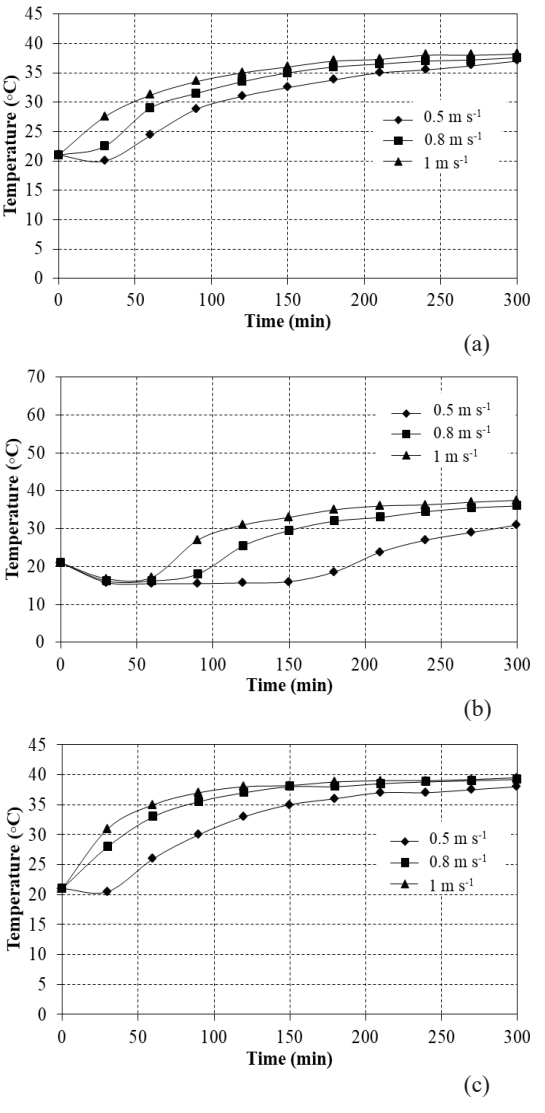

Figure 6- Surface temperature distributions obtained at different air velocities of foods with different geometric shapes at air temperature 40 °C (a, rectangle; b, cylinder; c, square)

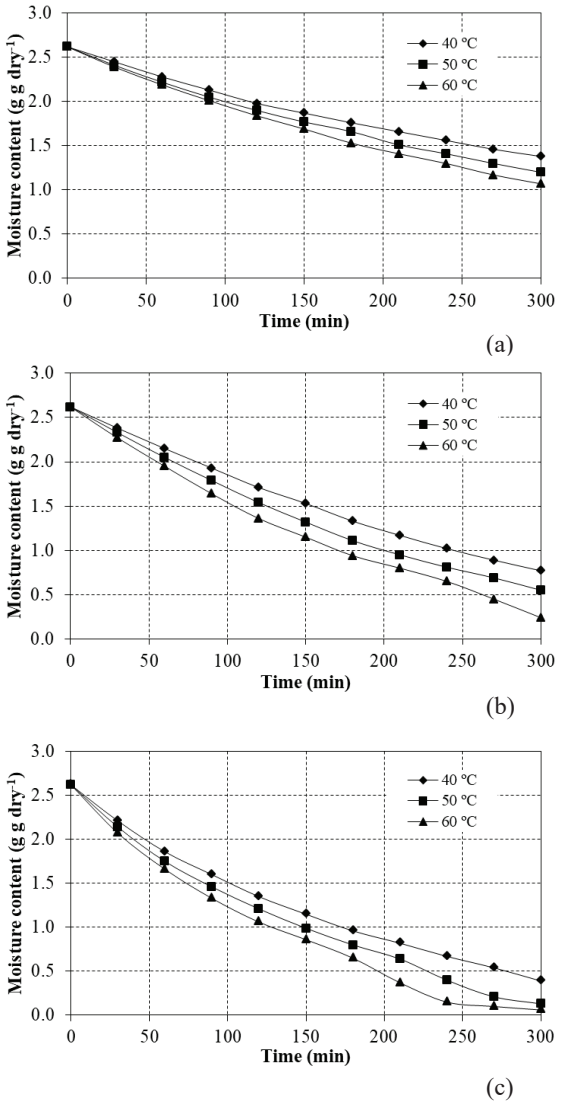

Figure 7- Moisture contents at different air temperatures of foods with different geometric shapes at air velocity 0.5 m s^{-1} (a, rectangle; b, cylinder; c, square)

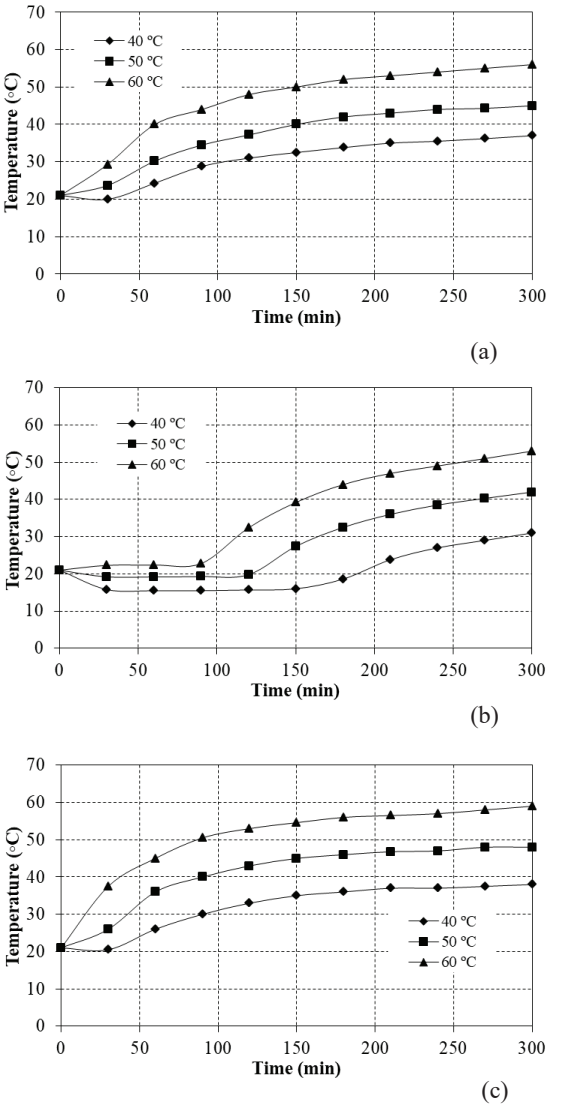

Figure 8- Surface temperature distributions at different air temperatures of foods with different geometric shapes at air velocity 0.5 m s^{-1} (a, rectangle; b, cylinder; c, square)

As the drying air temperature increased, the moisture content decreased and the drying time was shortened (Aversa et al 2007). The moisture content values of the rectangular, cylinder and square shape food at the drying air temperature of 60 °C at the end of the fifth hour, were calculated to be 1.07, 0.24 and 0.06 g g dry^{-1}, respectively. It was observed that the lowest moisture content among the geometric shapes was obtained from square shape food during the drying process at different air temperatures. 50% increase of air temperature caused 97% decrease in moisture content of square-shaped food at the end of the fifth hour at 0.5 m s^{-1} air velocity. Surface temperatures at different drying air temperatures for rectangular, cylinder and square shapes food are shown in Figure 8. The surface temperature values increased over time as the air temperature increased. The surface temperature values of rectangular, cylinder and square shape food material were determined to be 56, 53 and 59 °C, respectively, after the five-hour period at the air temperature of 60 °C. The highest temperature increase occurred in the square shape food. It is clear that increasing drying air velocity and temperature improved considerably drying rate. Similar moisture profiles were obtained by some studies in the literature (Karim & Hawlader 2005; Nguyen & Price 2007; Curcio et al 2008; Thuwapanichayanan et al 2011; Kumar et al 2015). Parametric analysis showed that the square shape food material had the fastest drying characteristic compared with the other food shapes. Evaporative cooling occurred at the beginning of the drying process in Figure 6 and 8. Temperature decrease was observed in the first 30 minutes of the drying of the food. The food surface initially covered with a moisture layer. The amount of moisture evaporated from the food was high at the first stage of the drying period compared to as shown in Figure 5, 6 and 7. At the beginning of the drying, the decreased temperature profile was also obtained in some studies in the literature including (Turner & Jolly 1991; Zhang & Mujumdar 1992; Curcio et al 2008; Golestani et al 2013; Kumar et al 2015). The increased temperature in the food may cause quality deterioration. Therefore, it is necessary to optimize the drying process to increase food quality. Thus, with intermittent drying, the process can be restarted after each temperature period, and more evaporation can be provided (Kumar et al 2015).

4. Conclusions

In this study, firstly, two different theoretical models with and without shrinkage effect were developed in order to define the drying characteristics (temperature and moisture distributions). The numerical results obtained from these two theoretical models were compared with the obtained experimental results. The numerical results of both theoretical models were found to be in considerable agreement with experimental results. Furthermore, a parametric analysis was carried out to investigate the surface temperature and moisture content of the foodstuff for different air velocity (0.5, 0.8 and 1 m s^{-1}) temperature (40, 50 and 60 °C) and different geometric shapes. The conclusions can be summarized as follows:

According to the obtained results it was determined that the fastest drying occurred in the square shape food at the air temperature of 60 °C and the air velocity of 0.5 m s^{-1}. Therefore, the square slice can be preferred to carry out a faster-drying process. It was shown that the moisture content of rectangular shape food at the end of the fifth hour was higher than other cylinder and square shapes food. Temperature values obtained for cylindrical shape food at the air temperature of 40 °C and the air velocity of 0.5 m s^{-1} in the first 150 minutes of the drying were constant and under 20 °C. Cylindrical shape food should be used to achieve more evaporation at low surface temperatures. As expected, it was observed that the increased air velocity and temperature on drying decreased the drying time. The analysis result showed that the air velocity and temperature are important parameters on the evaporating moisture content. The remaining moisture content may cause food deterioration and microorganism activity. For this reason, minimizing the moisture content is vital. It is difficult to determine how much moisture remains at any point in the food during drying, experimentally. Therefore, numerical analysis of drying process is

very important. By using the numerical method, the temperature and moisture distributions of the food can be easily estimated depending on the time. For three geometric forms (rectangle, cylindrical and square) it was shown that when the temperature and velocity of air 40 °C and 0.5 m s^{-1}, respectively, in the first 30 minutes of the temperature of the food was decreased slightly. This situation was occurred due to the evaporative cooling phenomenon during the initial stages of drying. Thus keeping the product temperature in low level is important for the quality of the food. Finally, the drying period should be optimized by using numerical method. The developed numerical model should be also used for three-dimensional geometries in future drying research.

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Identification of Barley Landraces and Wild Barley (*Hordeum spontaneum*) Genotypes Resistant to *Rhynchosporium commune*

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ABSTRACT

Barley scald is caused by the fungal pathogen *Rhynchosporium commune*. This disease causes substantial losses in barley production areas of the world. In this study, seedling resistance of 198 barley landraces, 104 wild barley (*Hordeum spontaneum*) genotypes and two susceptible Turkish cultivars (Bülbül 89 and Efes 3) to 6 *R. commune* isolates was assessed in greenhouse experiments. Virulence differences among the *R. commune* isolates were observed. One six-rowed barley landrace (Yeşilköy 9052) was resistant to all six isolates. Another six-rowed barley landrace (genotype no 17) showed resistant reaction to 5 isolates. Fourteen barley landraces were resistant and susceptible to 4 and 2 isolates, respectively. Twenty seven genotypes of *H. spontaneum* numbered as 5, 6, 7, 8, 9, 11, 16, 20, 30, 31, 36, 37, 48, 50, 51, 56, 58, 59, 60, 62, 67, 79, 80, 83, 90, 94 and 101 were found resistant to all six isolates of *R. commune*. Apart from these genotypes, 19 genotypes numbered as 1, 2, 32, 33, 34, 42, 43, 49, 52, 64, 66, 76, 77, 78, 96, 97, 102, 104 and 107 showed resistance to 5 isolates and susceptibility to only 1 isolate. Two susceptible Turkish cultivars Bülbül 89 and Efes 3 were found susceptible to 96% of the scald isolates. Resistant barley landraces and *Hordeum spontaneum* genotypes can be used as sources of resistance against *R. commune*.

Keywords: Scald; *Rhynchosporium commune;* Barley landraces; Wild barley; *Hordeum spontaneum*; Disease resistance

1. Introduction

Rhynchosporium commune (formerly known as *Rhynchosporium secalis*) (Zaffarano et al 2011) is a haploid pathogen that attack different barley species including cultivated and wild barley. This pathogen can attack to barley plants and produce disease symptoms on leaves, leaf sheaths and ears of barley (Avora & Knogge 2012). The disease is common in the world and disease losses on cultivated barley due to this pathogen have been reported from 10% to 70% (Shipton et al 1974; Eshetu 1985; Zhang et al 1992; Sheikh Jabbari 2008; Karakaya et al 2014). Control of barley scald disease is achieved by a variety of control measures including chemical, agronomical and biological control methods. Using resistant cultivars, due to environmentally friendly implementation of this control method, is desirable.

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However, to obtain scald disease resistance sources in barley, extensive studies of both fungus pathogenicity and barley cultivars/lines screening for resistance are required. Turkey is considered as one of the precious gene pools of barley including disease resistance sources in Fertile Crescent (Kün 1996; Badr et al 2000; Karakaya et al 2016). Barley landraces and wild barleys are important sources of genetic variation and disease resistance (Yitbarek et al 1998; Ceccarelli & Grando 2000; Ellis et al 2000). Barley landraces and wild barley (*H. spontaneum*) genotypes are widely distributed in Turkey (Kün 1996; Badr et al 2000; Karakaya et al 2016; Ergün et al 2017). Due to rich heritage of barley landraces and wild barley (*H. spontaneum*) genotypes in different sites of Turkey, finding new sources of scald disease resistance using barley landraces and wild barley genotypes and transferring resistance traits into cultivated barley could have a great value in scald disease resistance studies. In this study, seedling reactions of 198 barley landraces and 104 wild barley (*H. spontaneum*) genotypes were determined using 6 *R. commune* isolates.

2. Material and Methods

2.1. Plant materials

Two hundred barley landraces and 107 *Hordeum spontaneum* genotypes were obtained from Central Research Institute for Field Crops located in Ankara, Turkey. These barley landraces and wild barley seeds were multiplied in the field for use in this study and in future studies. In the seed propagation process insufficient seeds were obtained from barley landraces no. 43 and no. 116 and wild barley genotypes numbered as 4, 15 and 41 and therefore, they were not used in the experiments.

2.2. Rhynchosporium commune isolates

Six *Rhynchosporium commune* isolates [(4 most virulent isolates (GPS 71-U, 13GPS203, 13GPS207 and 13GPS 109), one isolate with high virulence value (13GPS149) and 1 isolate representing the most common pathotype (E4)] selected from Azamparsa (2015) study were used in determination of seedling stage resistance of 198 barley landraces, 104 *H. spontaneum* genotypes and susceptible Turkish cultivars Bülbül 89 and Efes 3 under greenhouse conditions. Isolates GPS 71-U, 13GPS203, 13GPS207, 13GPS109, 13GPS149 and E4 were obtained from Gaziantep-Subağı, Manisa-Kula, İzmir-Bergama, Ankara-Şereflikoçhisar, Mardin-Midyat and Eskişehir-Tepebaşı regions of Turkey, respectively (Azamparsa 2015).

2.3. Making single spore isolates, inoculation, incubation and disease assessment

Infected leaf samples obtained from different regions of Turkey were cut into small sizes, surface sterilized with 70% ethyl alcohol in 15 seconds followed by 0.5% sodium hypochlorite in 90 seconds and placed on sterilized filter paper for 1 minute (Azamparsa 2015). These dried samples were placed on autoclaved Bean Agar (BA) medium (140 g fresh bean, 20 g dextrose, 18 g agar, 1 L distilled water) at 22 °C inside an incubator. Fungus colony was produced on this medium after 2-3 weeks. To obtain single spore of fungus, 1 mL of sterile water was placed in a small micro tube and by using a sterile needle small part of colony with spores was transferred into the micro tube. This micro tube was shaken well and with the use of a sterile loop spore suspension was dispersed on BA medium. After 2-3 days under a stereomicroscope germinated spores were taken to the other Petri dishes containing BA. Developed colonies of single spores were transformed to test tubes containing BA medium and stored at 4 °C in a refrigerator. To produce inoculum, each isolate was grown on BA medium for about 14 days and then distilled water was added onto this colony and spores were harvested. Harvested spore suspension was cleaned from large parts of colonies using a cheesecloth and spore concentration was adjusted to 1×10^6 spores mL^{-1}. One drop of Tween-20 was added to each 100 mL of inoculum. Five to 10 seeds of each genotype were seeded to 7 cm diameter plastic pots. These pots were placed in the greenhouse and watered when needed. Inoculation of plants was made when plants produced 1.5 leaves. After inoculation, plants

were transferred inside of a moist chamber with 100% relative humidity and 16-17 °C temperature range for 48 hours to insure infection of plants. After 2 days, plants were taken into the greenhouse with 20-25 °C temperature range. Disease symptoms started 8-10 days after inoculation and first disease assessment was made using El-Ahmed (1981) scale after 14 days of inoculation. The second disease assessment was made four days later (18 days after inoculation) and results of second assessment were used in disease evaluation. Three replications were used. Scale values of 0-2.0 were considered as resistant reaction and scale values of 2.1-4.0 were considered as susceptible reaction.

3. Results and Discussion

Among barley landraces only one genotype (genotype no: 200, Yeşilköy 9052) was resistant to all 6 tested isolates and therefore it was determined as the most resistant genotype. Barley landrace no: 17 was resistant to 5 isolates and moderately susceptible to GPS71-U isolate and this genotype was rated as the second most resistant genotype. Fourteen barley landraces were resistant and susceptible to 4 and 2 isolates, respectively. These barley landraces included the following landraces: 2, 15, 20, 23, 24, 79, 81, 82, 83, 120, 121, 132, 134 and 194.

Presence of resistance to barley scald in barley landraces and cultivars were reported previously. In Ethiopia, up to 100 single plant derived lines of 18 barley landraces were tested against *R. commune* and it was concluded that on average landraces with late maturity showed more resistance to barley scald (Alemayehu & Parlevliet 1997). In another study in Ethiopia, 180 barley landraces and 600 single head plants selected from 60 promising populations were evaluated for barley scald disease resistance. Barley landraces from higher altitude were more resistant to *R. commune* (Yitbarek et al 1998). In Spain, Silvar et al (2010) used 159 (148 six-rowed and 11 two-rowed) inbred lines from local landraces and 16 commercial cultivars (8 six-rowed and 8 two-rowed) and tested their resistance against several barley diseases including barley scald. They found resistant reaction in about 26% of Spanish lines (mostly winter barleys) to *R. commune*. In Turkey, some barley genotypes which were bred in breeding programs by Central Research Institute for Field Crops and International Center for Agriculture Research in Dry Areas (ICARDA) were tested against *R. commune*. One thousand and four hundred one and 1379 barley lines and cultivars were evaluated aginst barley scald in the field and greenhoue tests, respectively. From these tested lines and cultivars 432 genotypes in the field and 257 genotypes in the greenhouse were found resistant or moderately resistant to barley scald. From 15 barley cultivars only Erginel 90 cultivar was found resistant to *R. commune* in both field and greenhouse experiments (Albustan et al 1998). In the other experiment by Mert & Karakaya (2004) both differences for resistance among barley cultivars and pathogenicity among 5 isolates of *R. commune* were found. Among the tested cultivars Çetin 2000, Şahin 91, Erginel 90, Kıral 97, Akhisar 98, Çumra 2001 and Avcı 2002 were found resistant to all isolates. Cultivars Bülbül 89 and Efes 3 were susceptible to barley scald isolates. In another study, reactions of 36 barley cultivars and 683 barley genotypes to *R. commune* were determined in greenhouse and field experiments and from 683 barley genotypes, 44% in greenhouse and 39% in field tests were found resistant to *R. commune* (Düşünceli et al 2008). There was significant correlation (r= 0.53) between greenhouse and field test results (P= 0.0001). The cultivars Avcı 2002, Çetin 2000, Kıral 97, Erginel 90, Akhisar 98, Kaya 7794, Yeşilköy 387 and Zafer 160 were found as resistant under both greenhouse and field conditions. The cultivars Vamıkhoca, Çıldır 02 and Quantum were found as susceptible under greenhouse conditions; however, they were found as resistant under field conditions. Twenty five cultivars were susceptible to barley scald both in greenhouse and field tests. Azamparsa et al (2015a) reported virulence differences among the *R. commune* isolates. Isolate obtained from Gaziantep was the most virulent followed by isolates obtained from Manisa and Eskişehir provinces of Turkey. These results show differences in resistance among barley cultivars and genotypes as well as virulence

differences among isolates of *R. commune*. Similar results were obtained in our current study.

From 198 barley landraces which were tested in this study, 169 and 29 barley landraces were belonged to 2-rowed and 6-rowed barleys, respectively. While 6-rowed barley cultivars had medium disease value of 2.5 out of 4, two-rowed barley landraces had disease value of 3.3 out of 4. Based on these results it can be said that six-rowed barley landraces were more resistant than two-rowed barleys to *R. commune*. The same results were reached by many workers in previous studies. In these experiments six-rowed barley cultivars were found more resistant than two-rowed barley cultivars to *R. commune* (Zencirci & Hayes 1990; Mert & Karakaya 2004; Düşünceli et al 2008; Silvar et al 2010; Mert et al 2014).

Twenty seven genotypes of wild barley (*H. spontaneum*) numbered as 5, 6, 7, 8, 9, 11, 16, 20, 30, 31, 36, 37, 48, 50, 51, 56, 58, 59, 60, 62, 67, 79, 80, 83, 90, 94 and 101 were found resistant to all six isolates of *R. commune*. Apart from these genotypes, 19 genotypes numbered as 1, 2, 32, 33, 34, 42, 43, 49, 52, 64, 66, 76, 77, 78, 96, 97, 102, 104 and 107 showed resistance to 5 *R. commune* isolates and susceptibility to only 1 isolate.

Genotypes of *H. spontaneum* are used in breeding programs as resistance sources especially resistance to cold and dry weather and disease resistance (Chen et al 2008; Çelik & Karakaya 2017). In the current study 26% of wild barley genotypes showed resistance to barley scald disease in seedling stage. This result shows high potential of wild barley (*H. spontaneum*) genotypes for resistance to *R. commune*. Resistance genes were successfully transformed from wild barley to cultivated barley (Çelik & Karakaya 2017). Abbott et al (1992) screened accessions of *H. vulgare* ssp. *spontaneum* collected from Israel (70), Iran (15) and Turkey (6) against four isolates of *R. commune* in seedling stage and they found a high percentage of resistance (77%) among these wild barley accessions to barley scald. Cherif et al (2012) in Tunisia tested 56 accessions of wild barleys *(H. spontaneum, H. marinum* and *H. murinum*) and 7 varieties of *H. vulgare* against net blotch and scald to find new sources of resistance to these diseases and they found sources of resistance to *R. commune* in three wild species of barley. However, due to strong incompatibility between cultivated and two wild barleys (*H. marinum* and *H. murinum*) (Pickering & Johnston 2005), it seems that transfer of scald resistance genes to cultivated barley from *H. spontaneum* is more feasible. In their study, two *H. spontaneum* accessions collected from Afghanistan and Egypt showed resistance to barley scald both in seedling and adult growth stages.

Two susceptible Turkish cultivars Bülbül 89 and Efes 3 used in the current study were found susceptible to 96% of the scald isolates. Susceptibility of these cultivars were reported previously (Mert & Karakaya 2004; Azamparsa et al 2015b) and confirmed with this study. Resistant *H. spontaneum* genotypes and barley landraces identified in this study could be used in breeding programs for obtaining scald resistant genotypes or promising landraces could be used directly as the seeding materials.

4. Conclusions

In this study only one barley landrace out of 198 landraces (genotype no: 200) (0.5% of the genotypes) showed resistance to all six tested isolates to *R. commune*. Another six-rowed barley (genotype no: 17) landrace showed resistant reaction to 5 isolates and moderately susceptible reaction to 1 isolate. Fourteen barley landraces were resistant and susceptible to 4 and 2 isolates, respectively. Twenty seven lines of wild barley (*H. spontaneum*) out of 104 genotypes were resistant to all 6 isolates of *R. commune*. In addition, 19 genotypes showed resistance to 5 isolates and susceptibility to only 1 isolate. Resistance to *R. commune* among barley landraces was low. On the other hand, more wild barley (*H. spontaneum*) genotypes were resistant to *R. commune*. It appears that *Hordeum spontaneum* genotypes are good resistance sources of scald disease of barley caused by *R. commune*.

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