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Translucent and opaque bands in vertebral section
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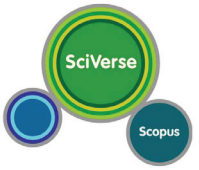

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Tarım Bilimleri Dergisi
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Yayın Ünitesi
06110 Dışkapı/Ankara-TÜRKiYE

Telefon : +90 (312) 596 14 24
Belge geçer: +90 (312) 317 67 24
E-posta: tbdeditor@ankara.edu.tr

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Ankara University Faculty of Agriculture
Publication Department
06110 Dışkapı/Ankara-TURKEY

Telephone : +90 (312) 596 14 24
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Effect of Supplementation with Green and Black Tea on Microbiological Characteristics, Antimicrobial and Antioxidant Activities of Drinking Yoghurt

Gülfem ÜNAL[a], Cem KARAGÖZLÜ[a], Özer KINIK[a], Ecem AKAN[b], Ayşe Sibel AKALIN[a]

[a]*Ege University, Faculty of Agriculture, Department of Dairy Technology, 35100, Bornova, Izmir, TURKEY*

[b]*Adnan Menderes University, Faculty of Agriculture, Department of Dairy Technology, Aydın, TURKEY*

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Corresponding Author: Gülfem ÜNAL, E-mail: gulfem.unal@ege.edu.tr, Tel: +90 (232) 311 27 32
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ABSTRACT

The influence of supplementation with green and black tea on microbiological properties, antimicrobial and antioxidant activities of drinking yoghurt were investigated during 21 days of storage. The samples supplemented with 2% either green or black tea had higher viable counts of both yoghurt starter bacteria than those of infused by the ratio of 4%. Both green and black tea extracts showed antimicrobial activity on *E. coli*, *B. cereus*, *S. aureus* and *C. albicans* however this effect was detected higher in samples containing green tea. The samples added green tea extract had the highest DPPH scavenging activity when compared to those supplemented with black tea extract throughout the storage. Green tea had a superior effect than black tea in terms of total phenolic content of drinking yoghurt samples.

Keywords: Tea; Drinking yoghurt; Antimicrobial; Antioxidant; Viability

Yeşil ve Siyah Çay İlavesinin İçilebilir Yoğurdun Mikrobiyolojik Özellikleri ile Antimikrobiyal ve Antioksidan Aktivitesi Üzerine Etkisi

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Gülfem ÜNAL, E-posta: gulfem.unal@ege.edu.tr, Tel: +90 (232) 311 27 32
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ÖZET

Yeşil ve siyah çay ilavesinin içilebilir yoğurdun mikrobiyolojik özellikleri ile antimikrobiyal ve antioksidan aktiviteleri üzerine etkisi 21 günlük depolama boyunca araştırılmıştır. % 2 yeşil veya siyah çay ilave edilen örneklerde canlı yoğurt starter bakteri sayısı % 4 oranında çay ilave edilen örneklere göre daha yüksek bulunmuştur. Gerek yeşil gerekse siyah çay ekstraktı *E. coli*, *B. cereus*, *S. aureus* and *C. albicans* üzerinde antimikrobiyal aktivite gösterirken bu etkinin yeşil çay içeren örneklerde daha fazla olduğu tespit edilmiştir. Yeşil çay ekstraktı ilave edilen örnekler siyah çay ekstraktı içerenler ile kıyaslandıklarında depolama boyunca daha yüksek DPPH radikalini bağlama aktivitesi göstermişlerdir. Yeşil çay, içilebilir yoğurt örneklerinin toplam fenolik miktarları açısından siyah çaya göre daha üstün bir etki göstermiştir.

Anahtar Kelimeler: Çay; İçilebilir yoğurt; Antimikrobiyal; Antioksidan; Canlılık

1. Introduction

Tea (*Camellia sinensis*, family Theaceae) is commonly consumed worldwide having various health benefits and physiological functionalities, such as antioxidative, anticarcinogenic and antimicrobial effects (Michalczyk & Zawiślak 2008; Archana & Abraham 2011; Chan et al 2011). The most important bioactive substances responsible for these health effects present in tea are tea polyphenols. Among various activities of tea, antioxidant function is one of the most important activities and most frequently studied (Erol et al 2009; Chan et al 2011). Different types of tea have been known to have good antioxidant activity whereas green tea has been reported to be the tea most abundant in catechins (Najgebauer-Lejko et al 2011).

The antimicrobial activity of tea which inhibit many undesired microbial growth are mainly related to their polyphenolic components (Michalczyk & Zawiślak 2008). The extracts of *Camellia sinensis* have been determined to inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Candida albicans* and *Bacillus cereus* in many studies (Archana & Abraham 2011; Chan et al 2011; Kumar et al 2012).

In recent years, green and black teas had been used because of their benefits to human health and their popular consumption worldwide in some dairy products such as milk, yoghurt, fermented milk and some other probiotic dairy products (Jaziri et al 2009; Najgebauer-Lejko et al 2011; Marhamatizadeh et al 2013; Ye et al 2013; Najgebauer-Lejko 2014). However, the effect of tea on the characteristics of drinking yoghurt has not been studied.

The objective of this study was to investigate the viability of yoghurt starter bacteria (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*), antimicrobial and antioxidant properties in presence of green and black teas during refrigerated storage. In addition, two different ratios (2% or 4%) were applied in order to state the effect of tea on the properties of drinking yoghurt is dose-dependent or not.

2. Material and Methods

2.1. Material

UHT milk which was used in the manufacture of drinking yoghurt was obtained from Pinar Dairy Products, Izmir, Turkey. Green and black tea leaves were obtained from a national commercial brand (Caykur, Rize, Turkey). Yoghurt starter culture, a combination of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* obtained in freeze-dried form (Jointec 12) from CSL (Centro Sperimentale del Latte, Italy) company.

Folin-Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Cat: 23,881-3), DPPH (2,2-diphenyl-1-picrylhydrazyl, D-9132) and gallic acid (G-7384) were purchased from Sigma-Aldrich (Steinheim, Germany). All other reagents and solvents commercially obtained were of analytical grade. All spectrophotometric data were acquired using a Cary 50 Scan UV-Visible spectrophotometer (UK).

2.2. Drinking yoghurt manufacture

The freeze dried culture was propagated by inoculating in skim milk which was heated at 90 °C for 30 min before the inoculation. The inoculated milk was incubated at 45 °C until pH 4.6 was reached, then stored overnight at 40 °C in refrigerator.

The whole milk was heated to 85 °C and waited for 10 min, then divided into five lots and supplemented with 2% black tea (2BDY), 4% black tea (4BDY), 2% green tea (2GDY) or 4% green tea (4GDY). On the other hand, the control drinking yoghurt did not contain any tea extract (CDY). The teas were infused for 10 min then different batches were filtered through sterile cotton to remove the particles. The milk samples were then cooled to 45 °C and inoculated 3% yoghurt culture and divided into 200 mL plastic containers and incubated at 42 °C until a pH 4.6 was reached. After fermentation, the samples were cooled and stored at 4 °C for 21 days for the analyses.

2.3. pH

The pH value of drinking yoghurt samples was determined using a pH meter (Hanna Instruments Model pH: 211; Woonsocket, RI, USA). pH values were determined during 21 days of storage.

2.4. Microbiological analyses

S. thermophilus enumeration was performed on M17 agar and aerobically at 37 °C for 48 h whereas the counts of *L. bulgaricus* were detected on MRS agar and at microaerophilic conditions at 42 °C for 72 h using the pour plate technique (Jaziri et al 2009).

2.5. Antimicrobial activity

2.5.1. Bacterial strains

The studied microbial strains were *Bacillus cereus* (from Collection Española de Cultivas Tipo CECT 495), *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* (from American Type Culture Collection; ATCC 29252, ATCC 29213, ATCC 64550, respectively).

2.5.2. Antimicrobial activity analysis

Antibacterial susceptibility testing was done by using disc diffusion method (Radji et al 2013). The microorganisms were activated by inoculating a loopful of the strain in the Tripton Soy Broth and incubated at 37 °C for one night. Then 0.2 mL of inoculum size was used 10^8 cells as per McFarland Standard. Then Tripton Soy Agar was poured into Petri Plates. For agar disc diffusion technique, the test compound (0.2 mL) was introduced on the disc (0.7 cm) (Hi media) and then allowed to dry. The plates were incubated at 37 °C for 24 h. Microbial growth was determined by measuring the diameter of zone of inhibition. The experiment was done three times and the mean values are presented.

2.6. Antioxidant activity and total phenolic content (TPC)

The DPPH radical scavenging activity of the samples was estimated according to the procedure described by Unal & Akalın (2012). Trolox was used as a reference antioxidant at a concentration of 0.25 mg mL^{-1}. DPPH scavenging activity percent was calculated by Equation 1.

DPPH scavenging activity (%)= [(control absorbance-extract absorbance)/(control absorbance)]x100 (1)

TPC of each sample was determined according to Folin-Ciocalteu method (Singleton & Rossi 1965; Singleton et al 1999). The phenolic content was compared to a gallic acid standard curve and the total phenolic content of the samples was expressed as milligrams gallic acid equivalents (GAE) per liter of sample. The equation for the gallic acid calibration curve was y= 0.0012x+0.0359 and the correlation coefficient was R^2= 0.9983.

2.7. Statistical analysis

The experiments were performed in twice with three parallel. Six values for each sample were averaged (n= 6). The data obtained was processed by one-way ANOVA using the general linear model procedure of the SPSS version 11.05 (SPSS Inc., Chicago, IL, USA). The means were compared with the Duncan test at $P<0.05$ level.

3. Results and Discussion

3.1. Changes in pH values

The changes in the pH values of drinking yoghurt samples during refrigerated storage are shown in Figure 1. The pH values of control drinking yoghurt (CDY) and sample with 4% green tea (4GDY) decreased at the end of the storage period ($P<0.05$). Similar results were obtained for control ayran (Turkish drinking yoghurt) and yoghurt supplemented with tea throughout the storage by other studies (Najgebauer-Lejko et al 2011; Marhamatizadeh et al 2013; Erkaya et al 2015).

The lowest pH values were detected in control sample during storage whereas samples supplemented with green tea generally had higher ($P<0.05$) values. Najgebauer-Lejko (2014) also determined higher pH values in acidophilus milk with 5% green tea infusion than that of plain acidophilus milk during 21 days of storage. In addition, parallel to our results the authors concluded that the pH of

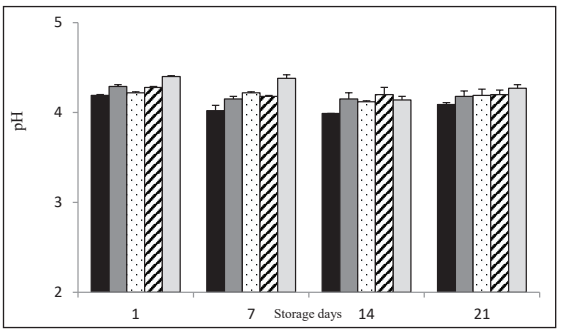

Figure 1- Changes in pH during 21 days of storage in drinking yoghurt samples; CDY, control drinking yoghurt (black bar); 2BDY, drinking yoghurt supplemented with 2% black tea (dark gray bar); 4BDY, drinking yoghurt supplemented with 4% black tea (dotted bar); 2GDY, drinking yoghurt supplemented with 2% green tea (hashed bar); 4GDY, drinking yoghurt supplemented with 4% green tea (light gray bar)

acidophilus milk was higher for higher levels of green tea supplementation, which was accompanied by the lower values of titratable acidity. In contrast, the pH value of yoghurt with 5% green tea infusion was found significantly lower than that of control yoghurt (without tea infusion) in another study (Najgebauer-Lejko et al 2011). Najgebauer-Lejko et al (2014) also detected higher titratable acidity in 5% supplemented yoghurt than that of plain yoghurt. This can be caused by the higher ratio of green tea when compared to the ratio used in our study.

3.2. Viability of Streptococcus thermophilus and Lactobacillus bulgaricus

The changes in the viable counts of *S. thermophilus* and *L. bulgaricus* in drinking yoghurts during refrigerated storage are presented in Table 1. The samples supplemented with 2% either green or black tea had higher ($P<0.05$) viable counts of both starter bacteria than those of infused by the ratio of 4%, in storage days which significant differences were observed.

The highest ($P<0.05$) viable counts of *S. thermophilus* were enumerated in control sample on the 1st and 14th day whereas there were no significant differences between all samples on the other days

Table 1- Changes in the viable counts of *S. thermophilus* and *L. bulgaricus* during refrigerated storage of drinking yoghurts (log cfu g^{-1})

Drinking yoghurt type[1]	*Storage day*			
	1	*7*	*14*	*21*
	S. thermophilus			
CDY	8.83 ± 0.01^{Aa}	8.58 ± 0.03^{Ab}	7.83 ± 0.02^{Ac}	7.41 ± 0.06^{Ad}
2BDY	8.75 ± 0.02^{Ba}	8.41 ± 0.65^{Aa}	7.62 ± 0.03^{Ba}	7.39 ± 0.72^{Aa}
4BDY	8.67 ± 0.04^{Ca}	7.91 ± 0.01^{Ab}	7.51 ± 0.03^{Cc}	6.82 ± 0.01^{Ad}
2GDY	8.70 ± 0.00^{BCa}	7.79 ± 0.21^{Ab}	7.54 ± 0.00^{Cb}	6.86 ± 0.01^{Ac}
4GDY	8.55 ± 0.03^{Da}	7.86 ± 0.01^{Ab}	7.38 ± 0.01^{Dc}	6.73 ± 0.03^{Ad}
	L. bulgaricus			
CDY	8.84 ± 0.01^{Aa}	8.65 ± 0.01^{Ab}	7.85 ± 0.01^{Ac}	7.46 ± 0.01^{Ad}
2BDY	8.76 ± 0.02^{Ba}	8.48 ± 0.04^{Bb}	7.64 ± 0.08^{ABc}	6.91 ± 0.01^{Ad}
4BDY	8.64 ± 0.04^{Ca}	7.96 ± 0.01^{Cb}	7.48 ± 0.21^{ABc}	6.80 ± 0.04^{Ad}
2GDY	8.74 ± 0.01^{Ba}	8.45 ± 0.04^{Ba}	7.59 ± 0.13^{ABa}	8.38 ± 2.09^{Aa}
4GDY	8.62 ± 0.01^{Ca}	7.95 ± 0.01^{Cb}	7.25 ± 0.40^{Bc}	6.74 ± 0.03^{Ac}

[1]CDY, control drinking yoghurt; 2BDY, drinking yoghurt supplemented with 2% black tea; 4BDY, drinking yoghurt supplemented with 4% black tea; 2GDY, drinking yoghurt supplemented with 2% green tea; 4GDY, drinking yoghurt supplemented with 4% green tea; $^{a-d}$, means±standard deviations in the same row with different superscript lowercase letters are significantly different ($P<0.05$); $^{A-D}$, means±standard deviations in the same column with different superscript uppercase letters are significantly different ($P<0.05$)

of storage. The control samples showed the highest viability of *L. bulgaricus* on 1st and 7th day whereas there were no significant differences between all samples at the end of the storage. It can be concluded that addition of tea to drinking yoghurt did not increase the growth and survival of both yoghurt starter bacteria during storage. Jaziri et al (2009) also reported that green or black tea extract fortification has no effect on the development of either *S. thermophilus* or *L. bulgaricus* in yoghurt that can be caused by insignificant changes in acid development. Similar to our results, Najgebauer-Lejko et al (2011) enumerated lower viable counts of both *S. thermophilus* and *L. bulgaricus* in yoghurt supplemented with 5% green tea when compared to control yoghurt without tea infusion. In contrast, enhancing effect of green tea addition on the viability of probiotic bacteria such as *L. casei*, *L. acidophilus* and *B. bifidum* was observed in another study (Marhamatizadeh et al 2013).

The viability of yoghurt bacteria in drinking yoghurt samples supplemented with tea extracts did not significantly ($P>0.05$) changed at the end of the storage when compared to the beginning of the period. Although some fluctuations were observed in streptococci counts, Najgebauer-Lejko (2014) also could not determine any significant change in acidophilus milk infused by 5% green tea within 21 days of storage period.

3.3. Antimicrobial activity

It was found that tea extracts have antimicrobial effect on *E.coli*, *B. cereus*, *S. aureus* and *C. albicans* at both 2% and 4% ratios however this effect was detected higher in samples containing green tea extracts (Table 2). Michalczyk & Zawiślak (2008)

Table 2- Antimicrobial activity of drinking yoghurt samples during storage given as the diameter of inhibited zone (mm)

		Bacterial strains			
Product[1]	*Storage days*	*E. coli*	*B. cereus*	*S. aureus*	*C. albicans*
CDY	1	3.10±0.11	4.35±0.17	2.75±0.29	3.30±0.34
	7	2.35±0.24	4.20±0.22	nzd	4.20±0.21
	14	1.83±0.24	2.12±0.09	nzd	2.55±0.06
	21	nzd	0.37±0.75	nzd	2.10±0.20
2BDY	1	4.92±0.09	6.33±0.22	7.90±0.27	10.62±0.12
	7	6.45±0.06	5.45±0.31	6.50±0.26	6.67±0.15
	14	5.50±0.09	7.25±0.19	5.25±0.10	8.25±0.25
	21	3.25±0.29	5.22±0.20	4.87±0.12	7.42±0.28
4BDY	1	9.22±0.20	9.22±0.20	11.72±0.09	12.50±0.08
	7	7.97±0.17	8.67±0.09	9.82±0.05	9.72±0.12
	14	6.36±0.15	7.35±0.19	8.15±0.19	10.40±0.16
	21	4.32±0.15	5.27±0.22	5.75±0.29	9.17±0.20
2GDY	1	8.02±0.26	8.20±0.20	9.27±0.32	12.25±0.30
	7	6.10±0.11	7.65±0.17	8.20±0.16	11.25±0.35
	14	6.37±0.26	6.55±0.07	7.55±0.05	9.27±0.32
	21	3.80±0.24	4.10±0.10	6.27±0.25	7.62±0.09
4GDY	1	11.55±0.34	12.75±0.64	13.20±0.19	15.60±0.43
	7	9.30±0.26	11.37±0.32	12.60±0.08	13.55±0.05
	14	8.45±0.24	9.37±0.25	10.65±0.30	12.27±0.32
	21	5.37±0.20	8.00±0.00	7.95±0.05	9.85±0.19

[1]CDY, control drinking yoghurt; 2BDY, drinking yoghurt supplemented with 2% black tea; 4BDY, drinking yoghurt supplemented with 4% black tea; 2GDY, drinking yoghurt supplemented with 2% green tea; 4GDY, drinking yoghurt supplemented with 4% green tea; nzd, no zone detected

and Chan et al (2011) reported that green tea extract inhibited various Gram positive bacteria but *S. aureus* was the least susceptible. Kumar et al (2012) also investigated the antibacterial activity of green tea leaves against environmental sources originated *S. aureus*, *Streptococcus*, *Pseudomonas aeruginosa*, *Bacillus*, *E.coli* and *Proteus* species and detected significant activity.

In addition, antimicrobial activity showed reduction during the whole storage period. Increase in the ratio of green tea extract used also cause an enhancement in the antimicrobial activity of the samples. Drinking yoghurt samples supplemented by black tea extract also show antimicrobial activity against *C. albicans* but lower than green tea extract in our study. Similarly, Chou et al (1999) detected a lower antimicrobial activity against *B. subtilis*, *E. coli*, *S. aureus*, Salmonella sp. and *Proteus vulgaris* in black tea extract when compared to green tea extract. Archana & Abraham (2011) also reported that *E. coli*, *Enterococccus faecalis*, *S. aureus*, *Pseudomonas aeruginosa* and *C. albicans* were very sensitive to fresh green tea extracts.

Wu et al (2007) found that water extracts of various tea types including green tea showed an antimicrobial activity against *S. aureus* and *B. subtilis* at 2 mg mL^{-1} concentration, however no antimicrobial effect was observed on Gram (-) *E. coli*. On the other hand, some other authors have reported that the level of resistance of Gram (-) bacteria against the extracts were related to the lipopolysaccharides in the cell membrane and the antimicrobial activity was higher in fresh tea leaves due to their high polyphenol content (Chou et al 1999; Alzoreky & Nakahara 2003; Chan et al 2011).

3.4. DPPH radical scavenging activity

The DPPH scavenging activity of the drinking yoghurt samples ranged from 76.42% to 96.21% (Figure 2). Trolox at a concentration of 0.25 mg mL^{-1} showed a DPPH scavenging activity of 97.04%. McCue & Shetty (2005) also investigated the DPPH scavenging activity of soy yoghurt produced by kefir cultures and reported the activity as 92.3% after 48 h of production which is similar to our results obtained on the 1st day of storage. Unal & Akalın (2012) and Unal et al (2013) also determined the DPPH scavenging activity as approximately 90% in control yoghurt samples at the beginning of the refrigerated storage. Moreover, Farvin et al (2010) studied the antioxidant activity of different fractions of yoghurt and found the DDPH radical scavenging activity of crude yoghurt (0.2 mg mL^{-1}) to be 94.47%.

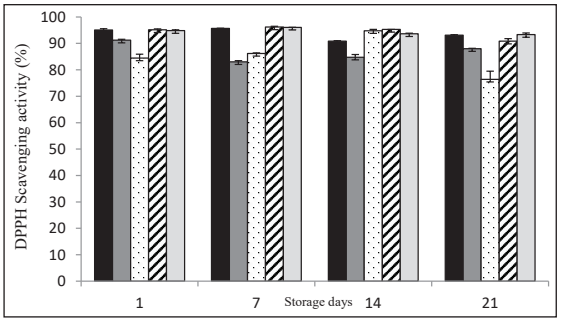

Figure 2- DPPH scavenging activity (%) of drinking yoghurt samples during 21 days of storage at 4 °C; CDY, control drinking yoghurt (black bar); 2BDY, drinking yoghurt supplemented with 2% black tea (dark gray bar); 4BDY, drinking yoghurt supplemented with 4% black tea (dotted bar); 2GDY, drinking yoghurt supplemented with 2% green tea (hashed bar); 4GDY, drinking yoghurt supplemented with 4% green tea (light gray bar)

The drinking yoghurt samples infused by green tea extract had the highest ($P<0.05$) scavenging activity when compared to those supplemented with black tea extract throughout the storage. This shows the superiority of green tea in terms of radical scavenging activity which can be attributed to containing higher amount of total phenolic content (Michalczyk & Zawiślak 2008; Jaziri et al 2009; Chan et al 2011). Similarly, Najgebauer-Lejko et al (2011) determined a higher anti radical power in yoghurt with 5% green tea infusion when compared to natural yoghurt. In another study, a higher DPPH radical scavenging activity (9-29 fold) was detected in fermented milks infused by 5%, 10%

and 15% green tea than fermented milk without any supplementation (Najgebauer-Lejko 2014).

The scavenging activity of all samples showed a fluctuation throughout the storage period and the activity significantly decreased ($P<0.05$) at the end of the storage according to the beginning of the period. The reduction in the activity has been attributed to the ability of starter bacteria (especially lactobacilli) to utilize phenolic components by producing phytase enzyme (Subrota et al 2013). In our study, similar fluctuation was observed in the viability of *L. bulgaricus* during 21 days of storage. On the other hand, synergistic effect of phenolic compounds with each other or other compounds can result an enhancement of antioxidant activity within such a fluctuation (Shahidi et al 1994).

3.5. Total phenolic content (TPC)

TPC of drinking yoghurt samples changed in an order of CDY<2BDY<4BDY<2GDY<4GDY during whole storage period (Figure 3). This order showed that the type of tea and the infusion ratio were found statistically significant. Green tea infusion increased the total phenolic content of samples more than those supplemented with black tea. This is probably because green tea includes higher amount of both catechin and other phenolic compounds than black tea as reported by many researchers (Jaziri et al 2009; Chan et al 2011). Similarly, Komes et al (2007) investigated polyphenol content of some types of tea and reported that green tea is the richest source of unmodified polyphenols among all types of tea. On the other hand, the superiority effect of green tea on the phenolic content of samples can be attributed to the relationship between tea polyphenols and milk. Ye et al (2013) investigated the interactions of black tea polyphenols (BTP) and green tea polyphenols (GTP) with milk. The researchers reported that the interactions between individual catechins (e.g. (-)-epigallocatechin gallate, (-)-epigallocatechin and (-)-epicatechin gallate) and pure proteins (eg: β-casein, α-casein and β-lactoglobulin) or milk proteins occurred with the formation of catechin-protein complexes. They concluded that the structures of catechins affect the affinities of tea catechins for casein micelles in the GTP-milk system but no obvious impact for the BTP-milk system.

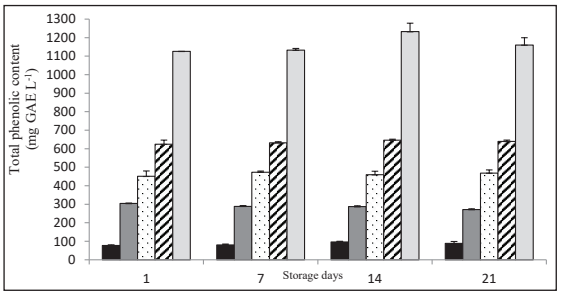

Figure 3- Total phenolic content (mg GAE L^{-1}) of drinking yoghurt samples during 21 days of storage at 4 °C; CDY, control drinking yoghurt (black bar); 2BDY, drinking yoghurt supplemented with 2% black tea (dark gray bar); 4BDY, drinking yoghurt supplemented with 4% black tea (dotted bar); 2GDY, drinking yoghurt supplemented with 2% green tea (hashed bar); 4GDY, drinking yoghurt supplemented with 4% green tea (light gray bar)

The superiority of green tea infusion on the TPC of the samples is in parallel to those of DPPH scavenging activity. This can be supported by the findings of Erol et al (2009) that the authors found a correlation between TPC and antioxidant activity of some types of tea.

The TPC of drinking yoghurt samples infused by tea extract did not generally changed ($P>0.05$) throughout the storage period. This can be caused by the ability of yoghurt starter bacteria to preserve the catechins from oxidation during both yoghurt fermentation and storage (Kachouri & Hamdi 2006).

4. Conclusions

The present study indicated that, addition of tea to drinking yoghurt did not increase the survival of both yoghurt starter bacteria probably due to the changes in pH values throughout the storage. On the other hand, green tea improves both antimicrobial and antioxidant activities of drinking yoghurt higher than black tea. This effect was stronger when the

supplementation ratio increased from 2% to 4%. Therefore, fortification of drinking yoghurt with green tea can be an alternative pathway to create a functional dairy product having both nutritional and health benefits. Furthermore, sensory and physical quality characteristics should be also evaluated before marketing such a product.

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Effect of Humic Substance Applications on Mineral Nutrition and Yield of Granny Smith and Jersey Mac Apple Varieties

Murat CANSU[a], İbrahim ERDAL[b]

[a]*Fruit Growing Research Station, Eğirdir, Isparta, TURKEY*

[b]*Suleyman Demirel University, Faculty of Agriculture, Department of Soil Science and Plant Nutrition, 32260, Isparta, TURKEY*

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Corresponding Author: İbrahim ERDAL, E-mail: ibrahimerdal@sdu.edu.tr, Tel: +90 (246) 211 85 91
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ABSTRACT

This study was conducted to examine the effect of soil, leaf and soil+leaf applications of humic substance on fruit yield, some quality parameters and mineral nutrition of Granny Smith and Jersey Mac apple varieties grafted on MM106 rootstock. The study was carried out for two consecutive years. According to the results obtained from both years, humic substance applications had no significant effect on fruit yield and quality parameters generally, but relative increases were recorded in yields. Leaf N, K, Ca, Fe and Zn concentrations were significantly affected from the applications. At the first year, humic substance application significantly affected only N and K concentrations of Jersey Mac variety, but in the second year, humic substance applications led to increase in leaf N, K, Ca, Fe and Zn concentrations in Granny Smith and N concentration in Jersey Mac apple variety. According to the results obtained, it can be said that the effects of humic substances were higher than the first year's effects mostly.

Keywords: Fruit productivity; Fruit quality; Humic material; Nutrient concentration

Humik Madde Uygulamalarının Granny Smith ve Jersey Mac Elma Çeşitlerinin Mineral Beslenmesine ve Verimine Etkisi

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: İbrahim ERDAL, E-posta: ibrahimerdal@sdu.edu.tr, Tel: +90 (246) 211 85 91
Geliş Tarihi: 25 Ekim 2016, Düzeltmelerin Gelişi: 06 Aralık 2016, Kabul: 14 Aralık 2016

ÖZET

Bu araştırma, MM106 anacına aşılanmış Granny Smith ve Jersey Mac elma çeşitlerinin verimi, kalitesi ve mineral beslenmesi üzerine humik maddenin toprak, yaprak ve toprak+yaprak uygulamalarının etkisini incelemek amaçlanmıştır. Deneme ardışık iki yıl yürütülmüştür. Araştırma sonunda, humik madde uygulamalarının meyvenin verim ve kalite ölçütleri üzerine genellikle anlamlı bir etkisi olmazken, meyve verimlerinde nisbi artışlar kaydedilmiştir.Yaprağın N, K, Ca, Fe ve Zn konsantrasyonları hümik madde uygulamalarından olumlu etkilenmiştir. İlk yıl, humik madde uygulamaları sadece Jersey Mac çeşidinin N ve K konsantrasyonlarını etkilemiştir. Buna karşılık ikinci yıl humik madde

uygulamaların Granny Smith çeşidinin N, K, Ca, Fe ve Zn konsantrasyonlarını, Jersey Mac çeşidinin ise N konsantrasyonunu artırdığı görülmüştür. Elde edilen sonuçlara göre, humik madde uygulamalarının ikinci yıldaki etkisinin, birinci yıla oranla daha fazla olduğu söylenebilir.

Anahtar Kelimeler: Meyve verimliliği; Meyve kalitesi; Humik madde; Besin elementi içeriği

1. Introduction

Turkey is one of the most important apple producing countries in the world. Apple production in Turkey was estimated as 3.1% of the world apple production. Although, Isparta is a very important apple growing region with the production nearly 26% of the country (TÜİK 2014), the yield and quality is not satisfactory. There might be several factors for this, but one of the main reasons of this is unfavorable soil condition due to low organic matter, some nutrient deficiencies and unavailability of nutrients (Erdal et al 2004).

Soil fertility is defined as condition or state which the soil can supply sufficient amount of nutrient for healthy plant growth. Soil organic matter is an important soil component that increases and keeps sustainability of soil fertility by means of many physical, chemical and biological effects. Soil organic matter increases water and heat holding capacity, improves drainage, aeration and aggregation, increases microbial activity by means of decomposition products, improves soil pH, lime content, cation exchange capacity etc. With these properties, organic matter has an indirect effect on soil fertility and plant growth. At the same time, in terms of nutrients that release with mineralization, organic matter has a direct effect on soil fertility and plant growth.

Humic acid (HA) and fulvic acid (FA) contain humic substances (HS), namely humus, which is an important component of the soil organic matter. Because of the different contribution ways on plant growth, HS are used in different areas of agriculture (Chen & Aviad 1990). Owing to chelating properties of HS with metallic cations, availability of many nutrients increase and thus plant growth is affected positively (Stevenson 1994). Additionally, HS increase root and root hair growth leading to expanded root surface area and thus nutrient uptake capacity increases (Marschner 1995; Pinton et al 1999; Cesco et al 2002). A small part of lower molecular weight components in HS can be taken up by plants. With the hormone-like function, HS may increase cell membrane permeability. Addition of organic material may increase plant growth due to the effect of HS on nutrient in the soils (Chen & Aviad 1990). Positive effects of HS on plant growth, yield and plant nutrient uptake can be supported by the previous findings of numerous researchers (Erdal et al 2000; Pilanalı & Kaplan 2003; Çelik et al 2008; Morard et al 2011; Tahir et al 2011; Çimrin et al 2013; Cunha et al 2015). Although there are many studies showing the positive effect of HS on plant growth and plant nutrient uptake, negative or no effects of HS have been reported (Tahir et al 2011; Leventoglu & Erdal 2014). Rauthan & Schnitzer (1981) indicated that more than 300 mg kg^{-1} of FA showed reducing effect on plant growth and nutrition uptake and these effects was below the control treatment at the levels of 1500 and 2000 mg kg^{-1} FA. Similarly, reduction of plant growth has been observed at the higher dose of HS applications (Chen & Aviad 1990). Nikbakht et al (2008) reported the non-significant effect of high levels of HS on fresh and dry weights of leaves. At the same study, nutrient concentrations increases with lower HA, but the higher levels of HA negatively affected some nutrient concentrations.

This study aimed to investigate the effects of HS applications on yield and mineral nutrition of Granny Smith and Jersey Mac apple varieties.

2. Material and Methods

The experiment was conducted as a field experiment for two consecutive years, 2012-

2013 at Horticultural Research Institute, Egirdir, Isparta-Turkey. The experimental soil was clayey loam (Bouyoucos 1951) having pH of 7.74 (1:2.5 soil to water ratio), 3% $CaCO_3$, 3.35% organic matter (Jackson 1962), 34 mg kg^{-1} $NaHCO_3$ extractable P (Olsen et al 1954), 565, 4811, 1235 mg kg^{-1} 1N NH_4OAC exchangeable K and Ca and Mg (Knudsen et al 1982). DTPA extractable Fe, Cu, Zn and Mn concentrations (Lindsay & Norwell 1978) were 14, 13, 5 and 14 mg kg^{-1}, respectively. As basal fertilization, 30 kg ha^{-1} N, 42.2 kg ha^{-1} P, 40 kg ha^{-1} K were applied using ammonium nitrate, mono ammonium phosphate and potassium nitrate. Thirteen year-old Granny Smith (GS) and Jersey Mac (JM) apple varieties grafted on MM106 which are planted as 3.0x3.5 m were used as plant materials. As humic substance, "TKİ HUMAS" containing 12% HA+FA (pH: 12) was used. The experiment was planned according to randomized blocks with 5 replications and each replicate consisted of one tree. For soil application (S) 4 levels of humic substances (-HS, S1, S2 and S3) corresponding to 0, 50, 100 and 200 kg ha^{-1} were given to each tree root zone around the tree canopy. As foliar application (L), 2% of HS was applied three times with one week intervals in June. Soil applications were made in early spring. Applications were repeated in the second years.

In order to determine leaf nutrient concentrations, samples were collected from the four sides of trees from the present year's shoots (Bergmann 1992). Then, samples were brought to laboratory and washed with water, dilute acid (0.2 N HCl) and distilled water. Later, samples were dried at 65±5 C^o for 2 days. Afterwards, samples were dried, grounded and wet digested with microwave oven. Total N was determined according to Kjeldahl method. Leaf P concentration was measured spectrophotometrically (Shimadzu UV-1208, 430 nm), K, Ca, Mg, Fe, Cu, Zn, and Mn concentrations were determined using atomic absorption spectrophotometer (Kacar & İnal 2008). Harvest of fruits was performed three times for JM in July and once for GS in October. Fruit weight, height and width were measured by digital scale and caliper with 20 randomly selected fruit from each tree. Fruit flesh firmness was detected from the two equatorial points of the fruit using hand penetrometer with 11.1 mm probe. Soluble solids content was measured using a digital refractometer. Pomological characteristics of the Jersey Mac apple fruits were conducted on the second harvest. Data was subjected to statistical analysis using Co Stat statistical software and the means were grouped using DUNCAN test.

3. Results

Although application of humic substance had no significant effect on fruit yield for both years, slight increases in fruit yield were recorded (Table 1). While the lowest yield was obtained from the control treatments for both varieties and two years, yields showed increment up to 19% and 8% for JM and GS cultivars, respectively. In general, fruit yields, fruit weights, heights and widths of JM and GS varieties were not affected from HS applications for both years, but in the second year fruit heights were negatively affected from soil+leaf applications (Table 2). According to the first year results, HS application did not affect fruit flesh firmness, soluble solids and pH for both varieties. However, fruit flesh firmness and soluble solids in JM variety were affected

Table 1- Effects of HS applications on yield

Applications	*Yield (kg tree^{-1})*			
	First year		*Second year*	
	JM	*GS*	*JM*	*GS*
-HS	26.8	40.0	27.8	41.8
S1	28.0	42.0	29.0	44.0
S2	28.9	42.4	30.0	44.4
S3	30.3	43.0	31.4	45.0
L	30.5	42.4	31.6	44.4
S1+L	31.6	43.2	32.8	45.2
S2+L	31.1	42.0	33.0	44.0
S3+L	31.2	41.6	32.4	43.6

JM, Jersey Mac; GS, Granny Smith

Table 2- Effects of HS applications on fruit weight, height and width

Applications	*Weight (g)*		*Height (mm)*		*Width (mm)*	
	JM	*GS*	*JM*	*GS*	*JM*	*GS*
	First year					
-HS	111	220	65	81	54	73
S1	122	210	68	80	56	70
S2	127	196	69	78	57	68
S3	132	219	69	80	58	71
L	124	225	69	82	52	70
S1+L	120	190	68	70	56	61
S2+L	111	191	65	73	53	67
S3+L	134	196	69	77	58	67
	Second year					
-HS	130	211	67	79 a*	56	70
S1	115	194	64	77 a	57	79
S2	126	205	68	79 a	58	69
S3	130	205	69	78 a	59	71
L	119	217	68	81 a	59	82
S1+L	127	201	68	69 d	61	73
S2+L	138	180	70	74 b	62	67
S3+L	120	202	66	73 c	58	64

JM, Jersey Mac; GS, Granny Smith; *, no significant differences between the same letters (P>0.05) in the same column; for each column, the numbers without letters indicate non-significance

Table 3- Effects of HS applications on fruit flesh firmness, soluble solids and pH

Applications	*Firmness (lb)*		*Soluble solids (%)*		*pH*	
	JM	*GS*	*JM*	*GS*	*JM*	*GS*
	First year					
-HS	7.7	8.7	9.8	9.3	3.1	2.1
S1	7.2	8.7	9.5	11.7	3.1	2.2
S2	7.2	9.1	9.1	11.4	3.0	2.2
S3	6.6	8.5	10.1	11.6	3.0	2.2
L	7.3	8.6	9.6	11.5	3.0	2.6
S1+L	7.6	9.4	9.8	12.9	3.0	2.3
S2+L	7.3	9.4	9.2	12.7	3.0	2.3
S3+L	7.2	8.3	9.9	11.5	3.0	2.3
	Second year					
-HS	6.2 b*	7.4	10.8 a	14.0	3.2	2.1
S1	7.1a	8.8	9.5 c	14.5	3.2	2.2
S2	6.8 a	7.4	9.1 c	13.5	3.3	2.2
S3	6.8 a	8.7	10.1 b	14.0	3.3	2.2
L	7.2 a	8.1	10.7 a	13.4	3.1	2.5
S1+L	5.9 b	7.9	10.7 a	13.4	3.1	2.3
S2+L	5.3 c	7.9	11.0 a	13.8	3.2	2.3
S3+L	6.0 b	8.3	10.6 a	13.8	3.1	2.3

JM, Jersey Mac; GS, Granny Smith; *; no significant differences between the same letters in the same column (P>0.05); for each column, the numbers without letters indicate non-significance

significantly from the applications at the second year (P<0.05). While individual effect of soil and leaf applications had a positive effect on fruit flesh firmness, negative effect was observed by combine application of soil and leaf. Soil applications had a negative effect on soluble solids amount, but leaf and soil+leaf combinations were ineffective. In the second year, HS applications did not affect flesh firmness, soluble solids and pH of GS apples (Table 3).

Effects of HS application on leaf N, P, K, Ca and Mg concentrations are summarized in Table 4. As can be seen from the table, only leaf N and K concentrations of JM were affected from the applications at the first year. In this variety, leaf N concentrations increased with the leaf and soil+leaf applications. In the second year, leaf N concentration of both varieties were affected positively from HS applications and leaf N concentrations of JM and GS varieties increased about 34% and 25% respectively (P<0.05).

For both varieties, soil+leaf combinations gave the best results in terms of leaf N concentrations generally. Leaf K concentration was affected positively from HS applications (P<0.05). For first year, it was observed that S2 and S3 applications and their combinations with leaf application had the higher effects than the other applications in JM variety. Furthermore, K concentration of GS

Table 4- Effects of HS applications on N, P, K, Ca and Mg concentrations of leaves (%)

Applications	*N*		*P*		*K*		*Ca*		*Mg*	
	JM	*GS*	*JM*	*GS*	*JM*	*GS*	*JM*	*GS*	*JM*	*GS*
					First year					
-HS	2.33 c*	2.36	0.27	0.25	1.33 b	1.35	1.28	0.73	0.35	0.27
S1	2.37 c	2.20	0.28	0.24	1.37 b	1.33	1.26	0.85	0.35	0.25
S2	2.38 c	2.27	0.28	0.25	1.50 a	1.30	1.38	0.89	0.33	0.26
S3	2.44 b	2.34	0.29	0.26	1.46 a	1.29	1.29	0.91	0.32	0.27
L	2.64 a	2.25	0.28	0.25	1.33 b	1.43	1.31	0.96	0.32	0.27
S1+L	2.55 a	2.03	0.27	0.23	1.40 b	1.21	1.09	0.74	0.32	0.24
S2+L	2.56 a	2.13	0.29	0.25	1.49 a	1.25	1.28	0.78	0.31	0.27
S3+L	2.52 a	2.29	0.29	0.24	1.51 a	1.30	1.29	0.81	0.29	0.26
					Second year					
-HS	1.46 c	1.50 c	0.29	0.20	1.46	0.58 b	1.63 a	1.09 c	0.42 a	0.26
S1	1.47 c	1.55 c	0.31	0.21	1.48	0.68 b	1.35 c	1.10 c	0.30 d	0.25
S2	1.84 a	1.72 b	0.33	0.28	1.44	1.06 a	1.87 a	1.26 b	0.36 b	0.29
S3	1.76 b	1.53 c	0.33	0.29	1.51	0.95 a	1.64 a	1.29 b	0.35 b	0.29
L	1.72 b	1.71 b	0.32	0.26	1.48	0.96 a	1.70 a	1.29 b	0.38 a	0.27
S1+L	1.86 a	1.87 a	0.30	0.25	1.55	0.96 a	1.34 d	1.30 b	0.33 c	0.25
S2+L	1.91 a	1.82 a	0.29	0.28	1.58	1.06 a	1.45 b	1.33 b	0.34 b	0.27
S3+L	1.96 a	1.83 a	0.29	0.25	1.57	1.15 a	1.56 b	1.38 a	0.38 a	0.26

JM, Jersey Mac; GS, Granny Smith; *; no significant differences between the same letters in the same column (P>0.05); for each column, the numbers without letters indicate non-significance

variety was not influenced from applications at the first year. Second year, effect of HS on K nutrition of GS was quite noticeable. As seen from the table, leaf K concentration of GS increased up to 2 fold with HS applications (except S1). Potassium concentration in JM variety was not affected from HS in this year. Additionally, in the second year, leaf Ca concentrations showed considerable increase with HS applications in GS variety. For leaf Ca and Mg concentrations of JM, it can be said that whether the effects of HS were similar to control or the effects of them were negative mostly. Effects of HS applications on Fe concentration of both apple variety in the first and the second years were significant (P<0.05). As can be seen from the first year's results, leaf Fe concentrations were the lowest at control treatments (-HS). However, leaf Fe concentrations with HS, especially soil+leaf applications, increased remarkably for GS and JM varieties. Effects of HS on Cu, Zn, Mn and B concentrations of two apple varieties were not significant in the first year. In the second year, leaf Fe concentrations increased with the some of the HS applications. In this year, leaf and soil+leaf applications had higher effects than that of others on leaf Fe concentration. Another important finding in the second year is that Zn concentration of GS showed noteworthy increment with soil+leaf combinations. Leaf Cu, Mn and B concentrations were not influenced significantly in this year (Table 5).

Table 5- Effects of HS applications on Fe, Cu, Zn, Mn and B concentrations of leaves (mg kg^{-1})

Applications	*Fe*		*Cu*		*Zn*		*Mn*		*B*	
	JM	*GS*	*JM*	*GS*	*JM*	*GS*	*JM*	*GS*	*JM*	*GS*
					First year					
-HS	73 c*	76 b	12	11	29	15	17	22	36	34
S1	75 c	79 b	10	10	28	14	15	25	36	31
S2	82 b	78 b	11	10	27	14	14	22	38	33
S3	83 b	82 b	11	10	26	13	15	23	37	32
L	83 b	81 b	11	10	26	13	14	23	37	31
S1+L	92 a	95 a	11	10	26	14	14	35	39	29
S2+L	93 a	89 a	11	10	25	13	14	25	40	33
S3+L	95 a	91 a	11	11	26	14	15	26	39	31
					Second year					
-HS	77 c	105 b	9	8	25	20 b	19	20	43	30
S1	76 c	100 b	7	8	19	20 b	21	20	35	30
S2	81 b	130 a	8	7	24	20 b	23	20	39	31
S3	75 c	109 b	7	7	18	22 b	18	22	38	30
L	92 a	124 a	8	6	19	23 b	23	23	39	30
S1+L	91 a	134 a	8	7	20	27 a	18	27	40	28
S2+L	86 b	132 a	7	7	20	27 a	21	27	39	29
S3+L	93 a	134 a	8	8	25	30 a	20	30	41	30

JM, Jersey Mac; GS, Granny Smith; *; no significant differences between the same letters in the same column (P>0.05); for each column, the numbers without letters indicate non-significance

4. Discussion

Except for the second year's fruit height of GS, humic substance applications did not affect fruit yield and quality parameters statistically for both years (P>0.05). Although relative yield increases were recorded for both varieties for two years, these increases were not significant. The effect of HS applications on mineral nutrition of apple varieties varied with the years. While only N, K and Fe were affected positively from HS applications in the first year, Ca and Zn were added to these nutrients in the second year. From these results it can be said that HS applications were more efficient in the second year. These results may be related to increment of organic matter content and mineralization at the second year (Demir & Çimrin 2011). In addition chelating properties of HS on some metals can play a role in increasing of Zn and Fe in leaf (Fallahi et al 2006). One reason for increasing of leaf K concentrations in leaves can be due to K concentrations that come from the KOH used for HS production. Increasing of Ca concentration in the leaves of GS variety can be explained with the promotion of Ca uptake with increasing of K uptakes by plants (Özkan & Yaman 2009). Some nutrient concentrations in leaves did not change with the HS applications. Similar results were found with the study conducted by Leventoglu & Erdal (2014) and they explained the ineffectiveness of HS under some soil conditions. Focusing on the nutrient concentrations of trees, it is clear that both varieties showed different respons to HS applications. As explained before, different plant varieties or even different genotypes of same variety can vary in terms of nutrient uptake even they grow in the same environment (Tsipouridis & Thomidis 2005; Jimenez et al 2007; Küçükyumuk & Erdal 2009; Küçükyumuk et al 2015). Although some nutrient concentrations of plants did not increase, residual nutrients in the soil decreased. One of these results can be related to

dilution of nutrient in plant tissues due to increased vegetative growth with HS applications (Kolsarıcı et al 2005). The other reason may be transforms of nutrient to the insoluble forms as organo-mineral complex due to soil HS applications (Strickland et al 1979; White & Chaney 1980; De Nobili et al 2002). Moreover, in some studies it is indicated that HS can compete with the nutrients for root uptake (Chen & Aviad 1990).

Humic materials are generally applied to the soil, and affect the some physical, chemical and microbiological properties of it. However as found in this study, foliar sprays of these substances under field conditions increased the concentrations of N, K, Fe and Zn in leaves (Fernandez-Escobar et al 1996). Additionally, Brownell et al (1987) and Katkat et al (2009) indicated that spraying of leonardite extracts promoted the growth of tomato, cotton, grape and wheat. This growth promoting functions of HS, may be due to plant hormone-like materials in the HS (O'Donnell 1973; Casenave de Sanfilippo et al 1990).

In conclusion, effect of HS showed different behaviors on nutrients availability thus nutrient concentrations in plants. Although HS applications did not have positive effect on leaf P, Mg, Mg, Cu, Mn and B concentrations, leaf N, K, Ca, Fe and Zn concentrations were affected positively from the HS applications. The shape and degree of influence of HS on mineral nutrition of apples showed variation depending on the variety. Even though there was no statistically effect; proportional yield increases were obtained from both varieties with HS applications.

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Effects of Waiting Period before Milking on Orotic, Uric and Hippuric Acid Contents of Milks from Shami and Kilis Goats

Zehra GÜLER[a], Mahmut KESKİN[b], Ahmet DURSUN[a], Sabri GÜL[b], Zühal GÜNDÜZ[b], Süleyman Ercüment ÖNEL[c]

[a]*Mustafa Kemal University, Faculty of Agriculture, Department of Food Engineering, Hatay, TURKEY*

[b]*Mustafa Kemal University, Faculty of Agriculture, Department of Animal Science, Hatay, TURKEY*

[c]*Mustafa Kemal University, Samandag Vocational School, Hatay, TURKEY*

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Corresponding Author: Zehra GÜLER, E-mail: zguler@mku.edu.tr, Tel: +90 (326) 245 58 45
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ABSTRACT

The organic acids present in milk in varying quantities can reflect the health condition of the animals and the nutritional quality of milk. In rural areas, goats are maintained in pasture during whole day and milking is being started as soon as goats arrive to farm. However, it is not known whether milking during feeding as soon as goats return from pasture or after 1 hour-waiting have any effect on nitrogen-containing organic acids are the body metabolism products. Therefore, in this study we objected to determine the effects of 1 hour-waiting period before milking on orotic, uric and hippuric acids of milks from "Shami (Damascus)" and "Kilis" goat breeds during lactation period. The trial was carried out with 40 goats. The 20 goats from each breed were randomly separated to two groups at equal number. Control and experimental groups of the both breeds were milked during feeding as soon as goats return from pasture and after 1 hour-waiting following feeding, respectively. The milk samples taken with interval 30 days from May to October were used for organic acid analysis at a reverse phase high performance liquid chromatography. During lactation hippuric acid was the most abundant organic acid, followed by orotic and uric acids. Experimental group of Kilis goats had the highest level of hippuric acid. Orotic acid was higher in Shami goats than that in Kilis breed. Uric acid was the highest in control group of Shami breed. The 1 hour-waiting period before milking resulted in a significant decrease in uric acid. In general, orotic and uric acid decreased towards the end of lactation whereas hippuric acid markedly increased in the last 3 months of lactation. It was concluded that the 1 hour-waiting before milking after returning from pasture may be especially suggested to Shami goat raisers due to the low uric acid content of the milk.

Keywords: Lactation; Goat breed; Organic acids containing non-protein nitrogen

Sağım Öncesi Bekleme Süresinin Şam ve Kilis Keçi Sütlerinin Orotik, Ürik ve Hippürik Asit İçeriği Üzerine Etkisi

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Zehra GÜLER, E-posta: zguler@mku.edu.tr, Tel: +90 (326) 245 58 45
Geliş Tarihi: 20 Ekim 2016, Düzeltmelerin Gelişi: 10 Ocak 2017, Kabul: 24 Ocak 2017

ÖZET

Değişen miktarlarda sütte bulunan organik asitler, hayvanın sağlık durumuna ve sütün kalitesine işaret edebilir. Kırsal alanlarda keçiler tüm gün boyunca merada otlatılmakta ve çiftliğe vardıklarında hemen sağıma başlanmaktadır. Ancak, hayvan meradan döner dönmez sağımın ve bir süre bekledikten sonra sağımın, vücut metabolizmasının ürünleri olan azot içeren organik asit içeriği üzerine herhangi bir etkiye sahip olup olmadığı bilinmemektedir. Bundan dolayı çalışmamızda laktasyon boyunca Şam ve Kilis keçi sütlerinde orotik, urik ve hippurik asit içerikleri üzerine sağım öncesi beklemenin etkilerinin araştırılması amaçlanmıştır. Deneme 40 keçi ile gerçekleştirilmiştir. Her bir ırktan 20 keçi rastgele eşit sayıda iki gruba ayrılmıştır. Kontrol ve deneme keçileri sırasıyla meradan döndükten hemen sonra yemleme sırasında ve 1 saat bekletmeden sonra sağılmışlardır. Mayıs ayından Ekim ayına kadar 30 gün aralıklarla alınan örnekler ters faz yüksek performanslı sıvı kromatografisinde organik asitlerin analizi için kullanılmıştır. Laktasyon boyunca hippürik asit en çok bulunan organik asit olmuş, onu orotik ve ürik asitler izlemiştir. Deneme grubundaki Kilis keçileri en yüksek hippürik asit içeriğine sahip olmuşlardır. Şam keçi sütleri Kilis keçi sütlerinden daha fazla orotik asit içermiştir. Sağım öncesi bekletme ürik asit içeriğinde önemli bir azalma sağlamıştır. Genellikle orotik ve ürik asit laktasyon sonuna doğru azalırken hippurik asit laktasyonun son üç ayında önemli bir artış göstermiştir. Sonuçta düşük ürik asit içeriğinden dolayı, keçi yetiştiricilerine özellikle Şam keçileri için meradan döndükten sonra sağım öncesi bekletme uygulaması önerilebilir.

Anahtar Kelimeler: Laktasyon; Keçi ırkı; Protein olmayan azot içeren organik asitler

1. Introduction

Due to the small fat globules and the differences in casein fraction and minor components, goat milk and its products have a higher digestive property than cows' milk (Jesse et al 1980; Slacanac et al 2010). This may be essential for babies and elders. Orotic, uric and hippuric acids, are carboxylic acids containing nitrogen, contribute to the non-protein nitrogen (NPN) pool of mammalian milk (Carver & Walker 1995; Gil & Uauy 1995). Their presence in milk is assumed to originate from either lactational cellular secretion, post-secretory metabolism in milk, and/or transfer across the blood-milk barrier (Robinson 1980; Schlimme et al 2000). Additionally, the various factors such as animal breed, species, feeding, milking time and the lactation period affect the organic acid content of milk (Buiarelli et al 2003; Carpio et al 2010). Goat milk had higher levels of uric and hippuric acid than cow and sheep milk whereas had lower orotic acid (Mahdi et al 1990; Indyk & Woollard 2004). The attention on nitrogen-containing organic acids of milk has recently been widely increased due to their potential physiological and nutritional importance to neonates and also technological importance to dairy products.

Orotic acid having neither phosphorus nor ribose is known as uracil-6-carboxylic acid and an intermediate pyrimidine nucleotides synthesis such as uridine nucleotide (Carver & Walker 1995). Thereby it is a component of all cells. Orotic acid is an essential growth factor of *Lactobacillus* subp. *bulgaricus* using for the fermented dairy products production (Tamime & Robinson 2001). With respect to physiological, orotic acid can decrease the level of cholesterol in human due to its interfering with the endogenous synthesis of cholesterol (Anastasi et al 2000). Like orotic acid, uric acid is the acid-soluble nucleotide of milk and a principal degradation product of the purine nucleosides adenosine, inosine, and guanosine (Robinson 1980; Resmini et al 1990). Increases in uric acid content of milk can indicate to increased rumen microbial protein synthesis in ruminant livestock as a microbial marker (Belenguer et al 2002). However, in animal metabolism uric acid is biodegraded to ammonia by urease and allantoicase enzymes. Such enzymes are lack in the human metabolism and uric acid is excreted in urine though the kidneys. If uric acid is elevated in the human body fluid as a result of consuming high uric acid containing-foods, it may cause gout disease (Najafpour 2015). On the other

hand, uric acid can increase the oxidative stability of milk and dairy products due to its potential antioxidant property (Ostdal et al 2000), which is an advantage for dairy technology. Both orotic acid and uric acid may be suitable indicators for the determination of the proportion of milk added to foods.

Hippuric acid (benzoyl glycine) is produced in mammals from the metabolism of benzoic acid. Carpio et al (2010) claimed that hippuric acid could be considered as a marker of feeding regime, which was markedly higher in milk from organically fed goats than conventionally fed ones. The concentration of hippuric acid in milk could also be an indicator for the health condition of the animals because gut microflora in animals produces hippuric acid (Buiarelli et al 2003). As mentioned above, the individual organic acid containing nitrogen has the different nutritional and technological properties.

Although several authors have examined orotic, uric and hippuric acids of goat milk taking into consideration various factors such as the effects of different lactation stages, organic and conventional feeding (Johke & Goto 1962; Gil & Medina 1981; Carpio et al 2010; 2013), no data in literature is about the effects of goat breed and waiting period before milking on orotic, uric and hippuric acid contents of milk. Johansson et al (1999) reported that the feeding to cows during milking compared to before and after milking resulted in higher milk production and higher protein and lactose yields, and also an increase in oxytocin and somatostatin secretion which are hormones relating to milk production. Goat raisers believe that the waiting after returning from the pasture result in an increase in milk yield. However, it is not known whether feeding to goats before and during milking after returning from pasture has any effect on orotic, uric and hippuric acids of milk from the different goat breeds. Therefore, the object of this study was to determine the effects of 1 hour-waiting period before milking on concentrations of orotic, uric and hippuric acids in milk from 'Shami' and 'Kilis' goat breeds during lactation period.

2. Material and Methods

2.1. Milk samples

The study was carried out with Shami (Damascus) and Kilis goats raised in Yalaz Village of Hatay Province. Twenty goats (3-4 years old) from each breed were randomly separated to two groups at equal number which were described as control and experimental. The goats were grazed on pasture during whole day and additionally concentrate feed (1 kg per goat day^{-1}) having 2600 kcal ME and 160 g crude protein kg^{-1} was offered to all the goats after returning from pasture. All the goats were milked by hand. Control group goats were immediately milked when reaching to the farm. The experimental group goats were milked after 1 hour-waiting. Milk samples were taken separately from the goats individually with interval 30 days from May to October. After milking, milk samples taken from two goats in each milking day were mixed at the equal volume and a 500 mL milk sample was analyzed for organic acid. In this manner, the five samples from each group at each lactation month were made and then the samples were immediately transferred to laboratory at Food Engineering Department, Mustafa Kemal University using ice boxes. A total of 20 milk samples at each analysis time were used for organic acid analysis.

2.2. Organic acid analysis

The organic acids (orotic, uric and hippuric) in milk were analyzed according to the procedure described by Guler (2014) with slight modifications. For this purpose, a 40 mL of 5 mM H_2SO_4 was added to 10 mL of milk. After mixing, the samples were centrifuged at 5697×*g* for 7 min at 5 °C. The upper layer was filtered using a filter paper (Whatman No. 1). The filtrate was filtered again through 0.45 µm syringe filters (Millex PVDF Millipore, Billerica, MA, USA). This filtrate was injected onto the high performance liquid chromatography (HPLC-20 AD Prominence, Shimadzu, Kyoto, Japan) with an ion exchange column (Aminex HPX-87 H, 300×7.8 mm, BIO-RAD, Hercules, CA, USA) since this

technique has recently been widely used for the analysis of components of the NPN fraction of milk (Indyk & Woollard 2004). Separation and determination of organic acids were carried out at an isocratic flow rate of 0.6 mL min^{-1} at 60 °C using 5 mM sulfuric acid as a mobile phase and at 210 nm with an UV/VIS detector (SPD-20 AV, Shimadzu, Kyoto, Japan), respectively.

Orotic acid, uric acid and hippuric acid standard (Sigma-Aldrich GmbH, Steinheim, Germany) solutions were prepared in 0.1 N NaOH at the eight different concentrations as the working solutions. As shown in Table 1, linear regression curve-based peak areas were calculated for the individual organic acid. As the linearity of the method is 0.999 for each organic acid it was suitable for quantification.

Table 1- Regression equations for the calibration curves and analysis of the linearity

Acids	*Range (mg L^{-1})*	*Regression equation (y= ax+b)*	R^2	*r*	*RSD* of *f (%)*
Orotic	3.2813-420	$y=9.87023.10^{-6}$ x - 1.86361	0.9996	0.9998	7.22
Uric	3.0625-392	$y=1.49714.10^{-5}$ x - 1.58686	0.9998	0.9999	5.92
Hippuric	3.3906-434	$y=1.25897.10^{-5}$ x + 2.40497	0.9993	0.9996	5.57

y, concentration; a, slope; x, response; b, intercept; R^2, coefficient of determination; f, response factor (f was calculated by dividing the area under the peak obtained in the chromatogram and the corresponding concentration); RSD, relative standard deviation

2.3. Statistical analysis

The experiment was a (2x2x6)x5 factorial design of two breeds (Shami and Kilis), waiting period (Control and experimental group) and lactation months (May, June, July, August, September, October). The General Linear Model (GLM) was applied to all data using SPSS statistical program (Version 22.00, SPSS, IBM, NY, USA). The paired comparisons of means were made using the Duncan's Multiple Range Test ($P<0.05$).

3. Results and Discussion

3.1. Validation of method

In order to the validation (accuracy and precision) of analytical method, recovery studies were carried out. Accuracy refers to how close a particular measure is to the true value. Precision is a measure of how reproducible. The firstly, the milk samples without adding standard were analyzed. The secondly, the spiked samples were analyzed. For this purpose, the standard solution (5 levels) of mix of orotic acid, uric acid and hippuric acid with the known amounts were added to the five milk samples (Table 2). For the each sample, concentration was calculated using the calibration curves (Table 1). Recovery values obtaining were close to 100% for all of organic acids (Table 2), which indicated a good accuracy. The precision of the method or its repeatability were expressed as the coefficient of variation (CV) for 10 replications of standard recovery at different concentrations (Table 2) for each organic acid. The coefficient of variation was between 2.75 to 5.02% which indicated a good repeatability. As a rule, a CV below 5% is considered acceptable, although it depends on the type of analysis. A chromatogram sample for the organic acids of goats' milk, standard organic acids used for calibration and spiked samples is shown in Figure 1.

3.2. Organic acids

To our best knowledge, this study is the first report on the effects of 1 hour-waiting period before milking on orotic, uric and hippuric acids in goat milk at the different lactation stages of the Shami and Kilis goat breeds. Therefore comparative data are scarce. As shown in Table 3, orotic acid in milk samples analyzed ranged from 18.09±1.00 mg L^{-1} to 130.57±3.80 mg L^{-1}, which was significantly

Table 2- Percentage recovery of organic acids added to milk and repeatability (as coefficient of variation) of the method

Organic acid	*Replicate*	*Added organic acid (mg L^{-1})*	*Original organic acid in milk (mg L^{-1})*	*Organic acid recovered (mg L^{-1})*	*Recovery of added organic acid*
	1	5.50	5.12	11.05	107.92±0.51
	2	12.39	3.64	15.59	96.44±0.02
Orotic	3	26.09	2.19	28.87	102.27±0.02
	4	51.79	1.56	54.89	102.98±1.06
	5	105.73	4.69	113.65	103.05±0.75
Mean					102.53±4.08
Repeatability*					3.97%
	1	5.24	2.97	8.67	108.84±0.03
	2	11.62	2.85	14.50	100.19±1.93
Uric	3	24.30	4.36	27.79	96.44±0.02
	4	48.23	2.89	50.26	98.22±0.95
	5	100.41	6.91	104.59	97.28±0.44
Mean					100.19±5.03
Repeatability*					5.02%
	1	8.88	10.35	18.59	92.83±0.03
	2	14.51	7.83	21.45	93.85±0.12
Hippuric	3	27.48	7.40	33.58	95.26±0.08
	4	50.29	7.32	56.36	97.51±1.78
	5	101.29	19.89	120.44	99.27±1.36
Mean					95.74±2.64
Repeatability*					2.75%

*, as coefficient of variation

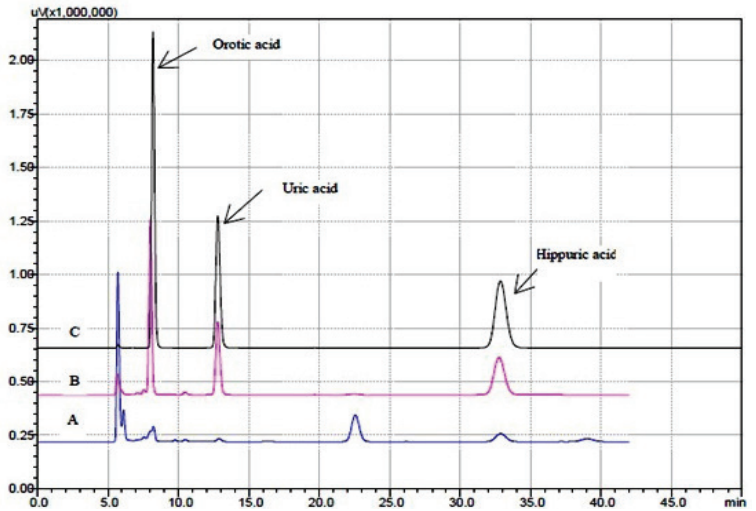

Figure 1- Chromatogram sample for milk (A), organic acid standard (B) and milk added organic acid (C)

(P<0.001) decreased towards the end of lactation. This trend was consistent with the findings of Jesse et al (1980) and Indyk & Woollard (2004). Decreasing in orotic acid may be due to the increases in fat content (data not shown) since there was an inverse relationship between fat and orotic acid contents of cows' milk (Anastasi et al 2000). Although the minimum value of orotic acid was within ranges (10-22 mg L^{-1}) reported by other researchers (Gil & Medina 1981; Indyk & Woollard 2004; Wehrmüller et al 2008), the maximum values obtained from lactation months between May and August were by far high. The changes in goats' metabolism and feed regime depending on lactation period may have favored high orotic acid production from May to August. The 1 hour-waiting period before milking and interaction between 1 hour-waiting before milking and lactation period had no effect on the mean concentration of orotic acid from both breeds whereas the interaction between breed and 1 hour-waiting period had a significant (P<0.001) effect on its. Independently of lactation period and 1 hour-waiting period before milking, breed had a significant (P<0.001) effect on orotic acid. Shami goats had higher orotic acid content (76.69±6.67 mg L^{-1}) than that (66.56±4.94 mg L^{-1}) of Kilis breed. This could be attributed to the differences in goats' amino acid metabolism depending on genotype since amino acids glutamine and aspartate are precursors for orotic acid synthesis (Brosnan & Brosnan 2007). The effect of interaction among lactation period, 1 hour-waiting period before milking and breed on orotic acid content was found to be statistically significant (P<0.001). While milk from experimental Kilis breed group at September month had the lowest level of orotic acid, milk from control Shami breed group at May month had the highest. In light of the present observations, it is probably that orotic acid content of goats' milk is markedly influenced by lactation stage and breed rather than the 1 hour-waiting period before milking. With respect to high orotic acid content, goat milks obtained from first- and mid-lactation terms may be more valuable for nutritional purposes.

Regarding on uric acid, it was significantly (P<0.001) affected by the interaction among lactation period, breed and 1 hour-waiting before milking. As shown in Table 3, uric acid was found in the range of 31-95 mg L^{-1}. This was markedly higher than that (10-26 mg L^{-1}) reported by Larson & Hegarty (1979) for goat milk stored at 4 °C or -18 °C. Becker (1993) reported that the cold storage could lead to a decrease in uric acid content of milk since it is converted to allantoin and glyoxylate at the cold conditions. In this study, the conversion may not be carried out as being used fresh raw goat milk. At July and August months the highest concentrations of uric acid in milk samples analyzed were obtained. This may be due to the conversion of xanthine to uric acid by xanthine oxidase which has a relatively high activation rate at high environmental temperatures (Fox & Kelly 2006). On the other hand, the high level of uric acid may be related to an increase in microbial nitrogen yield in duodenum of goats at high seasonal temperatures as reported by Tas & Susenbeth (2007). Regardless of the lactation period and 1 hour-waiting period before milking, the mean concentration of uric acid with a value of 61 mg L^{-1} was significantly (P<0.05) higher in milk from Shami breed than that (54 mg L^{-1}) from Kilis one. This may be due to the physiological differences as previously reported by Indyk & Woollard (2004). Independently lactation period and breeds, there was observed a significant (P<0.001) decrease in uric acid of experimental group compared to control group. This may be due to decrease in intake energy with the rest after returning from pasture since a significant increase in uric acid excretion in cows' milk with increase in energy intake was observed by Giesecke et al (1994). With respect to technological, high uric acid level may play an important role for milk products as an antioxidant but it may be indicator for poor hygienic quality of milk and also may carry a risk for health due to gout, urolithiasis and possible cardiovascular diseases.

Hippuric acid contents of all milk samples from both breeds showed a significant (P<0.001) variation during lactation whereas they were fluctuated as increase or decrease. A similar

Table 3- The effect of waiting before milking in Shami and Kilis goats on concentrations of nitrogen-containing organic acids (mg L^{-1}) in milk during lactation (n= 5)

Organic acids	*Lactation month*	*Shami*			*Kilis*				
		Control†	*Experimental*‡	*1P*	*Control*	*Experimental*	*1P*	*2P*	*3P*
Orotic	May	130.57±3.80^{a}	97.28±6.96^{ab}	**	72.72±5.97^{a}	97.23±8.16^{a}	*	***	ns
	June	111.68±5.27^{b}	82.73±13.12^{b}	ns	76.33±3.15^{a}	100.30±5.53^{a}	**	ns	ns
	July	94.30±7.45^{c}	109.90±7.06^{a}	ns	71.03±3.68^{a}	91.25±3.34^{a}	**	***	*
	August	84.66±6.16^{c}	77.66±7.21^{b}	ns	67.19±5.14^{ab}	72.73±4.83^{b}	ns	ns	ns
	September	24.54±1.70^{d}	32.96±3.45^{c}	ns	21.65±1.84^{c}	18.09±1.00^{d}	ns	***	ns
	October	38.57±4.26^{d}	35.37±2.65^{c}	ns	55.12±4.49^{b}	49.46±6.63^{c}	ns	**	ns
	Mean	80.72±7.27	72.65±6.09	ns	60.86±3.92	72.27±5.95	ns	***	ns
P value for lactation		***	***		***	***			
Uric	May	84.86±2.58^{b}	47.63±6.36^{ab}	***	44.52±3.80^{b}	44.11±4.58^{c}	*	ns	ns
	June	74.79±3.09^{b}	47.33±3.71^{ab}	***	59.01±4.16^{a}	58.55±5.63^{ab}	ns	ns	*
	July	95.71±3.60^{a}	62.56±8.87^{a}	**	71.74±4.47^{a}	67.48±3.84^{a}	**	ns	***
	August	82.31±2.51^{b}	46.19±3.26^{ab}	**	71.35±4.70^{a}	55.82±4.00^{abc}	**	ns	***
	September	58.84±2.43^{c}	38.78±3.40^{b}	**	32.34±2.84^{b}	31.20±1.21^{d}	***	*	ns
	October	42.22±6.37^{d}	54.80±4.87^{ab}	*	63.07±6.47^{a}	53.54±1.49^{bc}	*	ns	ns
	Mean	73.12±3.57	49.55±2.45^{ab}	***	56.79±3.19	51.72±2.65	**	*	***
P value for lactation		***	*		**	**			
Hippuric	May	112.61±2.63^{d}	73.96±5.68^{d}	***	70.72±14.87^{de}	80.31±5.20^{e}	ns	*	ns
	June	79.90±5.94^{de}	54.87±3.86^{d}	**	96.19±15.07^{d}	120.99±2.11^{d}	**	***	ns
	July	66.33±5.28^{e}	66.26±6.55^{d}	ns	62.15±12.03^{e}	83.63±2.02^{e}	**	ns	ns
	August	208.73±9.31^{b}	192.90±7.17^{c}	ns	181.29±16.57^{c}	215.49±11.8^{c}	*	ns	ns
	September	356.65±26.20^{a}	361.88±8.60^{a}	ns	406.08±45.05^{a}	339.37±16.07^{b}	ns	ns	ns
	October	155.48±16.76^{c}	235.04±13.21^{b}	**	216.36±12.32^{b}	395.36±11.26^{a}	***	**	**
	Mean	163.28±19.02	164.15±20.92	ns	170.61±124.59	199.32±23.06	*	*	*
P value for lactation		***	***		***	***			

†, control indicates the goats milked immediately after returning from pasture; ‡, experimental indicates the goats milked following 1 hour-waiting after returning from pasture; means in the same column followed by different letters are significantly different (at *, P<0.05; **, P<0.01; ***, P<0.001); [1]P, significance between control and experimental groups of each breed at each month; [2]P, significance between breeds irrespectively the control and the experimental groups; [3]P, significance between control and experimental groups at each month irrespectively goat breed

tendency was observed for Czech White Shorthaired breed (Hornickova et al 2014). Hippuric acid was significantly (P<0.001) influenced by interaction among 1 hour-waiting period before milking, breed and lactation period. Milk from experimental Shami breed group at June month had the lowest level of hippuric acid with a value of 54.87±3.86 mg L^{-1} but milk from control group of Kilis breed at September month had the highest hippuric acid value with 406.08±45.05 mg L^{-1}. Regardless of 1 hour-waiting period before milking and breeds, August, September and October months of lactation favored high hippuric acid production compared with May, June and July months. This could be attributed to the variations in feeding regime and goats' physiological as progressing lactation. Carpio et al (2010) reported

that hippuric acid is a marker of feeding regime and is significantly high in milk from organically fed goat. The 1 hour-waiting period before milking for Kilis goat breed resulted in a significant increase in hippuric acid but it had no significant effect on Shami goat breed. Regardless of 1 hour-waiting period before milking and lactation period, hippuric acid with a mean of 185 mg L^{-1} was significantly ($P<0.05$) higher in milk from Kilis breed than that (164 mg L^{-1}) in milk from Shami breed. This could be attributed to the mainly differences in genotype since the other conditions were almost identical. The mean result for hippuric acid were within ranges (72.59-188.96 mg L^{-1}) reported by Carpio et al (2010) for milk samples from organically fed goats but it was higher than values (5.78-25.11 mg L^{-1}) reported by Hornickova et al (2014) for milk from Czech White Shorthaired goat breed. This could be attributed to the differences in genotype and in feed regime between our studies and theirs.

Briefly, orotic, uric and hippuric acids were significantly influenced by the interaction among period of lactation, breed and 1 hour-waiting period before milking. The waiting period before milking had not effect on orotic acid only.

4. Conclusions

Determination of orotic, uric and hippuric acids in milk by HPLC method had a good repeatable for both breeds. These organic acids were significantly affected by the interaction among lactation period, 1 hour-waiting period before milking and breed. As conclusion; 1) lactation stages of the breeds affected the orotic, uric and hippuric content of milk produced in all groups, 2) irrespectively 1 hour-waiting before milking and lactation period, breed differences affected the nitrogen-containing organic acids of milk, 3) milk from Kilis goat breed had higher hippuric acid than that from Kilis goat breed whose milk contained more orotic acid and uric acid and 4) one hour-waiting before milking of goats is resulted in low uric acid level in milk that may have an advantage for human health. However, more studies are needed to elucidate the effects of waiting before milking on the other milk components.

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Comparison of Hens Reared in Free-Range and Deep-Litter Systems in Terms of Certain Production Characteristics

İsmail TÜRKER[a], Sezai ALKAN[b]

[a]*Bozok University, Faculty of Agriculture, Department of Animal Science, Yozgat, TURKEY*

[b]*Ordu University, Faculty of Agriculture, Department of Animal Science, Ordu, TURKEY*

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Corresponding Author: Ismail TÜRKER, E-mail: iturker37@hotmail.com, Tel: +90 (354) 242 10 28
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ABSTRACT

The study was carried out compare the hens reared in free range system and deep-litter system in terms of certain production characteristics. A total of 300 Lohmann Brown hens, with 150 hens in each of the groups were used in the study. In the free range system, 4 m^2 grazing ground was allocated for each hen. The hens were taken into research henhouse at 16 weeks of age and production characteristics were determined up to 52 weeks of age. It was determined that out of the characteristics focused in the study, there is no significant difference between the groups in terms of viability, 50% production age, egg weight, hen-day egg production, feed efficiency, feed consumption and body weight at 18 week of age, whereas there is a significant difference in terms of final body weight. In the study, it was concluded that hens in the deep-litter system gained more body weight compared to those in the free-range system but the other characteristics were not affected by the husbandry systems.

Keywords: Free range system; Deep-litter system; Egg production age; Feed efficiency; Body weight

Serbest ve Altlıklı Yer Sisteminde Yetiştirilen Tavukların Bazı Verim Özelliklerinin Karşılaştırılması

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: İsmail TÜRKER, E-posta:iturker37@hotmail.com, Tel: +90 (354) 242 10 28
Geliş Tarihi: 27 Aralık 2016, Düzeltmelerin Gelişi: 30 Ocak 2017, Kabul: 30 Ocak 2017

ÖZET

Araştırma serbest sistem ve altlıklı yer sisteminde yetiştirilen tavukların bazı verim özellikleri bakımından karşılaştırılması amacıyla yürütülmüştür. Her yetiştirme sisteminde 150 adet olmak üzere toplam 300 adet Lohmann Brown tavuk kullanılmıştır. Serbest sistemde tavuk başına 4 m^2 otlatma alanı ayrılmıştır. Tavuklar 16 haftalık yaşta araştırma kümesine alınmış ve 52 haftalık yaşa kadar verim özellikleri belirlenmiştir. Araştırmada üzerinde durulan özelliklerden yaşama gücü, % 50 verim yaşı, yumurta ağırlığı, tavuk-gün yumurta verimi, yem tüketimi, yemden yararlanma oranı ve dönem başı canlı ağırlığı bakımından gruplar arasında farklılık bulunmadığı, dönem sonu canlı ağırlığı bakımından ise önemli farklılık olduğu belirlenmiştir. Araştırmada altlıklı yer sistemindeki tavukların serbest sisteme göre daha fazla canlı ağırlık kazandıkları, ancak diğer özelliklerin yetiştirme sistemlerinden etkilenmediği sonucuna varılmıştır.

Anahtar Kelimeler: Tavuk; Serbest sistem; Altlıklı sistem; Yumurta verimi; Yem tüketimi

1. Introduction

Most consumers prefer eggs produced through alternative production systems that apply food safety regulations since animal rights started to be considered important in egg production systems (Anderson 2009). There are various alternative production systems available such as free range, organic, enriched cage, aviary and deep-litter systems.

Hen strains and husbandry systems are effecting the egg quality traits (Doley et al 2010; Angelovičová et al 2014; Nistor et al 2014; Yang et al 2014; Hanusová et al 2015; Nistor et al 2015).

It has been determined that the strains used in production shown different reaction to husbandry systems (Leyendecker et al 2001). It has been reported by Doley et al (2010) that the body weight of hens reared in deep-litter systems are higher than those reared in the free range and semi-intensive systems. Hill (1986) stated that the egg eating habit is more common in deep-litter systems compared to aviary, strawyard, perchery and free range systems.

It is observed that producing eggs in the cage system is more hygienic and economic compared to the other systems, however, it creates the disadvantage of limited movements for hens. It has been reported that the eggs produced through cage system have darker yellow yolks and they are heavier in weight and the hens have lower mortality, feed consumption, body weight, dirty and cracked egg rate (Wegner 1982). Sekeroglu et al (2010) emphasized that husbandry systems have to be studied in detail.

Since egg production is important in alternative production systems in terms of both variation and animal welfare, the effects of these systems on productivity and quality should be investigated in detail. Although various studies on the subject exist, they are not sufficient.

In this research, it was aimed to compare hens reared in deep-litter systems and free range systems in terms of certain productivity traits.

2. Material and Methods

A total of 300 Lohmann Brown hens, with 150 hens in each of the groups were used in the study. The study was carried out with a research henhouse in Ordu city located in the Black Sea Coast which has a humid and warmish climate in the summer and temperate and rainy climate in the winter. This research was conducted between July 2015 and July 2016. In the free range system, a green area of 4 m^2 was allocated for each hen out of the henhouse. Green area is consist of *Trifolium repens, Trifolium pretense, Lolium perenne, Festuca rubra, Urtica dioica, Bellis perennis and Primula spp.* Five hens were placed in each meter square in henhouse. The chemical composition of feed materials was given in Table 1. The hens were placed in henhouse at 16 weeks of age. In the study, the lighting period was applied as 13 hours up to 18^{th} week and after the 18^{th} week it was stabilized at 16 hours with 1 hour increment per week increase. The water and feed were provided ad libitum. This study was conducted in accordance with the ethical rules concerning the animal rights in Turkey.

2.1. Measurements

2.1.1. Body weight at 18 week of age

Body weight of all hens in the research groups were weighted individually at 18 weeks of age using a scale with 0.01 g sensitivity.

2.1.2. Egg production age

Egg production ratio was calculated based on the age (day) of the hens reached 50% egg production.

2.1.3. Egg production

By considering the eggs produced in each replication, hen-day % was calculated.

2.1.4. Egg weight

From periods of 4 weeks, 20% of the eggs from each reproduction were selected randomly and weighed using a scale with 0.01 g sensitivity.

Table 1- Chemical compositions of feed material

Nutrients	*0-3 weeks*	*4-10 weeks*	*11-16 weeks*	*17-52 weeks*
Dry matter, min (%)	88.0	88.0	88.0	88.0
Crude ash, max (%)	8.0	8.0	8.0	8.0
Crude protein, min (%)	19.0	18.0	16.0	17.0
Metabolic energy, min (kcal kg^{-1})	2900	2800	2700	2800
Calcium, min-max (%)	1-1.2	1-1.1	0.9-1.0	3.5-4.0
Available phosphorus, min (%)	0.45	0.42	0.40	0.40
Lysine, min (%)	1.15	0.98	0.72	0.75
Methionin, min (%)	0.55	0.47	0.35	0.47
Methonin+cystein min (%)	0.85	0.76	0.58	0.78
Triptophan, min (%)	0.20	0.19	0.17	0.20
NaCl, min-max (%)	0.35-0.50	0.35-0.50	0.35-0.50	0.35-0.50
Crude cellulose, max (%)	4.5	5.0	6.0	6.0
Linoleic acid, min (%)	1.5	1.25	1.0	1.7
A vitamin (IU kg^{-1})	13000	13000	10000	12000
D_3 vitamin (IU kg^{-1})	3000	3000	2000	2500
E vitamin (mg kg^{-1})	20.0	20.0	20.0	20.0
K_3 vitamin (mg kg^{-1})	2.0	2.0	2.0	2.0
B_2 vitamin (mg kg^{-1})	5.0	5.0	5.0	5.0
B_{12} vitamin (mg kg^{-1})	0.02	0.02	0.01	0.01
Niacin (mg kg^{-1})	60.0	60.0	30.0	25.0
Mangan (mg kg^{-1})	100	100	100	60.0
Zinc (mg kg^{-1})	70.0	70.0	70.0	40.0
Iron (mg kg^{-1})	40.0	40.0	40.0	40.0
Cupper (mg kg^{-1})	7.0	7.0	7.0	7.0
Selenium (mg kg^{-1})	0.2	0.2	0.2	0.2
Cobalt (mg kg^{-1})	0.5	0.5	0.5	0.5

2.1.5. Feed consumption

Feed consumption was calculated with Equation 1.

Feed consumption (g)= (Given feed (g) - remaining feed (g))/(Number of hens x day) (1)

2.1.6. Feed efficiency

Feed efficiency was calculated with Equation 2.

Feed efficiency= Total given feed (kg)/Total produced eggs (kg) (2)

2.1.7. Final body weight

The body weights of all hens in the research groups were weighted individually at 52 weeks of age using a scale with 0.01 g sensitivity.

2.1.8. Viability

Viability was calculated from 18 to 52 weeks considering the dead hens at each replication with Equation 3.

Viability (%)= (Number of dead hens)/(Number of remaining hens) x 100 (3)

2.2. Statistical analysis

For all traits included in the study, the control of normal distribution was done by using Kolmogorov-Smirnov test. T-test was used in the evaluation of the traits which fulfill the assumptions. For the data expressed as rates and %, angle transformation was applied.

3. Results and Discussion

As a result of the evaluation of the data obtained in the research, findings regarding the body weight of beginning of period, 50% egg production age, egg production and egg weight are presented in Table 2. Feed consumption, feed efficiency, viability and final body weight values are presented in Table 3. Out of the traits considered in the study, it was determined that there is no significant difference in terms of body weight at 18 week of age, 50% egg production age, hen-day egg production, egg weight, feed efficiency and viability between husbandry systems ($P>0.05$), whereas there is a significant difference in terms of final body weight ($P<0.05$).

Since the hens had been reared in deep-litter system until they were transferred into the research henhouse a difference is not expected in terms of body weight at 18 week of age between husbandry systems. However Malik & Singh (2010) have reported that there are important differences between husbandry systems in terms of body weight at 18 week of age. In the study, the hens have similar body weight at 18 week of age showed that the hens used in this study were homogeneous. The hens carried similar traits in regard to reaching 50% production age in respect to husbandry systems in this study. In a study by Sekeroglu et al (2010) which using Atak-S hybrids were used, the 50% production age was reported to be 168.75 days in deep-litter system and 160 days in free-range system. In both husbandry systems, the hens had similar egg production traits. On the contrary, this could be regarded as on important for animal welfare. The free range system provides an environment where hens can move more freely compared to the deep-litter system. Senčic´ & Butko (2006) have reported that the egg production rate of hens reared in free range system is lower than that of those reared in cage system. On the other hand, Sekeroglu et al (2010) have reported that the egg production in deep-litter, free-range and cage systems are 96.44, 118.08 and 111 respectively, whereas Pavlovski et al (1992) have reported that with Isa Brown hens in a production period of 72 weeks in the same order egg production is 291, 255 and 248.

In our study, it was determined that the husbandry systems do not affect egg weight. However, Doley et al (2010), found that egg weight is higher in deep-

Table 2- Body weight at 18 week of age, 50% egg production age, hen-day egg production and average egg weight

Groups	*Body weight at 18 week of age*	*50% production age (day)*	*Hen-day egg production (%)*	*Average egg weight(g)*
	$\overline{X} \pm S_{\bar{x}}$	$\overline{X} \pm S_{\bar{x}}$	$\overline{X} \pm S_{\bar{x}}$	$\overline{X} \pm S_{\bar{x}}$
Free-range	1389.70±9.47	161.63±1.45	81.31±2.38	61.91±0.55
Deep-litter	1389.90±13.60	160.50±0.50	81.66±2.41	61.36±0.66
P	0.986	0.692	0.922	0.522

Table 3- Feed consumption, feed efficiency, viability and final body weight

Groups	*Feed consumption (g)*	*Feed efficiency*	*Viability (%)*	*Final body weight (g)*
	$\overline{X} \pm S_{\bar{x}}$	$\overline{X} \pm S_{\bar{x}}$	$\overline{X} \pm S_{\bar{x}}$	$\overline{X} \pm S_{\bar{x}}$
Free-range	131.82±1.23	2.39±0.05	95.39±3.76	1893±46.7
Deep-litter	131.83±2.77	2.48±0.06	93.24±1.35	2050±42.9
P	0.995	0.263	0.694	0.017

litter system, and Pavlovski et al (1992) pointed out that egg weights differ in cage, deep-litter and free range systems. Senčic´ & Butko (2006) reported that eggs in free range system are heavier than those in cage system whereas Torges & Matthes (1975), Wegner (1982), Pavlovski et al (2004), Clerici et al (2006) and Samiullah et al (2014) reported that eggs produced in free range system are lighter than those produced in cage system. It is believed that different findings reported in different studies could be due to the fact that the free range systems have not reached a standard structure like the other systems.

The husbandry systems focused in the study have affected feed consumption and feed efficiency similarly. Pointing out that husbandry systems are effective on feed consumption, Sekeroglu et al (2010) stated that in Atak-S hens feed consumption in deep-litter, free-range and cage systems are 157.21, 146.7 and 134.33 g respectively, Pavlovski et al (1992) reported that feed consumption for each egg in cage, deep-litter and free range system are 172, 166 and 178 g. In the study by Senčic´ & Butko (2006) it was found that hens reared in the free range system consume more feed compared to those reared in cage system. Although it was not reported in studies, it was predicted that there could be differences in findings also in terms of feed efficiency. In this study, that no difference was found in terms of feed consumption and feed efficiency could be due to the fact that green grazing grounds provided in the 4 m^2 area allocated to each hen. Moreover, the final body weights of hens reared in the deep-litter system was found to be higher than those reared in the free range system. It was predicted that, this stems from the fact that hens accommodated in the deep-litter system move less compared to the hens in the free range system. It was thought that, no difference was found in terms of feed consumption and feed efficiency between husbandry systems because the body weight of hens in the deep-litter system is higher than those in the free range system.

In a study conducted by Doley et al (2010) hens reared in the deep-litter system gain more body weight compared to those reared in the free range and semi intensive systems. This result supports the research findings of the present study. However, Pavlovski et al (1992) reported that the body weight of Isa Brown hens reared in cage, deep-litter and free range systems after 72 weeks of production are 1810, 1866 and 1912 g respectively. This result contradicts with the findings of the present study.

The viability values of the hens used in the study through 52 week production period were not affected by the husbandry systems. In a study comparing deep-litter, free range and cage husbandry systems, Wegner (1982) reported that mortality was lower in the cage system. In their study compared cage, deep-litter and free range systems using Isa Brown hens. Pavlovski et al (1992) reported the death rates through a production period of 72 weeks 10.8%, 4.3% and 7.7%, respectively. In this study, the lowest mortality was observed in the deep-litter system.

In this study, there was no difference in yield characteristics between husbandry systems. It is thought that this is caused by the fact that hens fed on the same feed.

4. Conclusions

When the research results are evaluated, it is seen that there is not an important difference between the two husbandry systems in terms of production traits. In the study, the difference emerging in terms of final body weight has pointed to positive results in favor of free range system. Considering animal rights and consumer preference, it could be said that free range husbandry is a more suitable option provided that the conditions specified in the study are met. However, the climate conditions of the region should be primarily taken into account in the application of free range system. It would be more suitable for free range egg production husbandry to be applied in regions with temperate climatic conditions which are neither too cold or too hot and at the same time where green grass is found in open areas throughout the year.

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A Visual Assessment of Roadside Poplar Plantings in Turkey

Engin EROĞLU[a], Cengiz ACAR[b]

[a]*Duzce University, Faculty of Forestry, Department of Landscape Architecture, Düzce, TURKEY*

[b]*Karadeniz Technical University, Faculty of Forestry, Department of Landscape Architecture, Trabzon, TURKEY*

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Corresponding Author: Engin EROĞLU, E-mail: engineroglu@duzce.edu.tr, Tel: +90 (533) 648 89 78
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ABSTRACT

Roadside plantings along urban and rural road corridors are designed to create visual effects as well as to provide functional benefits such as shading, screening or routing. Considering their potential for daily public use, roads and road corridors, as significant visual impact centers, can attract people's attention and affect their points of view. Poplar species, which grow rapidly and easily along road corridors and can adapt to different ecological conditions swiftly, are used widely in both urban and rural landscapes in Turkey. The main objective of this study was to determine the visual structures dependent on the road-plant relationships of the poplar compositions that play an important role in shaping the roadside landscapes. For this purpose, compositions of poplar trees in selected urban and rural road corridors throughout Turkey were photographed and visual analysis techniques (VATs) were applied to determine visual preferences. Participants (n= 35) were shown 30 photographs, selected from the nearly 1000 taken, of road corridor poplar plantings and their evaluations were recorded in a questionnaire. In this survey, the people were requested to evaluate their appreciation levels towards the plantings as well as to express their other visual preferences and to assess visual quality. In addition, the association of all these evaluations with the demographic characteristics of the participants were determined. A correlation analysis was then performed to identify the relationships among all the visual assessments and a cluster analysis was conducted according to the visual status of the photographs in order to determine their groups. In the results of the study, the poplar trees, either individually or in the form of compositions, were reported to have a significant visual diversity. Moreover, the cluster analysis found the resulting three groups to be associated with leaf density, seasonal conditions and the trees as individuals or in groups. The seasonal variation factor in particular was shown to be visually effective in the poplar compositions. It was determined that visual perception of the poplar plantations differed in accordance with the demographic differences. This work established that poplar plantations as single trees or in groups and their proximity to the road altered visual preferences, and consequently, some suggestions were made concerning the use of poplars in roadside landscape planning.

Keywords: Poplar plantings; Plant compositions; Rural and urban roadside environments; Roadside vegetation; Visual assessment techniques (VATs)

Türkiye'deki Yol Kenarı Kavak Bitkilendirmelerinin Görsel Değerlendirmesi

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Engin EROĞLU, E-posta: engineroglu@duzce.edu.tr, Tel: +90 (533) 648 89 78
Geliş Tarihi: 18 Mart 2015, Düzeltmelerin Gelişi: 07 Şubat 2017, Kabul: 08 Şubat 2017

ÖZET

Kentsel ve kırsal yol koridorları bitkilendirmeleri hem görsel etkiler oluşturmak hem de gölge, perdeleme ve yönlendirme oluşturma gibi işlevlerle gerçekleştirilmektedir. Kullanım potansiyeli dikkate alındığında yol ve yol koridorları günlük yaşamında önemli bir görsel etki merkezi ve manzara noktası olarak da dikkat çekmektedir. Türkiye'de yol koridorlarında kolay yetiştirilme, hızlı büyüme, farklı ekolojik koşullara hızlıca adapte olabilme özellikleri ile kavak türleri hem kentsel hem de kırsal peyzajlarda yaygın bir şekilde kullanılmaktadır. Çalışmanın temel amacı kırsal ve kentsel yol kenarı peyzajlarının şekillenmesinde önemli bir rol oynayan kavak kompozisyonlarının yol-bitki ilişkilerine bağlı görsel yapılarını belirlemektir. Bu amaçla, Türkiye'deki bazı kentsel ve kırsal yol koridorlarındaki kavak kompozisyonları fotoğraflanmış ve görsel tercihlerinin belirlenmesinde görsel değerlendirme teknikleri (GDT) uygulanmıştır. Yol koridoru boyunca kavak bitkilendirmelerinin görsel değerlendirme teknikleri içerisinde alandan çekilen fotoğrafların değerlendirmeleri için anket çalışması gerçekleştirilmiştir. Alandan çekilen yaklaşık 1000 fotoğraf içerisinden seçilen 30 fotoğraf toplam 35 katılımcıya değerlendirtilmiştir. Bu anket çalışmasında kişilerin bitkilendirmelere yönelik beğeni düzeylerinin yanı sıra diğer görsel tercihleri ve görsel kaliteye yönelik değerlendirmeleri istenmiştir. Tüm bu değerlendirmeler kişilerin demografik yapıları ile de ilişkilendirilmiştir. Ayrıca tüm görsel değerlendirmelerin aralarındaki ilişkilerin belirlenmesinde korelasyon analizi ve fotoğrafların görsel durumlarına göre oluşturdukları grupların belirlenmesinde kümeleme analizi yapılmıştır. Çalışmanın sonucunda, tek tek ya da kompozisyonlar halinde bulunan kavak ağaçları önemli bir görsel çeşitliliğe sahip oldukları belirlenmiştir. Kümeleme analizinde ortaya çıkan sonuçlarda da ortaya çıkan üç grup, bitkilerdeki yaprak yoğunluğu, mevsimsel durum ve bitkilerin tek ya da gruplar halinde olmaları ile ilişkilidir. Özellikle, mevsimsel değişim kavak kompozisyonlarının görsel tercihlerinde etkili olduğu görülmüştür. Demografik farklılıklara göre kavak bitkilendirmelerinin görsel algısında da değişimlerin olduğu belirlenmiştir. Bitkilendirmelerin tek tek ya da gruplar halinde olması, bitkilerin yola olan yakınlık ve uzaklıklarının görsel tercihleri değiştirdiğinin belirlendiği bu çalışmanın sonucunda yol kenarı peyzaj planlamalarında kavakların kullanımına yönelik öneriler getirilmiştir.

Anahtar Kelimeler: Kavak bitkilendirmeleri; Bitki kompozisyonları; Kentsel ve kırsal yol kenarları; Yol kenarı vejetasyonu; Görsel değerlendirme teknikleri (GDT)

1. Introduction

Roadside corridors are extremely important ecologically in terms of both human activity and vehicle movement. When considered in this context, roadside tree populations, both natural and planted, have emerged as an important subject of study, especially the poplar species, which are capable of providing a link between rural and urban settings.

Trees are closest and most useful to people when they are close to the road environment, especially in urban areas. Alongside the ecological approach, there are other elements important for roadside corridor plantings, including the environmental aesthetics and visual beauty considerations discussed in their visual evaluation (Akbar et al 2003). It has been demonstrated that the visual quality of the landscape aesthetics affects motorists and their operation of vehicles (Fathi & Masnavi 2014). Plants are the most important element contributing to visual quality along the highway corridor. The presence of plants on the road and in the immediate vicinity of the road was found to be preferable to unnatural elements such as lighting, litter, and barriers (Froment & Domon 2006). Likewise, for road users, groups of trees along the corridor were preferred to other plant groups (Todorova et al 2004; Spooner & Lisa 2009; Weber et al 2014).

The poplar is easily adapted to both rural and urban settings and is often preferred because of its rapid growth. Poplars have a place in many areas of landscape planning and design work, including amenity plantings in parks, along roads and in residential gardens, public areas and green spaces, as well as in landscape restoration work and for wind screens and noise reduction (Çepel 1998; Görcelioğlu 2002; Yavuzşefik & Uzun 2005; Dirik 2008).

In Turkey, the poplar (*Populus* L.) is one of the plant species used in urban and roadside tree plantings. *Populus* L. in the family Salicaceae has six native taxa in Turkey (Yaltırık 1993). The poplar taxa are largely foreign to the culture in question. In particular, *Populus nigra* L. and its hybrids are the most widely grown poplar in and around cities (Ürgenç 1998). However, problems concerning the use of poplar trees in the city have sometimes arisen. Most of these debates remain in a narrow framework and deal with allergy issues caused by spring pollen and the cottony seeds. Both the positive and the adverse effects on health resulting from the presence of poplar species in urban environments must be evaluated, and the versatility of a landscape dominated by poplar must at the same time be considered.

On the other hand, fast-growing species like poplar and eucalyptus planted on the side of the road are responsible for causing cracks in asphalt pavements because the roots have developed close to the soil surface. Next to buildings, these trees can damage foundations. Therefore, fast-growing species should not be planted along narrow roads or next to buildings (Trowbridge & Bassuk 2004; Yılmaz 2008; Avşar & Ok 2010).

A number of studies have visually evaluated roadside and corridor plantings and reported elements of the visual preferences and values of effective visual quality such as plant species, plant age and size, proximity to the road, diversity and seasonal changes (Mader & Neubert-Mader 2004; Wolf 2006; Bulut & Yilmaz 2008; Eroğlu et al 2012; Fathi & Masnavi 2014; Demir & Eroğlu 2015). Hence, the present study aimed to determine the type of poplar plantations most visually preferred alongside roads in Turkey, especially in Anatolia. The parameters subjected to evaluation included visual preference and visual quality, potential of seasonal variation and proximity to the road.

Thus, the basic purposes of the study were to determine the visual value of roadside poplar plantings in Turkey and to utilize this research to design roadside landscapes, to determine the visual status of roadside poplar plantings, to compare visual preferences and visual values of roadside poplar plantings, to identify which visual descriptor groups affect visual preferences of roadside poplar plantings and to assess which landscapes and visual components are important for determining the visual effects of roadside poplar stands.

2. Material and Methods

2.1. Study areas

In selecting the study areas, the geographical region in Turkey of the roadside poplar planting sites was not taken into account. The most important reason for choosing these areas for the study was that they can represent different climatic conditions in Turkey, and poplar stands are most frequently found along the roadsides. Consequently, the research was carried out using roadside poplar plantings in the vicinity of the Turkish cities of Samsun, Antalya, Erzurum, Karabük, Ankara, Isparta and Kastamonu from February to September 2012. While Samsun and Antalya are coastal cities, Ankara, Isparta, Kastamonu, Karabük and Erzurum are located in the interior regions of Turkey (Figure 1). In selection of the road corridors that make up the research areas, rather than the systematic sampling approach, the conscious or random sampling of road corridors having poplar plantings was adopted. The dependent variables were kept simple and included topography, weather or road geometry. For this research, only the road-plant relationship was important.

Figure 1- Roadside poplar planting sites selected for the study

2.2. Methods

In the research, an analytical approach was adopted in order to determine the visual structure of the road corridor and to attempt to explain the road corridor-plant relationship. Many visual assessment techniques (VATs) for determining the visual value of a landscape are found in the literature, and include surveys, in situ observation, creation of observation maps and on-site assessment by participants. In this study, the survey approach was chosen for the visual evaluation of the study areas. For visual assessment especially, most studies are carried out using this method (Akbar et al 2003; Acar et al 2006; Demir & Eroğlu 2015)

2.2.1. Landscape photographs and participant selection

Many approaches have been employed for visual landscape assessment. The use of visuals depicting the area is a frequently referenced method in the determination of the visual value and quality of a landscape (Clay & Daniel 2000; Müderrisoğlu & Eroğlu 2006; Acar et al 2008; Eroğlu & Acar 2011; Eroğlu et al 2012; Acar et al 2013).

In this study, the visual landscape assessment of roadside plantings was accomplished by photographic analysis. Clay & Smidt (2004) examined the reliability of using color photos and slides to elucidate visual differences of landscape conditions and for the development of quantitative relationships between photo-based assessment and landscape attributes (Daniel & Vining 1983). The present study investigated different roadside poplar groups by representing the study sites in photographs. This procedure was selected in order to determine the roadside corridor structures and the visual qualities of the poplar plantings. The photos were intended to capture the most relevant features within each selected corridor.

In this study, a large number of landscape photographs were taken of poplar plantings along the roadsides in a number of sites near cities throughout Turkey using the Canon EOS 500D equipped with an 18-55 mm standard lens. The photos were taken between 10.00 a.m. and 2.30 p.m. The photography techniques implemented in this study correspond to those in the studies of other researchers (Clay & Smidt 2004; Acar et al 2006). Between 50 and 100 photos of each corridor were taken and a set of nearly 1000 samples in total was created. The 30 test photos were evaluated and selected by Turkish BSc, MSc and PhD landscape architects. In the study, a participatory survey approach was adopted for the photo evaluations. Rather than different user groups in the vicinity of the road corridor, a single user group was considered for evaluating several corridors (Acar et al 2006).

In order to determine whether the selected photographs were grouped according to the similarity of their visual landscape features, visual perception variables (natural, varied, impressive, eye-catching, harmonious, interesting and exciting) were used.

2.2.2. Survey of visual assessment

The visual values (VVs) of preferences for the roadside poplar plantings were rated using a participatory approach (Brown & Daniel 1990; Ode et al 2009). The 35 participants (90% community contribution level, 95% confidence interval and 85% confidence level) (Kalıpsız 1981) were randomly selected and consisted of 20 men and 15 women between the ages of 22 and 50 working in several Turkish universities as academic faculty. In order to ascertain whether any relation existed between visual preferences and participant demographic features, in the correlation analysis, age, gender, work and education were used as demographic features along with the visual values. The participants were shown the 30 test slides from the study sites and asked to rate each slide on a 7-point scale: -3(1), -2(2), -1(3), 0(4), 1(5), 2(6), 3(7), where a 1 indicated a very low visual value and a 7 indicated a very high one. Aided by the pictures, the visual preferences were assessed according to the semantic differential scale (SDS) proposed by Osgood et al (1957). This scale is used in the subject field of landscape and environmental psychology research and is carried out with pairs of 5 or 7 items (Özgen 1984; Acar & Gülez 2002).

In this study, correlations ($-1 \leq r < -0.9$-strong (-), $-0.9 \leq r < -0.5$-medium (-), $-0.5 \leq r < 0$-weak (-), $0.9 < r \leq 1$-

strong (+), 0.5<r≤0.9-medium (+), 0<r≤05-weak (+) and correlations significant at the 0.01 and 0.05 levels) between the rating levels of visual values and the assessment of semantic descriptors assumed some descriptors to be unique or independent information about preference for a scenic composition. The participants were surveyed using the semantic descriptors correlated with the visual values. In this way, the 30 test slides were presented and the participants used nine the descriptor variables along with visual preference and visual quality (Table 1).

2.2.3. Statistical analysis

A data matrix was completed in Excel, including VV preference scores, VV aims, semantic descriptors, landscape characteristics of each slide and participants' demographics. The data were analyzed using the SPSS (Statistical Package for Social Science) 16.0.

Quantitative and qualitative analytical techniques were used to analyze and interpret the data set extracted from the preferences scale system in the Excel Program; the data matrix was obtained by including the values of the preferences. Based on mean assessed values calculated from all values, the visual values were given to illustrate general patterns of planting preferences. The data matrix was subjected to basic descriptive statistics, analysis of variance and cluster analysis. In order to describe the explanatory variables of the corridor landscapes, a correlation analysis of the scale variables was carried out.

In the study, to differentiate the road corridors poplar views from each other, cluster analysis in SPSS 16.0 software was applied to determine common affairs point groupings. How the formation of groups were linked to the corridor-plant relationship and the possible significance were confirmed by cluster analysis. Cluster analysis was used to test whether poplar plantation corridors were connected or whether they had randomly formed a typology.

Of the hierarchical clustering techniques, the variance technique (Ward's method) was designated. This technique utilizes the total squared deviation and is based on the average distance from the observations found in the same cluster.

Cluster analysis is one of the multivariate analysis techniques and takes into account the basic features of an object or group of individuals and put them into groups. In other words, with cluster analysis, ungrouped data are grouped according to their similarities in order to provide cumulative information to researchers. Cluster analysis, by calculating the values of all the measured variables of the units seen in the research, groups the units

Table 1- Visual characteristics selected for study

Visual attributes	*General definitions*
Natural	- Directly correlated with landscape preferences. Nature is often appreciated more than man-made landscapes.
Varied	- Related to complexity, and defined as "the diversity and richness of a landscape and its features."
Impressive	- Related to the perceptual level of admiration shown to a landscape. The appreciation level of a landscape is a component affecting perception in a positive way.
Eye-catching	- Refers to landscapes that completely affect the perception level. Unremarkable landscapes do not convey a perceptual sense.
Harmonious	- Refers to all the elements of a landscape forming a whole, or also to each element being compatible with the others. It is a perceptual component that is more effective in directing the visual preferences.
Interesting	- Can refer to a landscape or composition found directly interesting, or else to the appreciation of the landscape. From time to time, the "interesting" designation can also show an inverse relationship with appreciation.
Exciting	- Refers unforgettable landscapes that are the most easily perceived by people. They are characterized by a high level of appreciation.

resembling each other in the same cluster. The analysis focuses on emerging groups and clusters; the obtained clusters are homogeneous in themselves, while being heterogeneous in relation to each other (Kalaycı 2010). Thus, cluster analysis is used to classify units or objects according to their basic features (Everitt et al 2001; Abonyi & Feil 2007).

Following this hierarchical cluster analysis, discriminant analysis was employed in order to test whether the levels of separation were sufficient. Discriminant or separation analysis is a multivariate statistical technique for dividing two or more previously classified groups. Observations taken from outside a group indicate the group to which it can be assigned. Cluster analysis, in statistical terms, creates groups which differ from each other. It does not state what criteria will be used to classify those members joining the groups later. Discriminant analysis learns how the cluster analysis units are grouped and can easily classify other units that would be joining the groups.

3. Results

3.1. Visual preferences

When the arithmetic mean of the images was calculated (Figure 2 and Table 2), the photos numbered 6 (4.66±1.51), 14 (4.60±1.58), 28 (4.69± 1.71), 29 (4.94±1.53) and 30 (4.57±1.40) had the highest values (Figure 3), while the photos numbered 2 (3.28±1.60), 12 (3.26±1.58), 15 (3.09± 1.96), 20 (3.00±1.70) and 21 (3.20±1.53) had the lowest (Figure 4). The photos and their descriptor groups were calculated according to their frequency. Accordingly, the most varied was photo number 29, the most impressive was 14 and the most eye-catching was 6, while the most harmonious, natural, interesting and exciting photo was number 8.

According to the results of the correlation analysis, all the semantic descriptor groups were influential in determining visual preferences (Table 3). As shown by the analysis, interesting and exciting were the visual preference pair determined to be the most effective at P= 0.588 and 0.01 significance levels, respectively. Roadside poplar plantings identified as exciting exhibited an effective visual preference.

As shown in Table 3, natural and varied had a positive relationship with visual preferences, while they showed no relation to visual quality levels. Overall, visual preferences related positively to natural, varied, impressive, eye-catching, harmonious, interesting and exciting, while visual quality levels related negatively to those same descriptors.

3.2. Effects of the socio-demographic characteristics of respondents

The demographics of the participants were examined when determining the visual preferences.

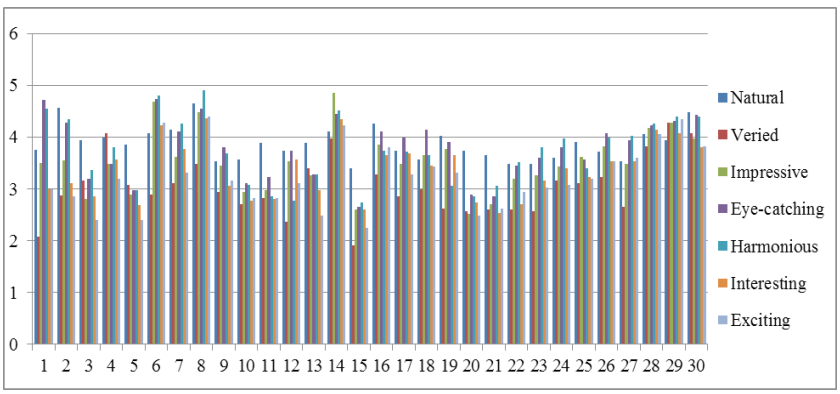

Figure 2- Relationship between images and semantic adjectives

Table 2- Arithmetic means of semantic meaning of photos

Photo numbers	*Natural*	*Varied*	*Impressive*	*Eye-Catching*	*Harmonious*	*Interesting*	*Exciting*	*Total mean*
1	3.75	2.08	3.50	4.72	4.56	3.00	3.00	3.52
2	4.56	2.88	3.55	4.28	4.34	3.11	2.86	3.65
3	3.94	3.17	2.80	3.20	3.37	2.86	2.40	3.11
4	4.00	4.09	3.49	3.49	3.80	3.57	3.20	3.66
5	3.86	3.09	2.89	2.97	2.97	2.69	2.40	2.98
6	4.09	2.89	4.69	4.74	4.80	4.23	4.29	4.24
7	4.14	3.11	3.63	4.11	4.26	3.77	3.31	3.76
8	4.66	3.49	4.49	4.54	4.91	4.37	4.40	4.41
9	3.54	2.94	3.46	3.80	3.69	3.06	3.17	3.38
10	3.57	2.71	2.94	3.11	3.09	2.77	2.83	3.00
11	3.89	2.83	2.97	3.23	2.86	2.80	2.83	3.06
12	3.74	2.37	3.54	3.74	2.77	3.57	3.11	3.27
13	3.89	3.40	3.26	3.29	3.29	2.97	2.49	3.22
14	4.11	3.97	4.86	4.46	4.51	4.34	4.23	4.36
15	3.40	1.91	2.60	2.66	2.74	2.60	2.26	2.60
16	4.26	3.29	3.86	4.11	3.74	3.66	3.80	3.82
17	3.74	2.86	3.49	4.00	3.71	3.69	3.29	3.54
18	3.57	3.00	3.66	4.14	3.66	3.46	3.43	3.56
19	4.03	2.63	3.77	3.91	3.06	3.66	3.31	3.48
20	3.74	2.57	2.51	2.89	2.86	2.74	2.49	2.83
21	3.66	2.60	2.71	2.86	3.06	2.54	2.63	2.87
22	3.49	2.60	3.20	3.46	3.51	2.71	2.94	3.13
23	3.49	2.57	3.26	3.60	3.80	3.17	3.03	3.27
24	3.60	3.17	3.43	3.80	3.97	3.40	3.09	3.49
25	3.91	3.11	3.63	3.57	3.40	3.23	3.20	3.44
26	3.71	3.23	3.83	4.09	4.00	3.54	3.54	3.71
27	3.54	2.66	3.49	3.94	4.03	3.54	3.60	3.54
28	4.06	3.83	4.17	4.23	4.26	4.14	4.06	4.11
29	3.94	4.29	4.29	4.31	4.40	4.09	4.34	4.24
30	4.49	4.09	3.97	4.43	4.40	3.80	3.83	4.14

Figure 3- High-rated photos

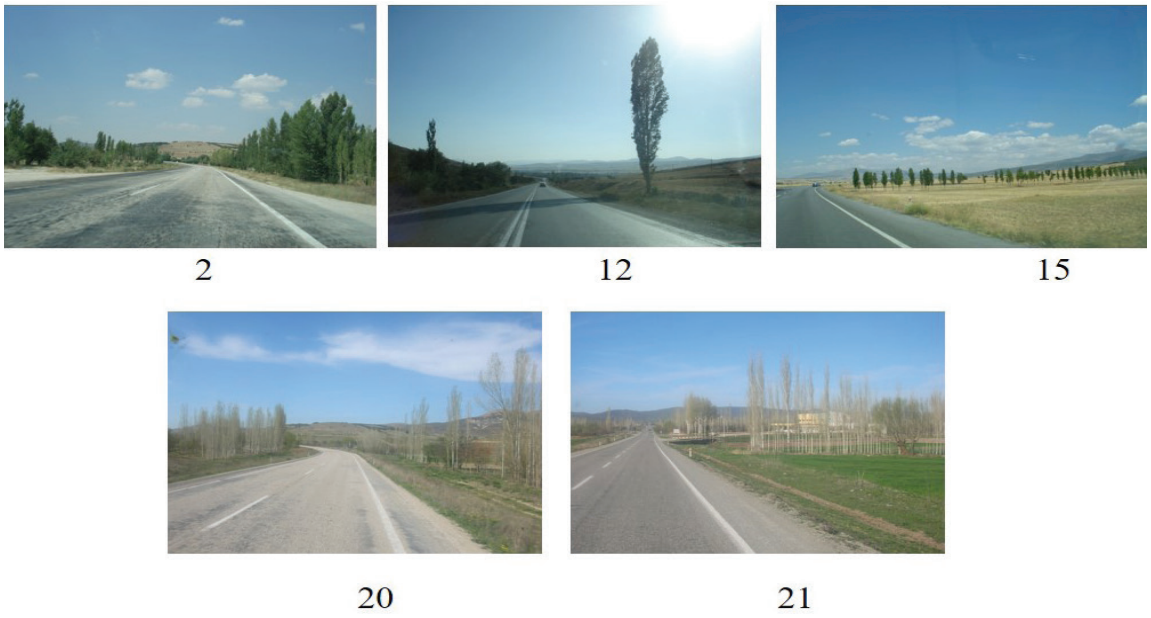

Figure 4- Low-rated photos

Table 3- Correlation analysis between visual values and adjective pairs

	Natural	*Varied*	*Impressive*	*Eye-Catching*	*Harmonious*	*Interesting*	*Exciting*
Visual preferences	0.25**	0.31**	0.58**	0.50**	0.52**	0.55**	0.59**
Visual quality levels	-0.02	-0.02	-0.16**	-0.13**	-0.12**	-0.18**	-0.16**

**, correlation significant at the 0.01 level; *, correlation significant at the 0.05 level

Accordingly, increasing age positively impacted the visual preference, while increase in the level of education adversely affected it. Moreover, the visual quality of the poplar plantings was observed to be related to age and gender (Table 4). As seen in Table 5, there was no relationship between descriptor groups and demographic features.

Table 4- Correlation analysis between visual values and demographic features

	Visual preferences	*Visual quality levels*
Age	0.14**	0.15**
Gender	-0.07*	0.15**
Education	-0.07*	-0.02
Work	0.06*	-0.06

**, correlation significant at the 0.01 level; *, correlation significant at the 0.05 level

Table 5- Correlation analysis between adjective pairs and demographic features

	Age	*Gender*	*Education*	*Work*
Natural	0.07*	0.19**	0.01	0.02
Varied	0.04	0.02	0.01	-0.04
Impressive	0.05	-0.02	-0.05	0.12**
Eye-catching	0.15**	0.01	0.10**	0.02
Harmonious	0.10**	0.03	0.07*	-0.05
Interesting	0.09**	-0.10**	0.00	0.10**
Exciting	0.11*	-0.04	0.04	0.05

**, correlation significant at the 0.01 level; *, correlation significant at the 0.05 level

3.3. Clustering the roadside environments based on visual attributes

In order to identify landscape visual perception values, the descriptors were divided into groups

according to the results of the cluster analysis. The first group included natural, eye-catching and harmonious, whereas the second one was comprised of varied, impressive, interesting and exciting (Figure 5).

The photos were then divided into three different groups (Figure 6) according to their visual and landscape perception values. Three homogeneous groups/clusters were obtained from the viewpoints of

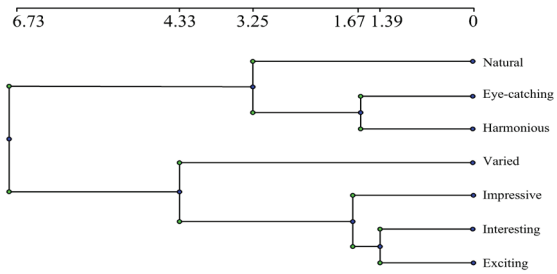

Figure 5- Cluster analysis of adjective pairs

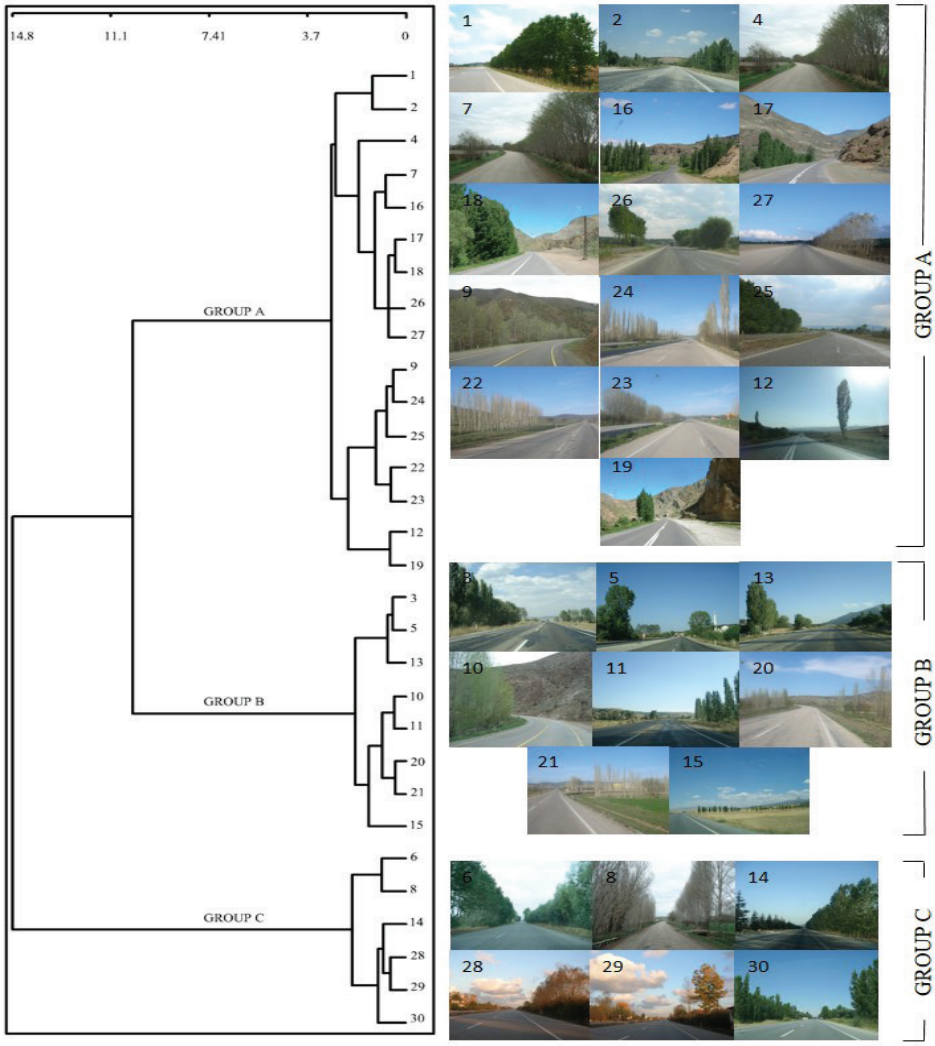

Figure 6- Cluster analysis of photos

35 participants measured on the basis of categorical variables for the 30 photographs.

According to the cluster analysis results; Group A included 1, 2, 4, 7, 9, 12, 16, 17, 18, 19, 22, 23, 24, 25, 26 and 27 (moderate grouping, moderate density, summer-autumn), Group B included 3, 5, 10, 11, 13, 15, 20 and 21 (less grouping (solitary), low density, autumn-winter) and Group C included 6, 8, 14, 28, 29 and 30 (greater grouping effect (more poplar in the composition), more plant and leaf density, spring-summer).

These clusters, especially the seasonal status of the poplar compositions, their density, and the case of a single species or groupings of trees, were observed to be effective.

We used discriminant analysis to test the accuracy of the resulting clusters in the cluster analysis. These three groups are shown in the discriminant function graph in Figure 7. An examination of Table 6 indicates the groups were correctly classified, with the high probability value of 93.4%. Thus, a consistent selection of visual and sensory parameters according to the populus viewpoint was demonstrated.

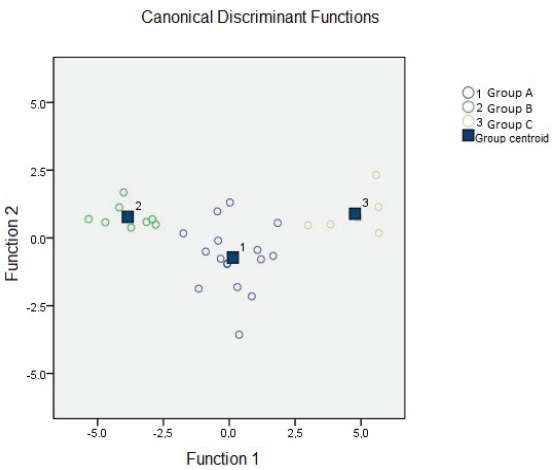

Figure 7- Discriminant analysis graph of the grouped photos

Table 6- Discriminant function analysis showing accuracy

Function	*Eigenvalue*	*% of Variance*	*Cumulative %*	*Canonical correlation*
1	9.466[a]	93.4	93.4	0.951
2	0.667[a]	6.6	100.0	0.633

4. Discussion

There is growing scientific evidence of the importance of the roadside landscape and the visual characteristics along the highways in terms of the perception of those who use the roads. As the landscapes become more interesting and the aesthetic quality of the roadside ambiance increases, relationships between the roadways and their surroundings may be more readily acknowledged (Acar & Gülez 2002; Cackowski & Nasar 2003; Froment & Domon 2006; Dell'Acqua & Russo 2010). Moreover, the roads and streets in urban spaces together with those in rural areas are known to be important elements of the transportation landscape (Acar & Günay 2014) and are credited with being new venues for bringing people and plants together (Pellegrini & Baudry 2014). Therefore, the aim of this study was to determine visual preferences for the composition of roadside poplar plantings in Turkey. The results have confirmed some significant relationships in the choice of planting poplar as the characteristic species in roadside landscape designs.

According to the findings obtained in this study, the most admired photographs, numbered 6, 14, 28, 29 and 30 have the common features of density and proximity to the road, while photographs 2, 12, 15, 20 and 21 picture remote and sparsely-spaced plantings. Indeed, in the studies of Mader & Neubert-Mader (2004), Wolf (2006) and Fathi & Masnavi (2014), it is stated that the plants closest to the road are much more appreciated and that the

increasing number of plants in the composition strengthen the visual impact.

In the assessments, there appears to be an intermediate level of naturalness for virtually all groups of photos. However, in the photographs numbered 2, 8, 16 and 30, where the poplar species are tall and close to the road and the area is effectively rural, the naturalness value is even higher. It is thought that naturalness values are not very high mainly because the trees are not naturally grown, but planted there, whereas the values are not very low because of the recognition factor. Atik et al (2013) stated that commonly recognized vegetation is associated with the feeling of naturalness.

Regarding the taste and visual assessment level of the photos made according to the results of the cluster analysis, in the emergence of the urban-rural relationship of the current groups, the increase in the number of trees and proximity to the road were seen to be effective. Therefore, plant group A was more urban, while group B included the more rural areas; the plantings of group B were sparse, while those of groups A and C were denser.

4.1. Roadside environment and trees as visual landscape elements

In the results of this study, it was observed that plants are more preferable when used in an effective manner that characterizes the road (Figures 3 and 4). This idea is compatible with environmental psychology studies in the literature because, according to the Gestalt theory, effective environments play a key role in human perception (Gürer 1990; Bell 2004; Güngör 2005). In this study, when the poplars close to the roadside were used in a manner to construct more mass-space and an effective environment, their preference levels rose. Additionally, according to evaluations using semantic descriptors for the relationship between road and plants, being sufficiently varied and eye-catching was important for the roadside environment (Figure 2). However, naturalness was also identified as an important element in the semantic evaluation because the roads in the study belonged to rural landscapes. On the other hand, road-dominated landscapes were less preferred by participants. In the correlation analysis in Table 3, road-dominated poplar plantings were qualified as exciting. According to Kaplan & Kaplan (1989), exciting is directly related to mysterious, so mysterious roads become exciting and their visual preference is increased. Therefore, in the study, varied, impressive, interesting and exciting were clustered together in one group and natural, eye-catching and harmonious in the other (Figure 5). This result is supported by previous studies (Akbar et al 2003; Clay & Smidt 2004; Fathi & Masnavi 2014) in which natural, eye-catching, varied and harmonious were indicated as identifying elements of visual preference for roadside vegetation, just as in this present research.

Serpa & Muhar (1999) stated that gender along with geographic and social origins affect how plants are perceived. Moreover, the gender, age, habitation and experiences of individuals also affect their visual preferences (Strumse 1996). As shown in the results of the present study, the visual preferences for poplar plantings were also related to age and gender.

Eroğlu et al (2012) reported that the potential of seasonal variations in plant composition affects visual preference positively. As a result of this study, it was also observed that higher values of visual preference were obtained with photograph groups which included much more seasonal variation and autumn coloring.

The types of plant composition are important, whether plants are used singly or in groups and plant density, and seasonal change in the visual perception and assessment of plant composition (Eroğlu & Acar 2011; Eroğlu et al 2012). In this study, the resulting situation was similar in character, especially with the cluster analysis.

The descriptor groups are significant visual assessment parameters for the explanation of components belonging to the landscape (Osgood et al 1975; Clay & Smidt 2004; Müderrisoğlu et al 2006; Ode et al 2009; Eroğlu et al 2012; Acar et al 2013). In the present study, the groups of descriptors were also effective in the determination of the value

of the poplar plantings in the visual landscape. The areas of high landscape value were those consisting of groups with intensive plantings and vegetation.

4.2. Poplar trees as tools for roadside greenery

Although roadside planting had its beginnings in ancient times, this type of planting activity took off during the Renaissance in Europe, where it continued to flourishing in the 17th Century and reached its peak in the 18th Century (Mader & Neubert-Mader 2004). In Turkey, roadside planting has been rapidly developing since the establishment of the Republic. Roadside poplar planting has been widely used by private individuals and public institutions up to the present day because of the ease in planting and the cheap maintenance costs of the poplar species.

According to Yılmaz (2012), poplar has many ecological and economic advantages. In addition to contributing to the visual enhancement of the rural Turkish landscape, the versatile poplar species should be given a place in planting design projects along with other native Turkish plant species. In this work, it can be seen that poplar exhibits one of the essential characteristics of natural landscape design by providing the important feature of linking rural and urban environments. Moreover, all these poplar plantings along roads in the cities and in the countryside represent the Anatolian cultural heritage of the people living there (Yılmaz 2012).

5. Conclusions

According to the findings of this study, poplars are important roadside trees for their rural-urban linking and ecological contributions as well as for their demonstrated natural effect and their visual diversity, effectiveness, and suppleness. The following conclusions were reached regarding a number of roadside poplar plantings in Turkey. Proximity to the road corridor and abundance of trees in the composition increase the level of visual appreciation of the roadside poplar species. As a commonly recognized species in rural areas, the roadside poplar is perceived as more natural. Roadside vegetation affects the visual value of landscape perception. In addition, this effect can vary according to demographic features. The richness, density and variety of roadside vegetation increase the visual landscape value and quality. As vegetation groups are found nearer to roadsides, visual landscape perception, quality and preference values rise. Poplars planted on roadsides should not create traffic hazards that endanger human safety. In determining visual preferences, the appreciation of roadside poplar plantings rose with the increasing age of the participants, while increasing educational levels affected appreciation in a negative way. In this case, the higher the education level, the more selective the participants were in their visual preferences. Interest and excitement were the most important factors in determining the visual preferences of the roadside poplar plantings. Poplar planting schemes and their seasonal variations affect visual preferences.

Thus, it was concluded that vegetation groups, especially roadside poplar plantings, have significant value in the visual landscape. This is particularly true of roadside vegetation including poplar plantings, which create an ecological corridor connecting the rural and urban landscapes.

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A Comparative Study on Age Determination of Carp (*Cyprinus carpio* Linnaeus, 1758) in Lake Eğirdir Using Otolith, Vertebrae and Scale Counts

Yıldız BOLAT[a], Abdulkadir YAĞCI[b]

[a]*Suleyman Demirel University, Faculty of Fisheries, Department of Fisheries and Seafood Processing Technology, Isparta, TURKEY*

[b]*Fisheries Research Institute, Egirdir, TURKEY*

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Corresponding Author: Yıldız BOLAT, E-mail: yildizbolat@sdu.edu.tr, Tel: +90 (543) 729 03 42
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ABSTRACT

This study, a comparative age determination study was carried out between May and November of 2013 in order to estimate most reliable bony tissue for ageing of the common carp in Lake Eğirdir. A total of 78 specimens aged between 1 and 11 were used in the study, having weights between 191 and 8685 g and fork lengths between 19.5 and 76 cm. Scales, vertebrae and otoliths were used by one reader via 5 replicates for ageing. Highest agreement among the bony tissues was found in otoliths (10.67%) and most reliable bony tissue was determined as otoliths for age determination in Lake Eğirdir carps.

Keywords: *Cyprinus carpio*; Age; Otolith; Vertebrae; Scale; Eğirdir Lake

Eğirdir Gölü Sazan (*Cyprinus carpio* Linnaeus, 1758) Balıklarında Otolit, Omur ve Pullardan Karşılaştırmalı Yaş Tayini Üzerine Bir Araştırma

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Yıldız BOLAT, E-posta: yildizbolat@sdu.edu.tr, Tel: +90 (543) 729 03 42
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ÖZET

Bu çalışma, Eğirdir Gölü sazan balıklarının yaş tayininde en güvenilir kemiksi dokunun belirlenmesi amacıyla karşılaştırmalı bir yaş tayin çalışması olarak Mayıs-Kasım 2013 tarihleri arasında gerçekleştirilmiştir. Çalışmada toplam 78 adet yaşları 1 ile 11, ağırlıkları 191 g ile 8685 g ve çatal boyları 19.5 cm ile 76 cm arasında değişen bireyler kullanılmıştır. Yaş tayini tespitinde pullar, omur ve otolitler kullanılmış ve 5 tekrarlı okuma yapılmıştır. Kemiksi yapılar arasında en yüksek uyum (% 10.67), otolit olarak belirlenmiş ve Eğirdir Gölü sazan balıklarında yaş tayini için en güvenilir kemiksi yapının otolit olduğu tespit edilmiştir.

Anahtar Kelimeler: *Cyprinus carpio*; Yaş; Otolit; Omur; Pul; Eğirdir Gölü

1. Introduction

Various bony structures including scales, otolith, vertebrae, fin rays and spines, opercular bones, cleithra have been utilized to estimate the age of different fish species (Das 1994; Worthington et al 1995; Göçer & Ekingen 2005; Khan et al 2011; Başusta et al 2014). There are numerous studies on usage of this practice for age determination of the common carp also (Lubinski et al 1984; Yerli 1997; Brown et al 2004; Balık et al 2006; Demirkalp 2007; Phelps et al 2007; Yılmaz & Polat 2008; Apaydın Yağcı et al 2008a; 2008b; Amouei et al 2013; Mert & Bulut 2014).

Ambrose (1989) stated that age determination is the most critical issue in fisheries biology studies and Chalanchuk (1984) reported age estimation with high precision is among the things to obtain accurate data on population dynamics during fish biology studies (Türkmen et al 2005). However, it is reported that peculiar features of each species like the timing of annulus formation, latent period of growth, breeding and migration activities should be known prior to age determination, and since the ideal bony structure or age determination method may vary among species, even age classes and populations of the same species, it is advised to perform confirmation studies on age determination which can be affected by a number of variables for the sake of the reliability of the method applied (Polat 2000).

Being an indispensable data source in fisheries management, reliable and precise age determination is of utmost importance in monitoring fish populations. The present study focuses on age determination by using three different bony structures of common carp in Eğirdir Lake.

2. Material and Methods

2.1. Sampling of the fish

The study material consisted of 78 *Cyprinus carpio* specimens collected with trammel nets having the mesh sizes 55, 60, 65, 70 and 80 mm from different parts of Eğirdir Lake between May and November 2013. Fork lengths (FL, mm) and weights (g) were recorded for each specimen caught.

2.2. Scales preparation

After measurements, scales were removed from above left lateral line under dorsal fin from near head region (Chugunova 1963). Scales from each fish were kept in petri dishes with warm water for 10-12 hours. Following the cleansing of mucus and the pigment layer via a brush, the scales were placed into 5% NaOH for 2 hours, then washed with distilled water and put into 96% ethanol for minutes to get rid of the water. Afterwards, the scales preserving structural unity and intact nucleus under light were placed between two slides and preparations were made. The study was performed via a Nikon Profile Projector V-10 imaging device and under a constant magnification rate (10x) (Figure 1).

Figure 1- Scale preparations and annulus

2.3. Vertebra preparation

All vertebrae between the fifth to tenth were taken out after removal of the internal organs and cleansing (Chugunova 1963), kept in boiling water for 3 minutes, cleaned from tissue remains and air dried. The polyester molds prepared with 100 g polyester (styrene (100-42-5)) and 2 g hardener (methyl ethyl ketone peroxide) under 70 °C in a pre-heated incubator for two hours (Figure 2), instead of the suggested method using 100 g polyester with 20-40 drops of catalyst under 35 °C incubator treatment (Metin 2001).

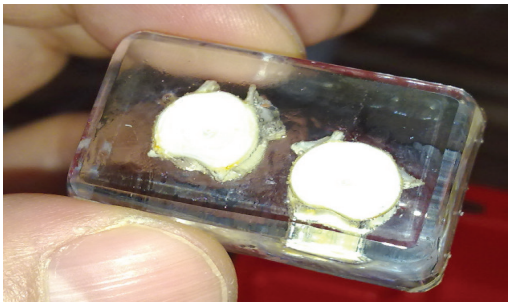

Figure 2- A mould filled with common carp vertebrae fixed in clear polyester resin

A Micracut 201 prevision cutter was used for sectioning. Prior to sectioning, centre of each vertebra was marked. 0.5 mm sections were cut via two blades with 40-50 µm sec^{-1} feed rate and 400-500 rpm speed (Figure 3).

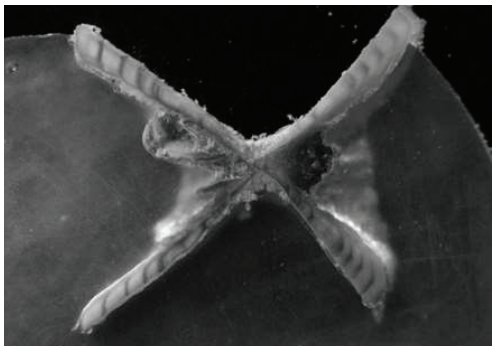

Figure 3- Translucent and opaque bands in vertebral section

Scratches were removed with 200 µ sandpaper. Readings were made under binocular microscope illuminated from the top and sides with the same magnification rate (10x). The images were captured imaging system and ageing was made through images.

2.4. Otolith preparation

Head portions of the fish were cut from midline and the otoliths were removed from otolith capsules with pincers (Chugunova 1963), the otoliths were cleaned in 96% ethanol and placed into Eppendorf tubes with glycerin. After three weeks, otoliths were studied under binocular microscope illuminated from the top and sides with the same magnification rate (10x). The images were captured imaging system and ageing was made through images (Figure 4).

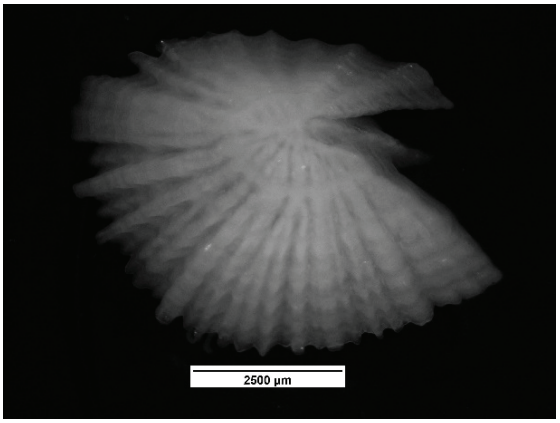

Figure 4- Translucent and opaque bands in carp otolith

Readings were made by a single reader and 5 replicates at different times. Last complete annulus were taken into account for ageing, determination of last annual ring based on 1st January accepted as the birthday for fishes of northern hemisphere.

2.5. Calculation of mean age

According to Baker and Timmons (1991), calculation of mean age aims to determine over-or underestimations. To calculate mean age Equation 1 was used (Bostancı 2005).

$$X_{kt} = \frac{\sum_i^n \sum_j^f x_{ijkt}}{n.f} \quad (1)$$

Where; x_{ijkt}, j fish; i, readings; l, age value of reading; *n*, replicate; *f*, total number of specimens. Although not without certainty about reliability of a certain structure over other, mean age gives a preliminary idea about presence of a reading over or below normal values (Bostancı 2005).

2.6. Agreement of otolith, vertebrae and scales

This value helps to determine the criteria to evaluate annuli. If all 3 readings give the same age in a study with 3 replicates, the similarity will be zero or in other words agreement is 100%. Higher agreement is preferred, although this doesn't prove the reliability of a certain structure. In short agreement is an important index for age analyses, although solely not enough the ensure reliability (Bostancı 2005). Rate of agreement over 5 readings can be expressed as 5/5, 4/5, 3/5, 2/5, 1/5, and demonstrated as a percentage (%) of the specimen number in the expression to all specimens. The structure with highest agreement is accepted as the most reliable (Yılmaz 2000). Five replicates were applied in our study.

2.7. Variance analysis of otolith, vertebrae and scales

Age estimation data obtained from 5 different readings for each structure was subjected to variance analysis (Equation 2). Fowler (1990) describes variance as the sum of squares mean age estimates minus mean age over degrees freedom (Yılmaz 2000).

$$V_t = \frac{\sum_i^n \left(X_i - \overline{X}\right)}{n-1} \quad (2)$$

Where; V_t, the variance for the structure t; *n,* number of replicates; X_t, first reading for each sample; $\overline{X}$, mean of replicates.

2.8. Ageing error

For each age estimation of a certain bony structure after 5 replicates, error rate was calculated after variance analysis. Ageing error (S_t, standard deviation) is expressed as the square root of the variance (Equation 3) (Yılmaz 2000). Lowest standard error gives the most reliable structure (Yılmaz 2000).

$$S_{t=\sqrt{V_t}} \quad (3)$$

2.9. Data analysis

Minimum age, maximum age, mean age, standard deviation, standard error, variance and CV values were analyzed using JMP 8 package program.

3. Results and Discussion

Distribution of weights in the 78 carps caught from Lake Eğirdir ranges between 191 and 8685 g, while the fork lengths range between 19.5 cm and 76 cm.

3.1. Age composition of otolith, vertebrae and scales

According to 5 reading replicates, age composition estimated for each bony structure is different. Scale readings of 78 carps give age distribution as 1-11, of 63 specimens (as in 15 samples no annulus formation was detected) vertebrae readings give it as 2-11 and according to readings from 75 specimens (as 3 otoliths were broken upon removal) it is found as 2-10 (Table 1).

Table 1- Age composition derived from different bony structures readings

Bony structure	*Age*											*Total*
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	
Scales	1	10	6	7	10	12	11	12	4	3	2	78
Vertebrae	-	7	9	2	4	11	13	11	3	2	1	63
Otolith	-	7	9	12	7	8	14	12	5	1	-	75

When the average estimates were compared for each bony structure, were found to be around 5, no statistical difference was observed (P>0.05). Standard errors and reading errors for bony structure-reader combinations are given in Table 2. Accordingly, lowest standard error and ageing errors are calculated in otoliths.

Table 2- Mean ages, standart and ageing errors in different bony structures for common carp

Bony structures	*Scales*	*Vertebrae*	*Otoliths*
Mean ages	5.76	5.88	5.64
Standard errors	0.28	0.30	0.25
Ageing errors	2.44	2.38	2.21

When the agreement among otoliths, vertebrae and scales of carps in Lake Eğirdir were investigated to find the most reliable bony structure, according to 5 different readings at different times, the evaluations of the percentages of shared readings for all five replicates over all readings are given in Table 3.

Table 3- Precision of readers on different bony structures (%)

Bony structure	*Aagreement of readers/number of total readings*					*Total*
	5/5	*4/5*	*3/5*	*2/5*	*1/5*	
Scales	7.69	29.49	38.46	24.36	-	100
Vertebrae	7.94	25.40	34.92	31.74	-	100
Otolith	10.67	28.00	36.00	25.33	-	100

This study is the first to be based on comparison of methodologically different bony structures in Lake Eğirdir fishes. As in similar studies, the most reliable age estimation is based on the lowest reading error and otolith readings had the lowest error (2.21) (Table 2). According to 5 different readings of the same reader, the highest agreement was found otolith (10.67) and the lowest in scales (7.69) (Table 3). Polat et al (2001) in Black Sea flounders and Polat et al (2004) in Derbent Dam perch found the highest reader agreement and the lowest reading error in vertebrae. Likewise, in a study on comparison of 6 different structures in carps of Altınkaya, Derbent Dams and Balık Lake (Bafra), Yılmaz & Polat (2008) have found the highest reader agreement and the lowest reading error in vertebrae for each lake. On the other hand, Temizer & Şen (2008) found among Keban Dam mirror carps the highest reader agreement between scales and vertebrae (86.68%).

There are many studies on use of scales to obtain the most suitable bony structure and process for age determination (Yerli 1997; Campana 2001; Balık et al 2006; Apaydın Yağcı et al 2008a, b). However, otoliths were found as the most reliable ageing structure in *Carassius gibelio* from Lake Eğirdir (Bostancı 2005), whereas vertebrae were more precise as compared to otoliths in *Alosa pontica* (Yılmaz 2000) and in *Carassius gibelio* in Lake Bafra (Bostancı 2005).

4. Conclusions

In conclusion, according to the highest agreement and lowest ageing error values, otolith is the ideal bony structure for precise age determination in Lake Eğirdir carps.

Acknowledgements

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Evaluating AquaCrop Model for Winter Wheat under Various Irrigation Conditions in Turkey

Sema KALE CELIK[a], Sevinç MADENOGLU[b], Bulent SONMEZ[b]

[a]*Suleyman Demirel University, Faculty of Agriculture, Department of Agricultural Structure and Irrigation, Isparta, TURKEY*

[b]*Soil, Fertilizer and Water Resources Central Research Institute, 06171, Ankara, TURKEY*

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Corresponding Author: Sema KALE CELIK, E-mail: semakale@sdu.edu.tr, Tel: +90 (246) 211 85 63
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ABSTRACT

Farming winter wheat in Central Anatolia of Turkey traditionally is rainfed. Crop yields are frequently affected in this region because of the drought events of varying severity. There is apparent necessary for an aim appraisal of the effect of dryness on this critical crop, to answer the contradiction whether irrigation is essential or not. For this reason the FAO-AquaCrop (Ver.5.0) crop water productivity model was preferred to predict attainable yields of winter wheat (*Triticum durum* L.) under four different irrigation regimes. Field experiment was conducted under four different irrigation treatments in Central Anatolia Region of Turkey during 2008-2010. The AquaCrop was calibrated with 2008-2009 field data and model validation was performed using 2009-2010 data. Model simulation results showed that model simulates soil water content in root zone (SWC), canopy cover (CC), grain yield (GY) and aboveground biomass (BM) of wheat reasonably well. The average root mean square error (RMSE) between simulated and observed SWC, CC, GY and BM were 21.1 mm, 7.1%, 0.32 t ha^{-1} and 0.34 t ha^{-1}. Nash-Sutcliffe efficiency (EF) and index of Willmott (d) also were obtained 0.89 and 0.98 for CC, 0.74 and 0.93 for SWC, 0.98 and 0.92 for BM, 0.95 and 0.82 for GY. Model predicted canopy cover, grain yields and biomass with high accuracy while soil water content at 90 cm soil depth was estimated in the moderate accuracy. The results presented that AquaCrop model can be suggested as a convenient model for decision-making whether irrigating wheat is in the priority or not at the limited water resources areas.

Keywords: AquaCrop; Grain yield; Irrigation; Canopy cover

Türkiye Koşullarında Kışlık Buğday için AquaCrop Modelinin Çeşitli Sulama Koşulları Altında Değerlendirilmesi

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Sema KALE CELIK, E-posta: semakale@sdu.edu.tr, Tel: +90 (246) 211 85 63
Geliş Tarihi: 14 Ekim 2016, Düzeltmelerin Gelişi: 07 Ocak 2017, Kabul: 21 Nisan 2017

ÖZET

Türkiye'de İç Anadolu Bölgesi'nde geleneksel olarak kışık buğday tarımı yağışa dayalı (susuz) olarak yapılmaktadır. Bu bölgede buğday verimi çeşitli seviyelerdeki kuraklık nedeniyle sıklıkla etkilenmektedir. Sulamanın gerekli olup

olmadığı çelişkisinin çözülebilmesi için bu stratejik ürün üzerinde, kuraklık etkisinin objektif olarak değerlendirilmesine belirgin bir ihtiyaç vardır. Bu amaca yönelik olarak FAO-AquaCrop (Ver.5.0) bitki su verimliliği modeli, farklı sulama rejimi altında elde edilecek kışlık buğday (*Triticum durum* L.) verimlerini tahmin etmek için seçilmiştir. Arazi denemesi, dört farklı sulama konusunda 2008-2010 yılları arasında İç Anadolu Bölgesi'nde yürütülmüştür. AquaCrop 2008-2009 arazi verileri ile kalibre edilmiştir ve modelin validasyonu için 2009-2010 verileri kullanılmıştır. Model simülasyon sonuçları, modelin bitki örtüsü (CC), kök bölgesindeki toprak su içeriği (SWC), biyokütle (BM) ve buğday verimini oldukça iyi tahmin ettiğini göstermektedir. Ölçülen ve tahmin edilen SWC, CC, GY ve BM arasındaki hata kareler ortalaması (RMSE) değerleri sırasıyla 21.1 mm, % 7.1, 0.32 t ha^{-1} and 0.34 t ha^{-1} olmuştur. Nash-Sutcliffe etkinliği (EF) ve Willmott indeksi (d) CC için 0.89 ve 0.98, SWC için 0.74 ve 0.93, BM için 0.98 ve 0.92, GY için ise 0.95 ve 0.82 olarak bulunmuştur. Model dane verimi ve biyokütleyi yüksek doğrulukta tahmin ederken, kök bölgesi toprak su içeriğini orta doğrulukta tahmin etmiştir. Sonuçlar AquaCrop modelinin su kaynaklarının kısıtlı olduğu alanlarda buğday sulamasının öncelikli olup olmadığının karar verilmesinde tavsiye edilebilir bir araç olduğunu göstermiştir.

Anahtar Kelimeler: AquaCrop; Dane verimi; Sulama; Bitki örtüsü

1. Introduction

Wheat is very significant agricultural crop in Turkey, and 9.5 million ha which is around 65-70% of the total arable land of 27 million ha is utilized for wheat production. Central Anatolia Region of Turkey has semi-arid climate (Altın et al 2012), and available water is a significant restriction for wheat production. Accordingly, irrigation is essential for this region to avoid water stress and maximize crop yield. Several researchers have reported that wheat is not evenly sensitive to drought at different growing stages (Zhang & Oweis 1999; Sezen & Yazar 2008).

Large amount of water resources are consumed by agriculture on worldwide (Geerts & Raes 2009; Andarzian et al 2011). Production depends almost entirely on irrigation especially dry conditions (Musick et al 1994; Steven & Tolk 2009). Investigation of plant responds to different irrigation strategies in the field is difficult and expensive taking into consideration this kind of restrictions, precise crop water productivity models are significant equipments in order to assess impacts of water upon crop production (Heng et al 2007; Farahani et al 2009; Andarzian et al 2011; Levidow et al 2014).

The FAO AquaCrop is simple, accurate, user friendly model which can be used by water managers, water use organizations, economists and policy makers to planning and analysis of irrigation scenarios. (Hsiao et al 2009). Besides, AquaCrop model predicts yield response to water of graminaceous crop (Heng et al 2009; Vanuytrecht et al 2014). Details of simulation processes are provided in irrigation and drainage paper number 66 (Steduto et al 2012). AquaCrop was tested for various crops under several environmental conditions (Heng et al 2009; Todorovic et al 2009; Araya et al 2010; Zeleke et al 2011; Amoah et al 2013; Ahmadi et al 2015; Trombetta et al 2016; Toumi et al 2016).

We focused on AquaCrop calibration and validation under several irrigation strategies using with experimental field data at this study.

2. Material and Methods

Experimental sites are located in Ankara, Murted Basin (40° 04' N and 32° 36' E, elevation 831 m) of Central Anatolia region of Turkey (Figure 1). A field experiment was conducted in two growing seasons of wheat between the years 2008 and 2010 in Research Farm Station of Soil, Fertilizer and Water Resources Central Research Institute in Murted Basin, Ankara, Turkey.

The climate is characterized as semi-arid in this region of Ankara. Long term monthly meteorological data was presented at Table 1. Daily data were obtained from meteorological station of the experimental site. The daily maximum-minimum temperature and ET_o, precipitation for growing season from 20th of October 2008 to 20th of July 2010 were given at Figure 2.

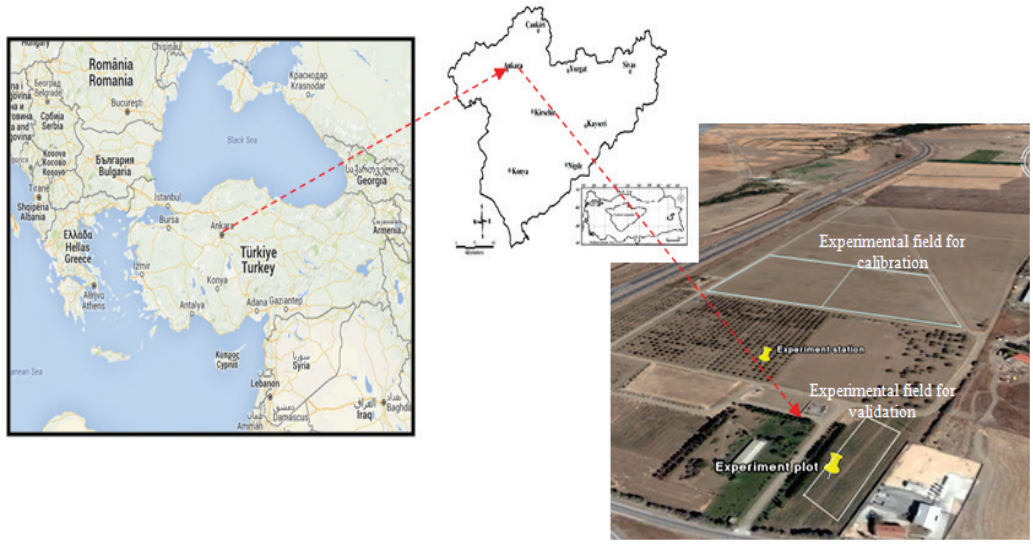

Figure 1- Location of experimental site

Table 1- Average climatological data of Ankara (1976-2011)

	Month												
Meteorological data	*X*	*XI*	*XII*	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*Annual*
Max temperature (°C)	27.6	20.4	14.4	9.3	12.1	20.6	25.3	28	30.4	32.1	34.3	32	34.3
Min temperature (°C)	-2.9	-10.5	-14.5	-14.8	-14.6	-9.7	-3.1	-1.8	3.9	9.6	4.9	4.9	-14.7
Precipitation (mm)	23	31.9	38.9	30.4	33.1	36.7	43.1	55.2	22.1	15	5.4	15.2	350
Relative humidity (mm)	57	70	79	72	71	60	58	58	50	37	35	41	57
Evaporation (mm)	95	44	-	-	-	-	103	147	225	243	276	167	1300
Wind speed (m s^{-1})	1.2	1.3	1.3	1.2	1.3	1.4	1.2	1.3	1.2	1.2	1.2	1.1	1.2

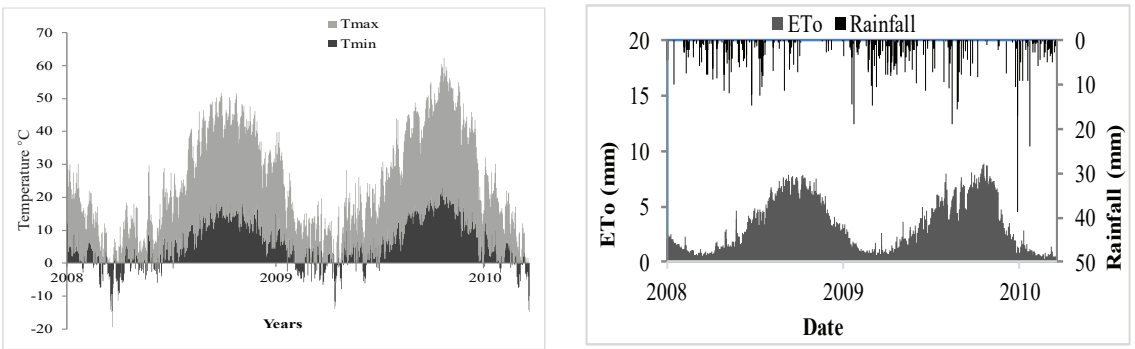

Figure 2- Daily maximum and minimum temperature ET_o and precipitation values for experimental area during the growing period during 2008-2009 and 2009-2010

The soils are mostly silty clay texture about 0.30 m soil depth, whereas clay is dominant texture approximately from 0.30 m to 1.5 m in the soil profile of experimental area. Field capacity on the volume basis of the top and basement soil layer is described to be 33 and 37%, and wilting point, 17 and 23% respectively. Some physical and chemical soil properties were given Table 2.

Table 2- Some physical characteristics of the soil in experimental sites

Depth (m)	*Moisture content*				*Bulk density (g cm^{-3})*	*K_{sat} (mm day^{-1})*
	FC (%, vol)	*PWP (%, vol)*	*Sat (%, vol)*	*TAW*		
0.0-0.3	33.8	17.4	45.0	164	1.24	230
0.3-0.6	36.2	22.1	47.0	141	1.27	175
0.6-0.9	36.9	22.2	47.0	147	1.21	125
0.9-1.50	37.4	23.0	50.0	144	1.20	125

FC, field capacity; PWP, permanent wilting point; TAW, total available water; Sat, water content at saturation; Ksat, saturated hydraulic conductivity

A locally adapted major wheat variety (Bayraktar-2000) was grown during the experimental studies. Wheat seeds were obtained from National Seeds Research Institute. Grain yield and biomass per plot was measured after harvesting. Weight of straws and grain (t ha^{-1}) were taken as dry biomass and grain yield, respectively.

The study was carried out through two growing seasons from 2008 to 2010. 2008-2009 growing season values were used for calibration and 2009-2010 for validation processes. The experiment consists of 4 irrigation regimes with 4 replications. Irrigation treatments were given at Table 3. The experimental design was as a complete randomized block design with a split plot layout. Plot dimensions were taken 20 m^2 (5 m x 4 m). There was 2 m distance between all plots. The plots have almost zero slope and were surrounded about 0.30 m high soil bunds (Figure 3).

Table 3- Irrigation treatments of experiment

Treatments	*Growing periods*			
	Germination	*Tillering*	*Heading*	*Grain filling*
I_1	-	-	-	-
I_2	x	x	x	x
I_3	-	x	-	x
I_4	-	-	x	x

I_1, rainfed; I_2, full irrigation

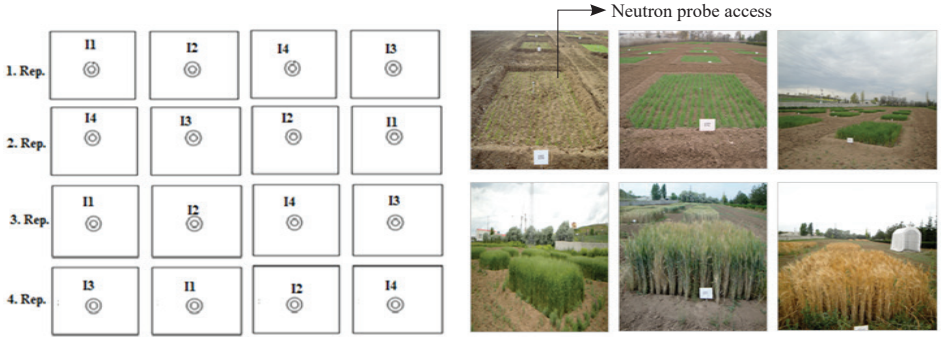

Figure 3- Experimental design

Winter wheat was planted around 20th October and harvested 20 of July for each year. The seed rate was 430 seed m^{-2} with 1.7 cm row spacing. According to soil fertility analysis results commercial N fertilizers were applied in a band about 10 cm near to the seed row (225 kg ha^{-1}, ammonium sulfate 21% were applied before sowing and 350 kg ha^{-1} ammonium sulfate were applied in second week of March). Sufficient rate of phosphorus was applied (178 kg ha^{-1}, DAP 18-46-0) to ensure adequate P nutrition.

2.1. Field observations

Soil volumetric moisture contents were measured by neutron probe (CPN-503DR Hydroprobe) in 20 cm interval from a depth of 0-100 cm at two times a week. Evapotranspiration of wheat was calculated based on Equation 1 (Allen et al 1998).

$$ET = I + P \pm \Delta S - R - D \quad (1)$$

Where; I, irrigation water amount (mm); P, precipitation (mm); ΔS, change in soil water content (mm); R, surface flow (mm, negligible; precision leveled to zero-grade); D, deep seepage (mm, negligible; irrigated until field capacity and water table depth is about 4 m).

For irrigation treatments, soil moisture was reached to the field capacity in the 0-90 cm depth. Irrigation water was applied 250 mm for I_2 treatment at 2008-2009 growing season. Irrigation water amount was 255 mm for I_2, 153 mm for I_3 and for 141 mm for I_4 at 2009-2010 growing season.

Emergence date, flowering length, beginning of senescence, maturity, maximum canopy cover and rooting depth were recorded during the experiment at the field.

Overhead photographs of canopy were taken with commercially available digital camera (Sony CyberShot DSC-H55 with a resolution of 14.1 mega pixels) at an invariable height of 1.5 m, between 11:00 and 15:00 every month from emergency to late senescence stage of wheat (Figure 4). Taken photographs were processed with the GreenCrop tracker software (Figure 5) which was freely distributed software from website. GreenCrop, tracker image processing software, is segmenting the green canopy from the background material. Several research results showed that the digital cameras images can be used for predicting canopy cover (Laliberte et al 2004; Guevara-Escobar et al 2005; Lee & Lee 2011).

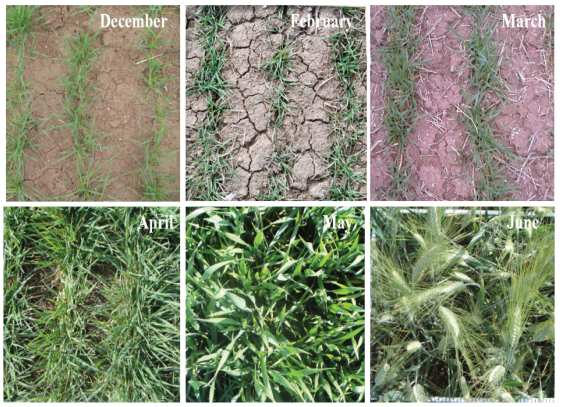

Figure 4- Canopy photographs of wheat from December to June

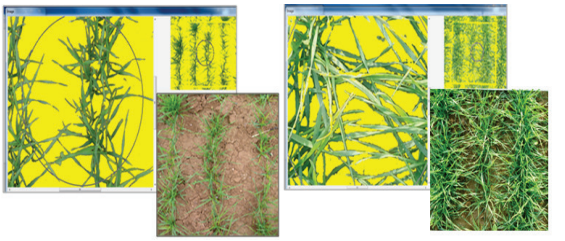

Figure 5- Some processed photographs with the GreenCrop tracker software to calculate canopy cover percent

2.2. Description of AquaCrop (Version 5.0) model

AquaCrop model was developed by FAO to predict yield response to water. The overall structure of the model and comprehensive information presented at Steduto et al (2008) and Raes et al (2009). AquaCrop predicts green canopy cover (CC) in place of leaf are index (LAI) and it calculates evapotranspiration (ET) from the flow water in and out of a system at the daily bases and partition ET into evaporation (E) and transpiration (T) (Araya et al 2010; Toumi et al 2016).

Input data for AquaCrop are climate file (minimum-maximum air temperature, ET_o, precipitation and CO_2), soil file (field capacity, permanent wilting points, saturated hydraulic conductivity), crop file (emergence date, start of flowering, length of flowering, max. canopy cover, canopy senescence, physiological maturity), management file (irrigation, field management practices) and initial condition file (initial soil water content) (Steduto et al 2012).

2.3. Methods of model calibration, validation

The AquaCrop (5.0) was calibrated for the full irrigation trail in 2008-2009. Canopy cover (CC) calculation parameters which highest canopy cover (CC_X), canopy decline and canopy growth coefficients (CDC and CGC, respectively) were used for calibration. A trial and error approach were used to minimize the difference between the simulated and measured data. The process of calibration was complied when the lowest root mean squared error between simulated and measured CC, soil water content and grain yield was obtained.

Field data set of all treatments at 2009-2010 growing season was used for model validation. Canopy cover, soil water content, biomass and grain yield were considered.

AquaCrop uses growing degree day (GDD) as a thermal time to calculate temperature values (Steduto et al 2009; Hsiao et al 2009). In AquaCrop model, base and the upper temperature are used to calculate GDD. In this study, the value 0 for base and 27 °C for upper temperature were used for the Bayraktar-2000 winter wheat cultivar (Tatar & Yazgan 2002). Two types of crop parameters are described in the model as conservative (not change with time, climate, management etc) and non-conservative (cultivar and conditions dependent) (Hsiao et al 2009; Raes et al 2009; Steduto et al 2012). These parameters used in the model for calibration and validation were presented in Table 4. Some of the data were obtained from conducted experiment between at the 2008 and 2010 cropping season, some of them were taken local experience, some of them were used from the reference manual for AquaCrop as a default (Raes et al 2012).

2.4. Data analysis

Measured and simulated data including soil water content, dry biomass and grain yield were compared statistically for evaluating model reliability. The agreement between predicted and measured values was defined by calculating coefficient of determination, the root mean square error (RMSE), normalized root mean square error (NRMSE) (Jacovides & Kontoyiannis 1995), Nash-Sutcliffe efficiency (EF) (Nash & Sutcliffe 1970) and index of Willmott (d) (Willmott 1982). Statistical parameters were expressed in Equation 2-5.

$$RMSE(\%) = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(S_i - O_i)^2} \quad (2)$$

$$NRMSE(\%) = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(S_i - O_i)^2}\, x\, \frac{100}{O_{avg}} \quad (3)$$

$$EF = 1 - \frac{\sum_{i=1}^{n}(O_i - S_i)^2}{\sum_{i=1}^{n}(O_i - O_{avg})^2} \quad (4)$$

$$d = 1 - \frac{\sum_{i=1}^{n}(S_i - O_i)^2}{\sum_{i=1}^{n}\left(|S_i - O_{avg}| + |O_i - O_{avg}|\right)^2} \quad (5)$$

Where; n, total observation numbers; O_i, observed value of the i^{th} observation; S_i, estimated value of the i^{th} observation; O_{avg}, mean of the observed values (i= 1 to n).

If RMSE is close to zero, the model performance can be described acceptable. If NRMSE is less than 10%, model simulation can be considered perfect (between 10 and 20%; acceptable, 20 and 30%; fair, greater than 30%; poor) (Jamieson et al 1991; Mohammadi et al 2016). EF values ranges from minus infinity (-∞) to 1.0. If EF value is 1.0 it is represents a perfect prediction and on the contrary lower values representing a gradually unsatisfactory prediction. Values of EF between 0.50 and 1.00 are assumed admissible. The index of agreement (d)

Table 4- Conservative and non-conservative crop inputs used in AquaCrop for winter wheat

Conservative parameters		
Description	*Value*	*Units or meaning*
Crop growth and development		
Base temperature[LE]	0	˚C
Cut-off temperature[LE]	27	˚C
Canopy cover (CC_o)[M]	6.47	% at 90% emergence
Maximum canopy cover, CCx (%)[C]	90	%
Canopy growth coefficient (CGC)[C]	2.7	% inc. in CC relative to existi. CC per GDD*
Canopy decline coeff. (CDC) at senescence[C]	0.34	%, decrease in CC relative to CC_x per GDD
Water stresses[D]		
Upper threshold of leaf growth	0.20	as frac. of TAW, above leaf growth is inhibi.
Lower threshold of leaf growth	0.65	leaf growth stops completely at this p
Curve shape of leaf growth stress coefficient	5.0	moderately convex curve
Upper threshold of stomatal conductance	0.65	above this stomata begin to close
Curve shape of stomatal stress coefficient	2.5	highly convex curve
Upper threshold of senescence stress	0.70	above this early canopy senescence begins
Curve shape of senescence stress coefficient	2.5	moderately convex curve
Biomass production and yield formation		
Harvest index[M]	36	%
Water productivity normal. for ET_o and CO_2[D]	15	g (biomass) m^{-2}
Non-conservative parameters		
Management dependent[M]		
Sowing rate	170	kg seed ha^{-1}
1000 seed mass	33.50	g
Germination rate	85	%
Cover per seeding	1.5	cm^2 $plant^{-1}$
Plant density	431.3	plant m^{-2}
Phenology (cultivar specific)[M]		
Sowing	20 October	date
Time from sowing to emergence	31 October, 11(123)	date,day, GDD
Time to reach max canopy cover	12 May, 204 (1276)	date,day, GDD
Time from sowing to maximum root depth	16 March, 146 (775)	date,day, GDD
Time to start senescence	10 June, 233 (1768)	date,day, GDD
Time from sowing to reach maturity	20 July, 274 (2605)	date,day, GDD
Time to reach flowering	15 May, 207(1320)	date,day, GDD
Duration of flowering stage	25 May, 10 (179)	date,day, GDD
Soil dependent[M]		
Minimum effective root depth	0.3	m
Maximum effective root depth	1.5	m
Hydraulic conductivity	125-230	(0-30 and 30-150 cm soil depth) mm day^{-1}

*GDD, growing degree days (°C); LE, local experience; M, measured; D, default (Steduto et al 2012); C, calibrated

value is varies between 0 and +1 (Andarzian et al 2011; Tavakoli et al 2015; Mohammadi et al 2016). According to d values the closer to one indicates that estimated and observed values are identical.

3. Results and Discussion

3.1. Model calibration results

Data set (full irrigation treatment) in the 2008-2009 growing was used for calibration season. Canopy cover, total soil water content, grain yield and final aboveground biomass have been calibrated. Maximum canopy cover, canopy growth coefficient and canopy decline coefficients were modified and re-modified to simulate the measured canopy cover. Figure 6 showed that there was a good agreement between the observed and simulated canopy cover development and soil water content at 90 cm soil depth. It was also approved by statistical values at Table 5.

EF, d and R^2 values are close to 1 which indicates simulated canopy cover and soil water content agreed well with observed. NRMSE values obtained with calibration are in the range 10 and 20% for canopy cover which indicated that simulation can be acceptable and smaller than 10% for soil water content which means that simulation can be considered as perfect. The results of this study are collaborated by other research studies (Andarzian et al 2011; Tavakoli et al 2015; Toumi et al 2016).

Table 6 shows both grain yield and aboveground biomass were sufficiently predicted by AquaCrop. The deviation of the predicted grain yield and biomass from observed calibration data set in 2008-2009 was 1.4% and 1.3%, respectively.

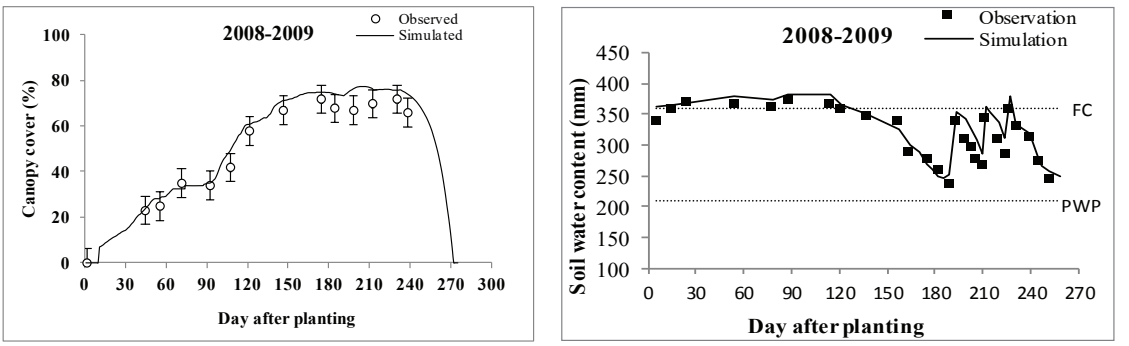

Figure 6- The observed and simulated canopy covers percent during the 2008-2009 growing season (vertical bars represents standard deviations)

Table 5- Statistical values belonging to simulated and observed canopy cover and soil water content for calibration under full irrigation of winter wheat

Year	*Variables*	*RMSE*	*NRMSE*	*EF*	*d*	R^2
2008-2009	Canopy cover (%)	5.6	10.9	0.90	0.98	0.99
	Soil water content (mm)	5.8	9.6	0.93	0.98	0.98

Table 6- Simulated and measured grain yield and biomass results for calibration under full irrigation of wheat

Year	*Yield (t ha^{-1})*			*Biomass (t ha^{-1})*		
2008-2009	*Measured*	*Simulated*	*Deviation (%)*	*Measured*	*Simulated*	*Deviation (%)*
	5.15	5.49	1.4	14.9	15.5	1.3

3.2. Model validation and testing results

In this study, the performance of the model was validated with simulating grain yield, biomass, canopy cover and soil water content. Validation was conducted with data for different irrigation treatments (rainfed, full irrigation, irrigation at tillering and grain filling, irrigation at heading and grain filling stage) in the 2009-2010 growing seasons.

3.2.1. Soil water content

The comparison of simulated and observed soil water content was presented in Figure 7. According to this figure, predicted soil water content has similarity of the measured values with slightly over-estimated for all treatments. Statistical results such as RMSE, NRMSE, EF, d and R^2 for four irrigation treatments were given in Table 7. According to statistical values the simulated soil water agreed with their corresponding observed values. Root zone soil water content is estimated in moderate accuracy by the model. The best fit was obtained between measured and simulated soil moisture at rainfed treatment. Similar observation results have been reported in various studies (Hussein et al 2011; Iqbal et al 2014; Toumi et al 2016).

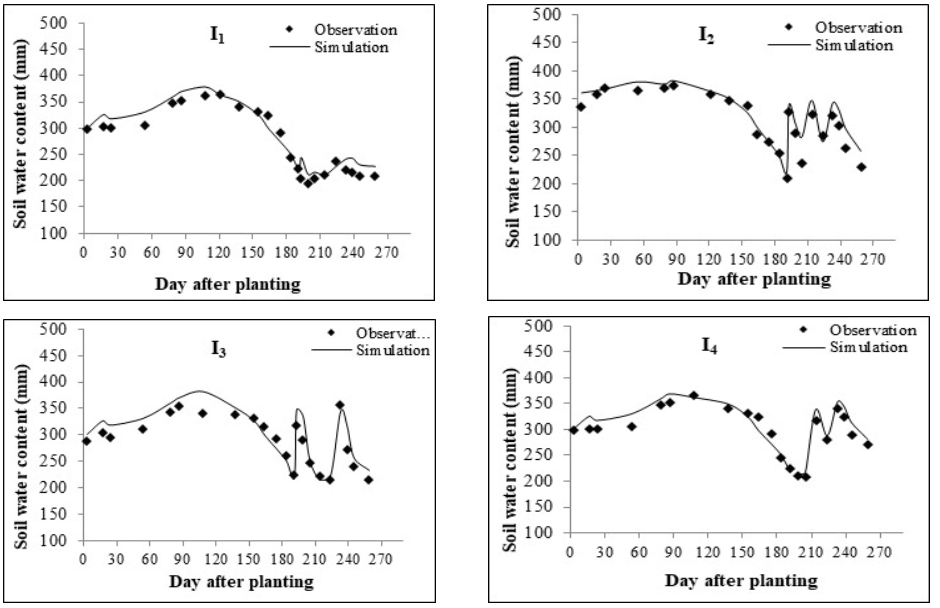

Figure 7- The observed and simulated water content at the top 0.90 m soil profile in the growing season 2009-2010 for four irrigation treatments

Table 7- Statistical values belonging to simulated and observed soil water content for validation during 2009-2010 growing season

Variables	*Treatment*	*RMSE*	*NRMSE*	*EF*	*d*	r^2
Soil water content (mm)	I_1	15.1	5.6	0.93	0.98	0.97
	I_2	25.2	7.9	0.73	0.94	0.94
	I_3	22.3	8.4	0.52	0.87	0.86
	I_4	21.8	7.7	0.78	0.94	0.90
	Average	21.1	7.4	0.74	0.93	0.92

3.2.2. Canopy cover

It is shown in Figure 8 that different irrigation treatments have slightly affected the canopy covers. Figure 8 shows the comparison between simulated and observed canopy development in irrigated and rainfed treatments. The results indicate that the simulated canopy cover was almost same to the observed values from sowing to senescence periods. But there was some inconsistency after senescence with measured CC. For all treatments CC values were mostly over estimated from the senescence to the end of the growing season which was also obtained by Andarzian et al (2011) and Toumi et al (2016) for wheat under different irrigation conditions.

Table 8 shows statistical analysis of the model performance for CC. According to results normalized deviation of predicted values from observed for CC percentage was between 10.3% and 18.5%, which is acceptable. Model efficiency and the index of agreement was in the range 0.84-0.93 and 0.97-0.98 which is close to 1.0 indicate the reliable performance of the model. The lowest CC was obtained at the rainfed condition whereas the highest value was in the full irrigation conditions. If we compare the average

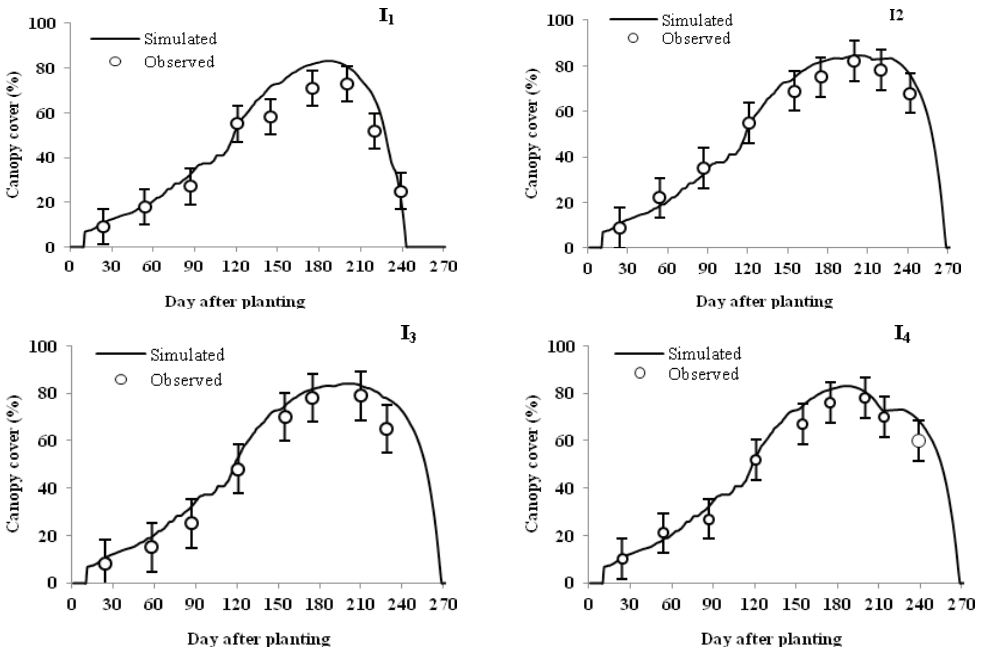

Figure 8- The observed and simulated canopy cover results in 2009-2010 growing season

Table 8- Statistical indices calculated for performing performance of AquaCrop model in predicting canopy cover and soil water content

Variables	*Treatment*	*RMSE*	*NRMSE*	*EF*	*d*	R^2
Canopy cover (%)	I_1	7.9	18.5	0.89	0.98	0.94
	I_2	6.9	11.4	0.88	0.98	0.96
	I_3	8.2	15.7	0.84	0.97	0.96
	I_4	5.7	10.3	0.93	0.98	0.98
	Average	7.1	13.9	0.89	0.98	0.96

canopy cover according to the treatments, the highest value was obtained from I_2 (full irrigation) and it was followed by I_4 and I_3 treatments. The lowest value was at I_1 (rainfed) treatment. Simulation of the results showed the same trend.

3.2.3. Grain yield and biomass

Observed and simulated grain yield values and final aboveground biomass were presented at Table 9. Table 9 shows a deviation of the simulated grain yield (1.8% to 11.4%) and biomass (1.3% to 3.5%) from their corresponding observed data. The highest positive deviation was simulated for grain yield in the case of treatment I_1 (rainfed). This could possibly be due to the fact that the senescence of the canopy accelerates under severe water stress at the field conditions. Iqbal et al (2014) reported much greater deviation (14.1%) under rainfed conditions. Similar results have been obtained by Araya et al (2010) and Zeleke et al (2011). They reported much greater deviation under rainfed or severe water stress treatments, as compared to full irrigation treatments for different crops simulated by the model. The highest grain yield and biomass (5.6 t ha^{-1} and 14.9 t ha^{-1}) were obtained from I_2 (full irrigation) treatment. Grain yield and above ground biomass values at I_1 (rainfed), I_3 (irrigated tillering+grain filing) and I_4 (irrigated heading+grain filing) treatments were 3.5 t ha^{-1}, 4.2 t ha^{-1} and 4.4 t ha^{-1}, respectively. The estimated values of grain yield and biomass for all treatments are in the range of the observed one. The model efficiency (EF) showed good performance for biomass (0.92), moderate performance for grain yield (0.75). Model simulated biomass more accurately than grain yield. This was also confirmed by Moderate EF and lower RMSE (0.32 t ha^{-1} and 0.34 t ha^{-1}) values indicate that the AquaCrop model is able to simulate grain yield and biomass well. Figure 9 shows linear correlation between simulated and observed grain yield and biomass. Determination coefficient show that the model simulated grain yield and biomass with a high degree of reliability has a R^2 of 0.99 for both of them. The AquaCrop model could very well predict

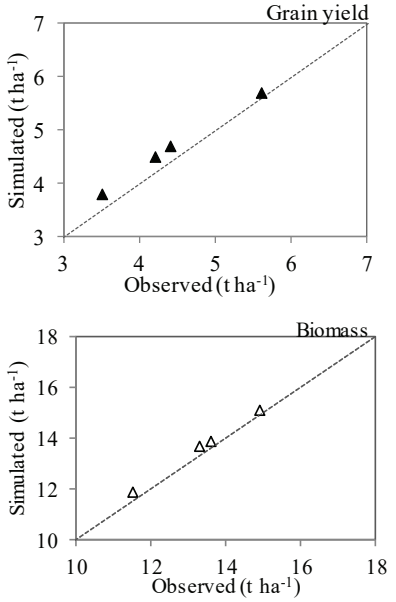

Figure 9- Relation between simulated and measured wheat grain yield and biomass

Table 9- Simulated and observed grain yield and biomass results for validated data set

Year	*Treatment*	*Yield (t ha^{-1})*			*Biomass (t ha^{-1})*		
		Observed	*Simulated*	*Deviation (%)*	*Observed*	*Simulated*	*Deviation (%)*
2009-2010	I_1	3.5	3.9	11.4	11.5	11.9	3.5
	I_2	5.6	5.7	1.8	14.9	15.1	1.3
	I_3	4.2	4.6	9.5	13.3	13.7	3.0
	I_4	4.4	4.7	6.8	13.6	13.9	2.2
RMSE (t ha^{-1})		0.32			0.34		
NRMSE (%)		7.32			2.52		
d		0.95			0.98		
EF		0.82			0.92		

grain yield and final aboveground biomass of winter wheat under semi-arid conditions.

It is important to note that in spite of the slight mismatching the overall results of this study intimate that AquaCrop model has adequately simulated grain yield, biomass, canopy cover as well as soil water content under various water availability conditions.

4. Conclusions

In this study AquaCrop model (5.0 version) was calibrated and validated for winter wheat crop grown under different irrigation treatments in the semi-arid region of Turkey (Central Anatolia). The results of the model for evaluation of simulate soil water content of root zone, seasonal canopy cover, grain yield and final harvested biomass showed sufficient accuracy of the model simulated and observed values. The average values of the root mean square error (RMSE) between observed and simulated CC, SWC, BM and GY were 7.1%, 21.1 mm, 0.34 t ha^{-1} and 0.32 t ha^{-1}, respectively. Nash-Sutcliffe efficiency (EF) and index of Willmott (d) also were obtained 0.89 and 0.98 for CC, 0.74 and 0.93 for SWC, 0.98 and 0.92 for BM, 0.95 and 0.82 for GY. Model predicted canopy cover, grain yields and biomass with high accuracy while soil water content in root zone is estimated in the moderate accuracy.

Despite model prediction slightly over-estimated, overall results of this study demonstrated that the AquaCrop model is a suitable tool for evaluating irrigation strategies of winter wheat in semi-arid regions.

Acknowledgements

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Multilevel Analysis for Repeated Measures Data in Lambs[1]

Suna AKKOL[a], Ferda KARAKUŞ[a], Fırat CENGİZ[a]

[a]*Van Yuzuncu Yil University, Faculty of Agriculture, Department of Animal Science, Van, TURKEY*

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Corresponding Author: Suna AKKOL, E-mail: sgakkol@yyu.edu.tr, Tel: +90 (506) 735 65 26
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ABSTRACT

The study was conducted to compare the individual growth curves models and to detect individual differences in the growth rate by a performing multilevel analysis. The data set used for this purpose consisted of live weight records of 52 crossbred lambs from birth to 182 days of age. There were 670 observations in level-1 units which were the repeated measurements over time, and there were 52 observations in level-2 units which were lambs. In the study, parameter estimation of time-independent covariate factors, such as gender, birth type and birth weight, was performed by using five different models within the framework of multilevel modeling. LRT, AIC and BIC were used for the selection of the best model. The "*Conditional Quadratic Growth Model-B*" provided the best fit to the data set. The multilevel analysis indicated that linear and quadratic growth in lambs was significant. According to the results of the study, individual growth curves can be investigated using multilevel modeling in animal studies which is an important parameter of the individual growth rate.

Keywords: Repeated data; Multilevel models; Intra-class correlation; Individual growth models

Kuzularda Tekrarlamalı Veriler için Çok Düzeyli Analiz

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Suna AKKOL, E-posta: sgakkol@yyu.edu.tr, Tel: +90 (506) 735 65 26
Geliş Tarihi: 25 Nisan 2016, Düzeltmelerin Gelişi: 31 Ekim 2016, Kabul: 01 Kasım 2016

ÖZET

Bu çalışma, çok düzeyli analizler kullanılarak bireysel büyüme eğrisi modellerini karşılaştırmak ve büyüme oranındaki bireysel farklılıkları belirlemek amacıyla yapıldı. Bu amaç için kullanılan veri seti, 52 baş melez kuzunun doğumdan 182 günlük yaşa kadar olan canlı ağırlık kayıtlarını içermektedir. Zaman içinde tekrarlanan ölçümlerin olduğu seviye-1'de toplamda 670 gözlem ve kuzuların olduğu seviye 2'de 52 gözlem bulunmaktadır. Bu çalışmada, çok seviyeli modelleme yapısı içinde beş farklı model kullanılarak cinsiyet, doğum tipi ve doğum ağırlığı gibi zamana bağlı olmayan kovaryet etkilere ilişkin parametre tahmini yapıldı. En iyi model seçimi için LRT, AIC ve BIC kullanıldı. Veri setini en iyi açıklayan "*Conditional Quadratic Growth Model-B*" olarak belirlendi. Çok düzeyli analiz, kuzularda doğrusal ve ikinci dereceden büyümenin önemli olduğunu gösterdi. Çalışmanın sonuçlarına göre, bireysel büyüme oranının önemli olduğu hayvancılık çalışmalarında bireysel büyüme eğrileri, çok düzeyli modelleme kullanılarak araştırılabilir.

Anahtar Kelimeler: Tekrarlamalı veri; Çok düzeyli modeller; Sınıf içi korelasyon; Bireysel büyüme modelleri

1 The part of the study was presented as a poster in conference named "Agriculture for Life, Life for Agriculture" in Romania.

1. Introduction

As in various study fields, hierarchical or clustered data structure is commonly encountered in animal studies. Some examples are as follows; milk production of the cows nested within sires (Simsek & Fırat 2011), milk fat ratio of the sheep nested within herds, egg production of the chicken nested within cocks, carcass weight of the sheep nested within sires. All are good examples for a two-level structure. In the example of carcass weight from the sheep nested within sires, sheep are level-1 units and sires are level-2 units. When compared with sheep of different sires, it is inevitable that carcass weights of the sheep from the same sire are more similar. In the last 20 years, multilevel modeling (Goldstein 1999) has become a standard approach in the analysis of such data. Multilevel models are mixed effects models containing both fixed effects and random effects which may take the form of either random intercepts or random coefficients, and are named random coefficient models (Leeuw & Kreft 1986) or hierarchical models (Bryk & Raudenbush 1986; Raudenbush & Bryk 2002) in the literature.

Repeated data, which are naturally dependent, represent all observations of a variable obtained from the same individual sequentially over time. It means that observations on the same individual are related to each other (Singer & Willett 2003; Hedeker 2004; Hedeker & Gibbons 2006). While the assumption of independence of the data is violated, predictions which are made using traditional statistical analyses such as ANOVA and ordinary least-square (OLS) are biased and Type 1 errors increase (Peugh 2010; Shek & Ma 2011). In the recent years, multilevel modelling has been used in the analysis of data in repeated structure (Chen & Cohen 2006; DeLucia & Pitts 2006; Peugh 2010; Shek & Ma 2011). Repeated data are analyzed as a two-level model in multilevel modelling. The measurements obtained repeatedly over time have been classified among individuals; repeated measurements represent level-1 units, while individuals/animals represent level-2 units (Singer 1998; Singer & Willett 2003; Hedeker 2004; Hedeker & Gibbons 2006). The fact that multilevel modelling is used for repeated measures data has some advantages over traditional methods. It does not require a balanced data structure. In other words, the same number of repeated measurements is not necessary for each individual (Green et al 1998). Both the mean change in the population over time and individual changes may be predicted. Parameter estimation is performed for both time-variant and time-invariant independent variables. The best covariance structure for the model may be chosen (Hedeker 2004; Hox 2010; Shek & Ma 2011). Furthermore, multilevel analysis can be easily performed for the models with three and more level (Lancelot et al 2000).

The aim of this study was to compare the individual growth curves models and to detect individual differences in the growth rate by a performing multilevel analysis. For this aim, firstly the appropriate covariance structure was selected, and then the models were compared to find the best model explaining the change in the individual growth curves of lambs.

2. Material and Methods

2.1. Material

The study was carried out at a Research and Application Farm of Yuzuncu Yil University in Van, Turkey. Animal material of the study consisted of (Ile de France x Akkaraman (B_1)) x Karakas crossbred lambs. The live weights of 52 lambs were recorded at two-weekly intervals from birth to 182 days of age. In the data set, 6 lambs had 12 and the remaining 46 lamb had 13 measurements obtained in different time points. Independent variables included in the model were gender, birth type and birth weight. The total number of observations was 670 in level-1 units which were repeated measurements, while level-2 which was the level of lambs which had 52 observations.

Five models were constructed for the data set in the study and were analyzed using SAS, 9.3 (SAS 2014) and MLwiN (2.02) statistics software (MLwiN 2009).

2.2. Method

Model I (Unconditional Mean or Null Model): In this model which is the baseline for 'Unconditional Linear Growth Model', only intercept is present and is known as 'null' model.

Level-1: $Y_{ti} = \pi_{0i} + e_{ti}$

Level-2: $\pi_{0i} = \beta_{\mathbf{0}} + u_{0i}$

The Equation 1 is obtained when level-2 items are put into their place in level-1.

$$Y_{ti} = \beta_{00} + u_{0i} + e_{ti} \quad (1)$$

Where; Y_{ti}, live weight of the lamb *i* at *t* time; β_{00}, grand-mean of live weight of the lambs; u_{0i}, deviations in a lamb's live weight mean around the grand-mean (β_{00}); e_{ti}, deviation between observed and expected live weights of the lamb. Assumptions made for level-2 (u_{0i}) and level-1(e_{ti}) error terms are $u_{0i} \sim N\left(0, \sigma^2_{u_0}\right)$ and $e_{ti} \sim N\left(0, \sigma^2_e\right)$, respectively. This model (Equation 2) is important to test the variance between level-2 units and calculate intra-class correlation coefficient (ICC) (Goldstein 1999; Raudenbush & Bryk 2002; Singer & Willett 2003).

$$ICC = \frac{\sigma^2_{u_0}}{\sigma^2_{u_0} + \sigma^2_e} \quad (2)$$

Model II (Unconditional Linear Growth Model): It is used to describe the time-dependent change in repeated measurements. Thus, individual variation in growth rate is examined and the model does not include any independent variable, except time (Equation 3).

Level-1: $Y_{ti} = \pi_{0i} + \pi_{1i} T_{ti} + e_{ti}$

Level-2: $\begin{aligned} \pi_{0i} &= \beta_{00} + u_{0i} \\ \pi_{1i} &= \beta_{10} + u_{1i} \end{aligned}$

$$Y_{ti} = \beta_{00} + \beta_{10} T_{ti} + u_{1i} T_{ti} + u_{0i} + e_{ti} \quad (3)$$

Where; Y_{ti}, described in Model I; T_{ti}, time variable showing *t*. measurement time for the lamb in *i*. order; β_{00}, intercept which is the grand-mean live weight of the lambs at the zero time point; β_{10}, regression coefficient namely slope which is the mean change in live weight over time; u_{0i} and u_{1i}, level-2 error term; e_{ti}, level-1 error term. All error terms have a normal distribution. $\mathfrak{u}_i$ and e_{ti} errors are independent of each other and error terms in the same level are correlated to each other. So, $e_{ti} \sim N(0, \sigma^2_e)$, $\mathfrak{u}_i = [u_{0i}, u_{1i}]^T \sim N(0, \Omega_u)$,

where $\Omega_u = \begin{pmatrix} \sigma^2_{u_0} & \sigma_{u_{01}} \\ \sigma_{u_{01}} & \sigma^2_{u_1} \end{pmatrix}$

Model III (Conditional Linear Growth Model): In addition to Model II, this model also has the variables that do not change over time and these are written in level-2 (Equation 4).

Level-1: $Y_{ti} = \pi_{0i} + \pi_{1i} T_{ti} + e_{ti}$

Level-2: $\begin{aligned} \pi_{0i} &= \beta_{00} + \beta_{01} Z_i + u_{0i} \\ \pi_{1i} &= \beta_{10} + \beta_{11} Z_i + u_{1i} \end{aligned}$

$$Y_{ti} = \beta_{00} + \beta_{10} T_{ti} + \beta_{01} Z_i + \beta_{11} T_{ti} Z_i + u_{1i} T_{ti} + u_{0i} + e_{ti} \quad (4)$$

Where; fixed and random effects described in Model II; Z_i, time-invariant variables; β_{01}, effect of the time-invariant variables; β_{11}, interaction effect of these effects and the time. In this study the variables are the genders of the lambs, birth type and birth weight.

Model IV (Conditional Quadratic Growth Model-A): This model includes linear end quadratic effects of the time. These effects take place in level-1 equation in Model IV.

Level-1: $Y_{ti} = \pi_{0i} + \pi_{1i} T_{ti} + \pi_{2i} T^2_{ti} + e_{ti}$

Level-2: $\begin{aligned} \pi_{0i} &= \beta_{00} + \beta_{01} Z_i + u_{0i} \\ \pi_{1i} &= \beta_{10} + \beta_{11} Z_i + u_{1i} \end{aligned}$

Generally for Model IV, a single equation is written using level-2 and level-1 (Equation 5).

$$Y_{ti} = \beta_{00} + \beta_{10}T_{ti} + \beta_{01}Z_i + \beta_{11}T_{ti}Z_i + \beta_{2i}T_{ti}^2 + u_{1i}T_{ti} + u_{0i} + e_{ti} \quad (5)$$

Where; T^2, quadratic effect of the measurement time; β_{2i}, average amount of change caused by the quadratic effect of time in the response variable.

Model V (Conditional Quadratic Growth Model-B): This model pays attention to the similar effects as Model IV. Its only difference is that the quadratic effect is a random effect in the model. This is given in level-2 equation.

Level-1: $Y_{ti} = \pi_{0i} + \pi_{1i}T_{ti} + \pi_{2i}T_{ti}^2 + e_{ti}$

$$\pi_{0i} = \beta_{00} + \beta_{01}Z_i + u_{0i}$$

Level-2: $\pi_{1i} = \beta_{10} + \beta_{11}Z_i + u_{1i}$

$$\pi_{2i} = \beta_{20} + \beta_{21}Z_i + u_{2i}$$

A general equation is written for Model V by using the equations of level-1 and level-2 (Equation 6).

$$Y_{ti} = \beta_{00} + \beta_{10}T_{ti} + \beta_{20}T_{ti}^2 + \beta_{01}Z_i + \beta_{11}T_{ti}Z_i + \beta_{21}T_{ti}^2Z_i + u_{1i}T_{ti} + u_{2i}T_{ti}^2 + u_{0i} + e_{ti} \quad (6)$$

Where; T^2, quadratic effect of time; β_{20}, mean change in response variable by quadratic effect of time; $T_{ti}^2Z_i$, interaction effect of quadratic effect of time and the time-invariant variables; β_{21}, mean change in response variable based on this interaction effect; u_{2i}, term of level-2 error recently added to the model. In this case, $\mathrm{u}_i = [u_{0i}, u_{1i}, u_{2i}]^T \sim N(0, \Omega_u)$ assumption is made for level-2 error types of

$\mathrm{u}_i = [u_{0i}, u_{1i}, u_{2i}]^T$. Where $\Omega_u = \begin{pmatrix} \sigma_{u_0}^2 & & \\ \sigma_{u_{01}} & \sigma_{u_1}^2 & \\ \sigma_{u_{02}} & \sigma_{u_{12}} & \sigma_{u_2}^2 \end{pmatrix}$

To obtain the variance-covariance matrix that is suitable for the study data, Unstructured (UN), Compound Symmetry (CS), first-order autoregressive (AR(1)) and Toeplitz covariance structures were used for each of the models (Littell et al 2000).

2.2.1. Model selection

The likelihood ratio test (LRT) has been used to compare two models. It is computed by the difference in deviance for the two models, which one of them is reduced and the other is current model (Equation 7).

$$LRT = [(-2LL_{Reduced\ Model}) - (-2LL_{Current\ Model})] \quad (7)$$

Akaike Information Criteria (AIC) and Bayesian Information Criteria (BIC) are the cohesion criteria used for the model selection. AIC uses log-likelihood and parameter number (Akaike 1974) and BIC uses also sample size together with log-likelihood and parameter (Equation 8) number (Schwarz 1978).

$$AIC = -2LL + 2k$$
$$BIC = -2LL + k\ln(n) \quad (8)$$

Where; *LL*, log-likelihood the model; *k*, number of estimated parameters in the model; *n*, number of observations.

The model having minimum AIC and BIC values is determined as the best model when selecting the model.

3. Results and Discussion

According to the results of Model I with UN and Toeplitz covariance structures having the minimum AIC and BIC values, both level-1 ($\sigma_e^2 = 91.58$) and level-2 variances ($\sigma_{u_0}^2 = 12.26$) were significant (P<0.05, P<0.0001). The ICC value was obtained using the Equation 2 (Goldstein 1999) indicated that the observations were not independent from each other and 12% of the change in the live weight was due to the difference between the lambs.

$$ICC = \frac{\sigma_{u_0}^2}{\sigma_{u_0}^2 + \sigma_e^2} = 0.1181 \cong 0.12$$

In studies with repeated measures data, *ICC* value was as expected (Ip et al 2011), but this criterion alone is not always sufficient. So, effective sample size ($n_{eff} = n/[1 + (n_{clus} - 1)]ICC$) and design effect ($DE = 1 + (n_{clus} - 1)ICC$) was calculated as 263 and

2.6, respectively. Since the design effect value was more than 2 (Peugh 2010; Simsek & Fırat 2011) and ICC value was different from zero (Gulliford et al 1999; Smeeth & Ng 2002; Ip et al 2011), the performance of multilevel analysis was required.

Based on the covariance structure (UN, CS, AR(1) and Toeplitz) the deviance and AIC and BIC values for Model II, III, IV and V are presented in Table 1. This table revealed that UN covariance structure for each model had the minimum deviance, and AIC and BIC values (Littell et al 2000). Thus, UN was accepted as the best covariance structure for all models. The goodness of fit statistics was sought for to find the best model among the models with UN covariance. The results of likelihood-ratio test for models are shown in Table 2. The difference in the deviance between the two models, $[(-2LL_{Reduced\ Model})-(-2LL_{Current\ Model})]$ proved that Model II was significantly better than Model I (P<0.001), Model III was better than Model II (P<0.01), Model IV was better than Model III (P<0.001) and Model V was better than Model IV ($\chi^2_3 = 324.6$, P<0.001). Additionally, it can be remarked that the Model V was the best fit to the data among other models. This result was also supported by AIC and BIC values given Table 1. Because the best model is the model which has the minimum AIC and BIC values, Model V with UN covariance structure was determined as the best model.

The multilevel analysis results obtained by using UN covariance structure for Model II, III, IV and V are presented in Table 2.

As shown in Table 2, while the intercept for Model V was not significant, random change of each lamb around the intercept was significant ($\sigma^2_{u_0} = 0.7270$, P<0.001). This finding was supported by previous studies conducted on multilevel models in various areas (Leeden 1998; Lancelot et al 2000; Kristjansson et al 2007; Peugh 2010). It revealed that initial weight (14 days of age) of each lamb was different from each other. The fact that linear effect of time was significant in

Table 1- The models used in the study and the goodness of fit statistics with respect to their covariance structures

Models	*Covariance structure*	*-2LL (Deviance)*	*AIC*	*BIC*
Model II	Unstructured (UN)	2940.1	2952.1	2963.8
	Compound symetry (CS)	3009.3	3017.3	3025.1
	First-order autoregressive AR(1)	3009.3	3017.3	3025.1
	Toeplitz (TOEP)	3009.1	3019.1	3028.9
Model III	Unstructured (UN)	2899.4	2919.4	2939.0
	Compound symetry (CS)	2915.1	2931.1	2946.7
	First-order autoregressive AR(1)	2915.1	2931.1	2946.7
	Toeplitz (TOEP)	2914.4	2932.4	2949.9
Model IV	Unstructured	2370.8	2392.8	2414.2
	Compound symetry (CS)	2430.8	2450.8	2470.3
	First-order autoregressive AR(1)	2430.8	2450.8	2470.3
	Toeplitz (TOEP)	2430.8	2450.8	2470.3
Model V	Unstructured	2046.2	2074.2	2101.6
	Compound symetry, (CS)	2740.6	2758.6	2776.1
	First-order autoregressive AR(1)	2740.6	2758.6	2776.1
	Toeplitz (TOEP)	2321.4	2343.4	2364.8

Table 2- The analysis results of multilevel modeling

	Model II estimate (SE)	*Model III estimate (SE)*	*Model IV estimate (SE)*	*Model V estimate (SE)*
Fixed effect				
Intercept	7.8629 (0.2760)***	-3.0477 (2.0884)	-5.2883 (2.0898)*	0.1258 (1.4181)
Time	2.5473 (0.0539)***	1.5502 (0.2944)***	2.7724 (0.2973)***	2.6227 (0.3047)***
BW		1.9307 (0.2900)***	1.9307 (0.2900)***	1.1918 (0.2015)***
Gender		0.6197 (0.4043)	0.6197 (0.4043)	0.04131 (0.2694)
BT		0.5710 (0.4747)	0.5710 (0.4747)	-0.09565 (0.3163)
Time* BW		0.2129 (0.06197)***	0.2129 (0.06197)***	0.2448 (0.06105)***
$Time^2$			-0.1018 (0.003454)***	-0.1018 (0.00856)***
Random effect				
$\sigma^2_{u_0}$	3.1016 (0.7786)***	1.1102 (0.3910)**	1.6288 (0.3883)***	0.7270 (0.2120)***
$\sigma_{u_{01}}$	0.4005 (0.1152)**	0.1994 (0.0729)**	0.1371 (0.07275)	0.06307 (0.1163)
$\sigma^2_{u_1}$	0.1341 (0.02969)***	0.1061 (0.0242)***	0.1165 (0.02419)***	0.5289 (0.1161)***
$\sigma_{u_{02}}$				0.008435 (0.01012)
$\sigma_{u_{12}}$				-0.03805 (0.008782)***
$\sigma^2_{u_2}$				0.00351 (0.00075)***
σ^2_e	3.1299 (0.1851)***	3.1299 (0.1851)***	1.2420 (0.07344)***	0.6037 (0.03744)***
Deviance	2940.1	2899.4	2370.8	2046.2
Number of parameter	6	10	11	14
χ^2	922.41***	40.7**	528***	324.6***
Degrees of Freedom	3	4	1	3

BW, birth weight; BT, birth type; *, P<0.05; **, P<0.01; ***, P<0.001

Model V meant that the lambs gained an average 2.6227 kg live weight every 14 days (P<0.001). The level-2 error term $\sigma^2_{u_1} = 0.5289$ showing the random change with respect to the linear effect of time was significant (P<0.001). Green et al (1998), Lancelot et al (2000), Dudley et al (2009) and Peugh (2010) proven that linear effect of time and its random change was significant. According to this model, the live weight gained every 14 days was not a stable value and varied from one lamb to another. In this model, quadratic effect of time was also examined and found significant (P<0.001). Supporting the current result, numerous authors (Green et al 1998; Lancelot et al 2000; Dudley et al 2009) also reported a significant quadratic time effect. This significant effect indicated that each lamb had an inflection point in the live weight gain and gradually decrease of weight after the point (-0.1018). The analysis clearly showed that linear growth trajectory (2.6227) was higher than the quadratic growth rate (-0.1018). So, initially, the live weight gain of the lambs increased linearly, and then then this linear increase slowed down. Quadratic effect, such as the linear effect of time, was also included in

random part of the model and the variation of this effect between lambs was detected to be significant ($\sigma^2_{u_2} = 0.00351$, P<0.001). The covariance of the linear and quadratic effect of time was significant ($\sigma_{u12} = -0.03805$, P<0.001). These results were supported by the Green et al (1998) and Lancelot et al (2000) who studied tree-level model. This means that quadratic growth started earlier in the lambs which had a faster linear growth. On the other hand, quadratic growth started later in the lambs which had a slower linear growth.

The lamb's birth weight, and the interaction between birth weight and linear effect of time was significant (P<0.001, P<0.001). Accordingly, every 1 kg increase in birth weight led to an average 1.1918 kg increase in the live weight. Each unit increase in time led to an average 2.6227 kg increase in the live weight. Each unit increase in interaction effect led to an average 0.2448 kg increase in the live weight. Since the value of the interaction (time*BW) is positive, significant interaction effect also indicated that high-birth weight lambs would have a higher live weight during the growth period.

Individual growth curves of lambs obtained based on Model V are presented in Figure 1. Figure 1 had three significant random coefficients because of Model V; the random coefficients of intercept, linear and quadratic effects. It means that the lambs had different intercept, linear, and quadratic effects from each other.

Figure 2 represents some details in the individual growth curves. The lamb with the id_13 had the highest initial live weight. Its live weight increased linearly until 182 days of age. Similarly, the lambs with the id_52 and id_6 had a linear increase in live weight up to 182 days of age. The live weight of lamb with the id_21 increased linearly up to 112 days of age (about 8. point). After this point the quadratic increase began for id _21. The lamb with id_32 had the lowest initial live weight and the increase in its live weight was slower than the others. Therefore, the lambs with the id_13 and id_15 may be preferred over the other lambs due to their late start of quadratic growth on the 182 days of age.

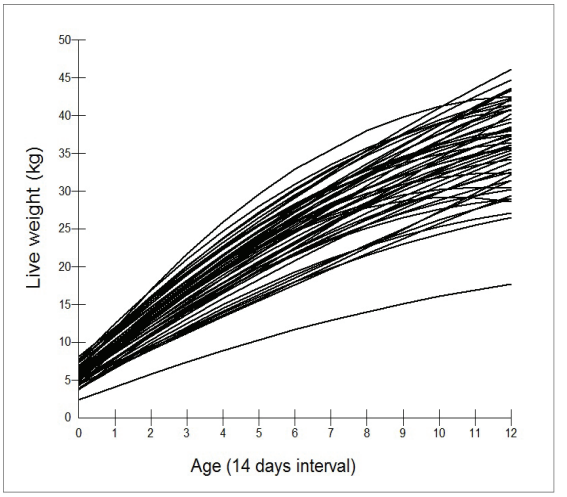

Figure 1- Individual growth curves of the lambs between 14 to 182 days of age

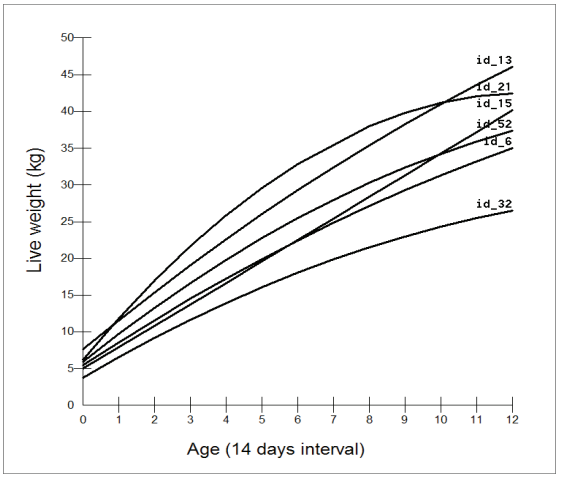

Figure 2- Individual growth curves of randomly selected lambs between 14 to 182 days of age

4. Conclusions

The data sets may have unbalanced repeated data structures, time-variant and time invariant variable, in which case, traditional methods become insufficient. A multilevel analysis is preferred by researchers because it has a powerful and flexible

procedure for such data set. The multilevel modeling can be used to investigate the individual growth rate of animals. The growth performance is one of the economically important traits in livestock. There are differences between breeds within a species, and individuals within a breed in respect of the rate of growth. An individual growth curve can be used to determine whether an animal's growth performance is improving, as well as whether the animal is growing faster or slower than other animals. In this study, possible applications of individual growth curves models for repeated data in crossbred lambs were discussed. It was determined that the best model was the Model V. According to the results of the study, the same-age lambs had different growth rates caused by their birth weight. Therefore, the individual growth curves may be indicators for animals which are at high risk of low performance. The multilevel analysis based on Model V indicated that linear and quadratic growth in lambs was significant. As a result, each individual's growth curve can be investigated using multilevel modeling in animal studies which is an important parameter of the individual growth rate.

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Antiviral and Antifungal Activity of Biologically Active Recombinant Bouganin Protein from *Bougainvillea spectabilis* Willd

Abdullah GÜLLER[a], Hikmet Murat SİPAHİOĞLU[b], Mustafa USTA[a], Emre DEMİRER DURAK[a]

[a]*Van Yuzuncu Yil University, Faculty of Agriculture, Department of Plant Protection, Van, TURKEY*

[b]*Inonu University, Faculty of Agriculture, Department of Plant Protection, Malatya, TURKEY*

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Corresponding Author: Hikmet Murat SİPAHİOĞLU, E-mail: murat.sipahioglu@inonu.edu.tr, Tel: +90 (422) 846 12 25
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ABSTRACT

Bouganin antiviral protein (BAP) gene, one of the ribosome inactivating proteins, isolated from *Bougainvillea spectabilis* Willd. was cloned, expressed and the antiviral and antifungal activities were investigated. The full-length bouganin antiviral protein gene was amplified by reverse transcription-PCR using mRNA as template extracted from mature leaves. The coding region of bouganin gene was cloned into prokaryotic expression vector pETDuet-1 after amplification with end to end gene specific primers. The recombinant plasmid was transformed into *Escherichia coli* cells BL21(DE3)pLysS and the expression of BAP gene was induced by isopropyl β-D thiogalactopyranoside (IPTG). Bouganin antiviral protein having a molecular mass of 28 kDa has been isolated from transformed bacterial colonies. Antiviral activity of bouganin was assayed against *Zucchini yellow mosaic virus* (ZYMV) by a mechanical inoculation test. The antifungal activity of purified recombinant protein was tested against pathogenic and non-pathogenic *Rhizoctonia solani, Trichoderma harzianum, and Fusarium oxysporum* fungi using disc diffusion method. The increased amount of antiviral protein reduced the disease severity caused by ZYMV. The bouganin antiviral protein was inhibited the growth of *R. solani* by 30.7% and of *T. harzianum* by 20% after 72 h compared to control. No growth inhibition was observed for *F. oxyporum*. All plants including controls treated with *in vitro* expressed BAP protein exhibited severe growth reduction compared with negative control (not treated) plants.

Keywords: *Bougainvillea spectabilis*; Antimicrobial protein; BAP gene; Expression; Antiviral and antifungal activities

Bougainvillea spectabilis Willd. Bitkisinin Biyolojik Olarak Aktif Rekombinant Bouganin Proteininin Antiviral ve Antifungal Aktivitesi

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Hikmet Murat SİPAHİOĞLU, E-posta: murat.sipahioglu@inonu.edu.tr, Tel: +90 (422) 846 12 25
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ÖZET

Bougainvillea spectabilis Willd. bitkisinden ribozom inaktive eden proteinlerden olan Bouganin antiviral protein (BAP*)* geni izole edilerek klonlanmış, ifade edilmiş, antiviral ve antifungal özellikleri araştırılmıştır. Bouganin proteinini

kodlayan genin tamamı bitkinin olgun yapraklarından ekstrakte edilen mRNA'dan Reverse Transkripsiyon-PCR yöntemi ile çoğaltılmıştır. Gen, uçtan uca bölgeyi kapsayacak şekilde tasarlanan spesifik primerler yardımı ile prokaryotik ekspresyon vektörü pETDuet-1'de klonlanmıştır. BAP geninin ifadesi rekombinant plazmitin *Escherichia coli* (BL21(DE3)pLysS) hücrelerine transferi sonrası isopropyl β-D thiogalactopyranoside (IPTG) ile teşvik edilmiştir. Molekül ağırlığı yaklaşık 28 kDa olan BAP transforme edilmiş bakterilerden izole edilmiştir. BAP 'ın antiviral aktivitesi *Zucchini yellow mosaic virus* (ZYMV) kullanılarak mekanik inokulasyon yöntemi ile araştırılmıştır. Antifungal aktivitenin belirlenmesi disk difüzyon metodu yardımıyla patojen ve patojen olmayan *Rhizoctonia solani, Trichoderma harzianum* ve *Fusarium oxysporum* fungusları ile araştırılmıştır. BAP uygulama miktarı arttıkça ZYMV'nin neden olduğu hastalık şiddetinin azaldığı tespit edilmiştir. BAP 72 saatlik kontrol uygulaması ile karşılaştırıldığında, *Rhizoctonia solani*'nin gelişimini % 30.7, *Trichoderma harzianum*'unun gelişimini ise % 20 oranında inhibe etmiştir. *Fusarium oxysporum*'un gelişiminde herhangi bir inhibisyon etkisi gözlenmemiştir. *In vitro*'da ifade edilen BAP proteininin uygulandığı tüm bitkiler (sadece BAP protieni uygulanan kontrol grubu da dahil) uygulama yapılmayan bitkilere oranla şiddetli gelişme geriliği sergilemiştir.

Anahtar Kelimeler: *Bougainvillea spectabilis*; Antimikrobiyal protein; BAP geni; Ekspresyon; Antiviral ve antifungal aktivite

1. Introduction

In nature, many plants contain proteins with antiviral activity (Barbieri et al 1993). Antiviral proteins (AVPs) are unable to bind the elongation factors. This leads to the interruption of protein translation step and then, it directly inhibits the protein synthesis on ribosomes (Bolognesi et al 2000; Qi et al 2004). Ribosome-inactivating proteins (RIPs) possess site-specific RNA N-glycosidase activity catalyzing the removal of a single adenine base from a conserved loop of the 28S rRNA of eukaryotic ribosomes (Endo et al 1987) and the 23S rRNA of prokaryotic ribosomes (Hartley et al 1991). Some of these AVPs are belong to the family of RIPs (Barbieri et al 1993).

RIPs have been reported in fungi, bacteria, plants, and algae (Girbés et al 2004). RIPs are common among the plants and generally found in families, such as *Poaceae, Euphorbiaceae, Cucurbitaceae*, and particularly to *Caryophyllales* superorder (Grasso & Shepherd 1978; Stirpe et al 1983; Stirpe & Barbieri 1986; Kwon et al 2000). In plants, the presence of the RIPs has been reported to occur in leaves, roots, seeds, and tubers in different concentrations (Stirpe et al 1992).

Based on their functions and structures, the RIPs are commonly classified into two subgroups. Type I proteins, which their molecular masses ranges between 28 and 35 kDa, consist a single polypeptide chain (Stirpe et al 1983). Type II proteins, which is linked to a cell-binding B chain, consist of a catalytically active A chain (Barbieri & Stirpe 1982; Stirpe et al 1992).

AVPs have been the focus of many researches because of their selective toxicity function. Therefore, enzymatic activities of RIPs have been used; in plant defence (Logemann et al 1992; Lodge et al 1993; Madin et al 2000), AIDS (Scadden et al 1998; Donayre Torres et al 2009), therapeutics against tumors (Schnell et al 1996; Wang et al 1998a), anti-fungal activities (Wang et al 1998b) and even biological weapon (Weiner 1996; Christopher et al 1997). RIPs have also been reported to antiviral (Sadasivam et al 1991) and antibacterial (Hakuba et al 1991; Hartley et al 1991) properties.

Bougainvillea spectabilis Willd. is an ornamental plant belonging to Nyctaginaceae family and commonly known Bougainvillea or Great Bougainvillea (Kobayashi et al 2007). A ribosome inactivating protein gene, bouganin, has been isolated from *B. spectabilis*. Based on SDS/PAGE analysis, the molecular mass of single-chain bouganin is estimated approx. 29 kDa (Bolognesi et al 1997). With the present study, we targeted to clone and express the RIP gene from Bougainvillea and test antiviral and antifungal activities by constructing the prokaryotic expression plasmid

encoding BAP gene. The inhibitory effects of the recombinant BAP was documented.

2. Material and Methods

2.1. Plant material

Bougainvillea (*B. spectabilis*) plant obtained from western part of Turkey was grown in a growth chamber at Van Yuzuncu Yil University Department of Plant Protection. Seeds of squash (*Cucurbita pepo* L.) plant were obtained from the regional certified resources and the seedlings were grown in pots containing sterile torf. All plants were grown at 26 °C with a 16 h/8 h light/dark period.

2.2. Test microorganisms

A highly virulent strain of ZYMV was used to test antiviral activities. The virus isolate was maintained on squash (*Cucurbita pepo* L.) seedlings in climate chamber. Viral inoculum was prepared in sodium phosphate buffer (0.01 M, pH 7.2) from systematically infected leaves of *C. pepo.* Three fungal isolates, *Fusarium oxysporum* (pathogen), *Rhizoctonia solani* (pathogen) and *Trichoderma harzianum* (non-pathogen) were used to test for the antifungal assays. The fungal isolates were cultured and maintained on potato dextrose agar (DIFCO, USA) and kept at the temperature of 25 °C. All viral and fungal isolates used in this study were supplied from the Department of Plant Protection of Van Yuzuncu Yil University.

2.3. Isolation and molecular cloning of a full-length BAP gene

The extraction of mRNA was made from the mature leaves of *B. spectabilis* with silica-based method (Foissac et al 2001). Reverse transcription was carried out using a commercial kit (RevertAid First Strand cDNA kit, Vilnius, Thermo-Fermantas-Lithuania). The complete bouganin gene was isolated by PCR amplification. The gene specific primers (B-*Bam* HI-F-5'-*CAGT*GGATCCGAT GGGTTGGTGGGCTATCAT-3' and B-*Sac*I-R-5'-*CAGT*GAGCTCTTAGGCAATGTTTGGCT CTAGT-3') were designed for the amplification of full length bouganin gene based on registered sequences in GenBank (GenBank access number AF445416) using Vector NTI Software. The primers contained *BamHI* and *SacI* restriction sites and four unrelated nucleotide residues at their 5'end in order to facilitate the cloning into the pETDuet-1vector (Novagen, Darmstadt, Germany). The gene was then sub-cloned into pGEM-T Easy vector (Promega, USA). The following PCR cycle was used to amplify the BAP gene: denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 1 min, annealing at 50 °C for 1 min, and an extension of 72 °C for 1 min, with a final extension of 72 °C for 10 min. A 50 µL of PCR mixture contained; 1 µL of each primer (100 pmol), 1 µL of dNTPs (10 mM each), 3 µL of $MgCl_2$ (25 mM), 2 µL of cDNA, 0.4 µL of Go Taq G2 Hot Start DNA polymerase, 5 µL of 10× reaction buffer and 36.6 µL of DNase free water. The amplified DNA was electrophoresed in 1% agarose gel. DNA bands were purified using a gel extraction kit (Isolate II PCR and Gel Kit, Bioline, Germany).

2.4. Construction of vector for inducible expression of BAP gene and the purification of recombinant His-tagged bouganin protein

BamHI and *SacI* sites of pETDuet-1, containing 6× His Tag coding sequence, were used to construct the recombinant plasmid. The BAP bearing plasmids were then transformed into competent cells of *E. coli* BL21(DE3)pLysS cells using a micropulser (BioRad, USA). Luria-Bertani (LB) broth agar containing ampicillin was used to grow transformed cells at 37 °C overnight. BAP gene bearing clones were determined by colony-PCR, sequence analysis and by restriction endonucleases. One of the sequenced clones was used for further study. Positively identified clone was grown on an LB broth to OD 600 0.6. With a constant shaking at 25 °C for 16 h, the bacterial growth was stimulated by adding IPTG to a final concentration of 0.4 mM. The growth media was centrifuged and the bacterial cells were pelleted and resuspended with proteinase inhibitor (Roche, Germany). After adding 0.5 mL of TrisHCl (pH 7.5), 1 mL of NP40 (10%), 25 µL of $MgCl_2$ (1 M), 7 µL of 2-mercaptoethanol and

20 µL of DNase I (10 U mL^{-1}), the suspension was sonicated four times (3-5 s) in ice, then, the mixture was incubated at 4 °C for 45 min on a shaker. After adding 0.3 g of NaCl (final concentration 0.5 M) the protein extract was ultracentrifuged at 30,000 rpm for 30 min (4 °C). Recombinant His-tagged BAP protein was purified by chromatography on a Ni2+-NTA agarose resin column. After washing the column with TL buffer (2.5 mL 1M Tris-HCl pH 7.5, 1.46 g NaCl, 5 mL 10% NP40, 35 µL 2-mercaptoethanol, 1 mL 1M imidazole pH 8.0) BAP protein was eluted with TE buffer (50% of imidazole and 50% of TL buffer). Fraction containing BAP protein was analyzed by 12% SDS-PAGE electrophoresis gel along with molecular weight markers (Laemmli 1970; Sankian et al 2007). The protein concentrations were determined according to the method of Bradford (Bradford 1976)

2.5. Bioassay of BAP protein for antiviral activity

The purified BAP protein was suspended in 10% DMSO at final concentrations of 2, 4 and 8 µL and used as stock solutions and applied to squash cotyledons by using a sterile pipette tip periodically through 4 days. Then, for each treatment, ZYMV extract (100 µL) was inoculated on squash cotyledons (Schmitt et al 2001). The development of symptoms was observed for three weeks and the symptoms were recorded as scale value. The controls were consisted of "only BAP protein", "only DMSO 10%", "only elution buffer (TE) (50% imidazole pH 8.0+50% TL buffer)", "only ZYMV", and 10% DMSO+Elution buffer. No treated plants were also used as controls (Table 1). For each treatment, the cotyledons of three *C. pepo* plants were used. All of them were tested in three replicates in each run of the experiments.

Antiviral activity of BAP protein was measured by a disease severity index (DSI) assay using *C. pepo* cotyledons and *Zucchini yellow mosaic virus* (ZYMV). A 0 to 5 rating scale was adapted to score the disease severity index (DSI) described by Liu et al (1995), where 0= no symptoms, 1= leaf spotting at least fifty percent of the leaf surface area, 2= leaf spotting at least more than fifty percent of leaf surface area, 3= mottle and mosaics, 4= severe mottle, mosaics and leaf deformations, 5= severe disease symptoms on leaf surface including shoestring symptom. DSI was calculated based on the Equation 1.

Table 1- The groups created for determination of antiviral activity of bouganin protein and control groups

Groups	*Treatments*
1	10% DMSO+2 µL BAP
2	10% DMSO+4 µL BAP
3	10% DMSO+8 µL BAP
4	10% DMSO+2 µL BAP+ZYMV
5	10% DMSO+4 µL BAP+ZYMV
6	10% DMSO+8 µL BAP+ZYMV
7	Only ZYMV (PC)
8	No treatment (NC)
9	Only 10% DMSO
10	Only TE
11	10% DMSO+TE

$$\text{Disease severity index (DSI)} = \Sigma(A \times B)/(T \times D) \times 100 \quad (1)$$

Where; *A*, scale number; *B*, number of symptomatic leaves; *T*, total number of leaves; *D*, highest scale.

Statistical significance of the treated groups mean with that of control groups were analyzed by SAS 9.4 (SAS 2014) package program. The General Linear Model analysis was used to determine the differences between the groups in the study followed by Duncan's multiple range tests to separate means. Differences were considered statistically significant if $P<0.05$. To determine the presence of ZYMV, inoculated squash groups were tested by RT-PCR as described by Özer et al (2012). Before inoculation, the plants were exposed to recombinant BAP protein for 4 days and were kept in a growth chamber for 3 weeks. After BAP application, the squash plants were inoculated with ZYMV in the cotyledon leaf stage. The plants were then scored for ZYMV-induced symptom severity. The presence of viral RNA in the inoculated plants was tested by RT-PCR and fresh and dry weights of plants were measured.

2.6. Antifungal activity of BAP protein

The antifungal activity of BAP was tested *in vitro* by a radial growth inhibition as described by Schlumbaum et al (1986). The test was conducted using sterile 9 cm diameter petri dishes. Mycelial plugs (5 mm in diameter) were placed on to 2 cm aside from the outermost of each plate. A total of 50 µL filter sterilized antiviral protein and TE buffer was loaded onto the filter paper disks. As a group of control, different petri dishes were prepared for each fungus. The plates were incubated at 25 °C for 7 days for which the hyphae grew to outwards from the center. The inhibition effect was observed daily following the treatment (24, 48 and 72 h). The fungal growth was measured (mm) daily and percentage inhibition in growth calculated. Three replicates were formed for each fungus.

3. Results

3.1. Cloning and expression of the bouganin gene

The RT-PCR result revealed that only one specific DNA band, 893 bp in length, was visualized in agarose gel electrophoresis. After cloning in expression vector, the gene was sub-cloned in pGEM-T Easy vector. The purified recombinant plasmids were then sequenced bidirectionally to verify the authenticity of the amplicon. The sequencing report showed that the BAP gene shared 85% identity with other isoforms of BAP sequences from *Bougainvillae* species. The BAP gene has an initiation amino acid methionin (ATG), and terminated by alanin amino acid (GCC). The BAP gene contained a complete open reading frame and had no introns. The sequence obtained in this study has been assigned the GenBank with the accession no. KP096226.

3.2. SDS-PAGE analaysis of expressed and purified recombianant His-tagged bouganin protein

After the recombinant plasmid containing an N-Terminal 6× Histidin Tag sequence was transformed into the bacterial cells, the fused His-BAP protein was purified by utilizing Ni-NTA affinity column. Based on SDS-PAGE analysis it was shown that the BAP gene was expressed after IPTG induction. As expected, a 28 kDa fused BAP protein was observed. A very slight unrelated protein bands were seen on SDS-PAGE gel (Figure 1). The concentration of the purified His-tagged bouganin protein was determined as 46.8 µg mL^{-1}.

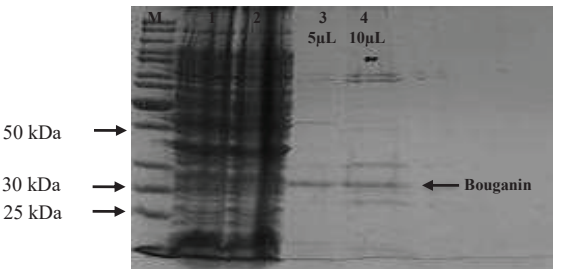


Figure 1- 12% SDS-PAGE electrophoresis gel analysis of the bouganin protein; 1, protein profile of IPTG-inducted *E. coli* containing recombinant plasmids; 2, protein profile of IPTG uninducted *E. coli* bacteria containing the recombinant plasmid; 3 and 4, purified bouganin protein profile; M, molecular protein size marker

3.3. Antiviral activity of BAP protein

The antiviral activity of recombinant BAP protein was examined by symptom expression on *C. pepo*. The results showed that the disease severity index (DSI) was 68.2%, 61.4% and 58.7% with the application of 2, 4 and 8 µL of purified BAP, respectively. The DSI of positive control (PC) was recorded as 72.9%. The most active amount was determined as 8 microliter of BAP protein, which exhibited a DSI of 58.7%. However, by using local lesion analysis Choudhary et al (2008) reported a high level of inhibition (~94%) against *Tobacco mosaic virus* (TMV). Our results demonstrate that the inhibitory activities of BAP against ZYMV are positively correlated with the amount. Therefore, the inhibition of infection and symptom expression by BAP were dose dependent between 2 and 8 µL (Figure 2).

Furthermore, pretreatment of squash leaves with BAP protein for four days before inoculation with ZYMV did not prevent the virus infection. All tested uninoculated squash leaves (controls) were negative while the all inoculated squash leaves

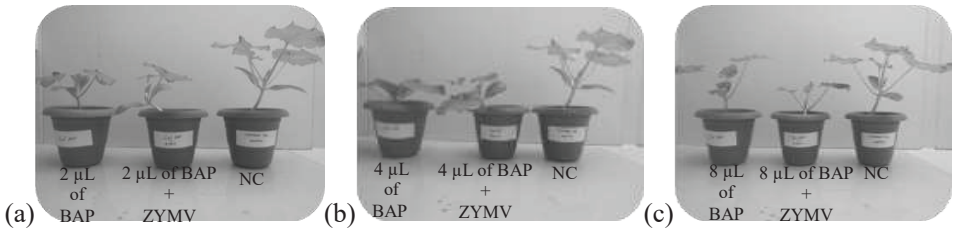


Figure 2- Experiment of bouganin antiviral protein (BAP) activity to *Zucchini yellow mosaic virus* (ZYMV); the cotyledon leaves were pretreated with 2, 4 and 8 µL of BAP in the presence of 10% DMSO for four days then, 100 µL of ZYMV inoculum were inoculated to the same leaves; the only purified BAP of 2, 4, and 8 µL were also kept as controls in the presence of 10% DMSO; NC, negative control

were reacted positive in RT-PCR tests 3 weeks after inoculation. DSI analysis of inoculated squash plants demonstrated that plants treated with 4 µL and 8 µL of BAP showed a significant reduction ($P<0.05$) in symptom expression compared with controls.

When BAP was applied with 10% DMSO to the leaves of squash plants, a severe reduction (50% or greater) occurred in plant size. The controls involving "only 10% DMSO", "only TE", and "only 10% DMSO+TE" showed no significant changes in plant size as compared to the control group (NC). Fresh and dry weights of BAP treated squash plants were also showed a severe decrease as compared to control plants. The obtained data was evaluated with the standard deviations and found significant statistically (Table 2). These results suggest that BAP protein blocks essential cellular functions needed for growth. The reduction was greater when ZYMV was inoculated after BAP treatment period. In those plants, besides delayed symptom expression as compared with positive controls, a severe growth reduction was also recorded.

3.4. Antifungal activity of BAP protein

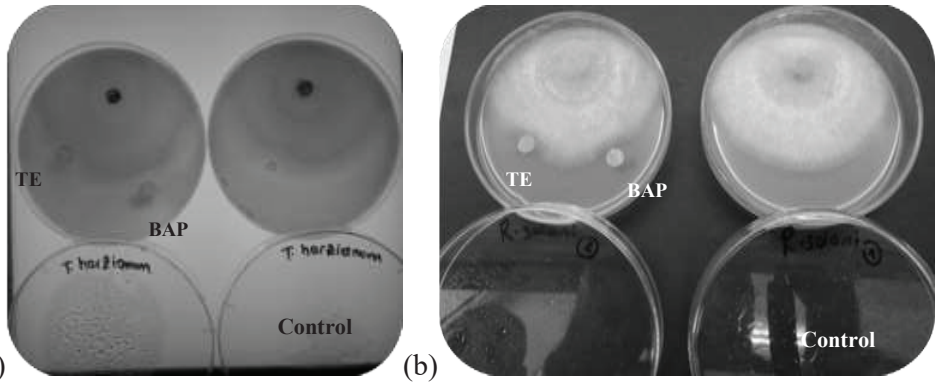
The results of antifungal activity of BAP against various fungi [*F. oxysporum* (pathogen), *R. solani* (pathogen), and a non-pathogenic fungus *T. harzianum,* (data not shown)] are shown in Figure 3. For the determination of antifungal activity, a 50 µL (46.8 µg mL^{-1} in 1 mM TE buffer, pH 7.5) of filter sterilized BAP suspension was used. It was shown that BAP inhibited the two of these fungi (Table 3).

Table 2- Average fresh and dry weights and statistical analysis of the squash plants used in this study

Groups[1]	*Dry weight (g)* $\left(\overline{X} \pm S_{\overline{x}}\right)^2$	*Fresh weight (g)* $\left(\overline{X} \pm S_{\overline{x}}\right)$
NC	4.54±0.12^{a}	21.40±0.34^{a}
Only ZYMV (PC)	3.87±0.08^{b}	14.40±0.23^{b}
Only TE	4.60±0.03^{a}	21.30±0.21^{a}
Only DMSO 10%	4.60±0.20^{a}	21.20±0.50^{a}
TE+DMSO	4.70±0.10^{a}	21.70±0.14^{a}
2 µL BAP	3.90±0.11^{b}	15.33±0.35^{b}
4 µL BAP	3.61±0.24^{bc}	14.56±0.34^{b}
8 µL BAP	3.40±0.09^{c}	13.81±0.79^{b}
2 µL BAP+ZYMV	3.72±0.05^{bc}	11.17±0.85^{c}
4 µL BAP+ZYMV	3.64±0.12^{bc}	10.47±1.43^{c}
8 µL BAP+ZYMV	3.55±0.12^{bc}	7.83±1.50^{d}

[1]; a, b, c, d means the difference between the averages with different letters in the same column is significant; [2], mean ± standard error of mean; BAP, Bouganin antiviral protein; DMSO, dimethylsulphoxide; ZYMV, Zucchini yellow mosaic virus; TE, Tris EDTAbuffer; PC, positive control; NC, no treatment

Based on time course experiment a moderate mycelia growth inhibition was observed with 50 µL purified BAP for up to 72 h of incubation. Two fungi *R. solani* and *T. harzianum*, exhibited a crescent-shaped zone of inhibition at their mycelial front (Figure 3). BAP was found to be most active on these two. No antifungal activity was detected against mycelial growth of *F. oxyporum*. TE buffer which was used as negative control did not showed any impact on the growth of fungi. Another remarkable finding in this study that bouganin protein was promoted the sporulation of T. *harzianum* (Figure 4).



(a) (b)

Figure 3- Antifungal activity of BAP against *T. harzianum* (a) and *R. solani* experiment (b); the dosage of the antiviral protein was 46.8 µg mL^{-1}; a crescent-shaped zone of inhibition at the mycelial front is seen; the controls consisted of 1 mM TE buffer, pH 7.5 and untreated separate inoculations of same fungi

Table 3- Inhibitory effect of Bouganin antiviral protein against some fungi in PDA

Fungal agent		*The average growth of the fungus mycelium (cm)*			*Inhibition (%)*
		24 h	*48 h*	*72 h*	
F. oxysporum	Control	-	1.9	2.9	-
	Treatment		1.6	2.9	
R. solani	Control	3.7	5.9	6.5	30.7
	Treatment	3.3	4.3	4.5	
T. harzianum	Control	2.8	3.7	5	20
	Treatment	2.3	3.7	4	

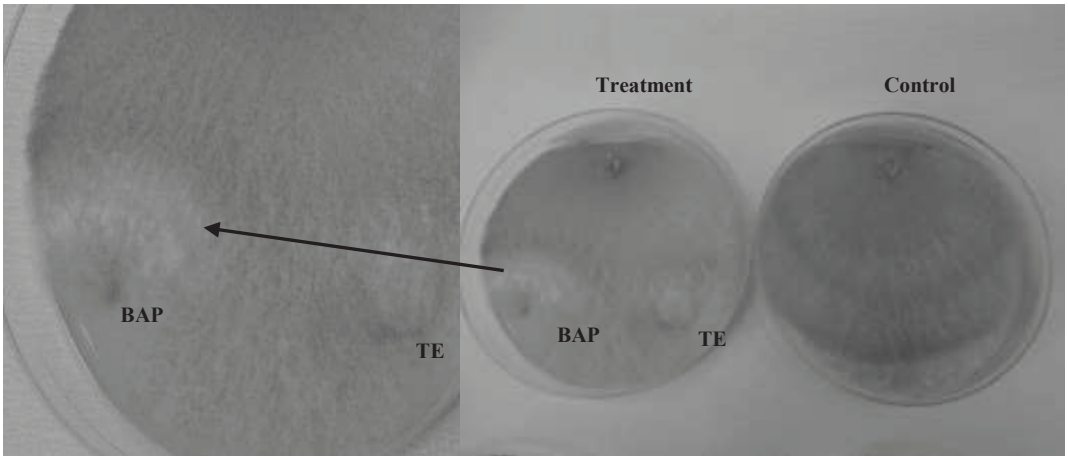


Figure 4- Sporulation of *T. harzianum* at the point where the fungus encounters with BAP protein

4. Discussion

The BAP I gene contains 297 amino acids, phylogenetic analysis of BAP I nucleotide sequence with those of other BAPs, on a consensus length of 893 nucleotide residues, established a maximum homology of 85%.

The *in vitro* tests showed that BAP proteins had activities against ZYMV symptom reduction, but its inhibitory activity on ZYMV infection was not confirmed. The relationship between the concentration of BAP protein and its antiviral activity toward tested virus was not established in this investigation. However, here we report that the inhibitory activity of BAP protein against ZYMV is a dose dependent manner. There was a steady decrease in the DSI with the increase in the amount of BAP for all the replicates tested for ZYMV. The present findings suggest that the antiviral activity of BAP could be related to its amount in diffused cells. It has been reported that the all types of RIPs have antiviral activity against plant viruses (Stevens et al 1981; Hartley et al 1996; Jackman et al 1999). Most of the studies shows that inhibit the symptoms of the virus but the presence or the absence of the virus have not been studied. Although disease severity has been decreased and is also important statically ($P<0.05$), when BAP protein applied to cotyledons of squash plants did not prevent the virus entry and it did not stop the ZYMV infection. BAP protein exhibits strong antiviral activity particularly toward *Tobacco mosaic virus* (Choudhary et al 2008). BBAP1 protein which derived from another Bougainvillea species (*B. xbuttiana*) is similar with the effect of inhibition showed against *Sunnhemp rosette virus* (SRV) (Choudhary et al 2007).

Antifungal activities of BAP protein have been performed using radial growth inhibition assay in *R. solani, T. harzianum and F. oxyporum.* This protein displayed an inhibitory activity on *R. solani,* and *T. harzianum*, but there was no obvious inhibitory effect against *F. oxyporum*. In our study, the bouganin protein has inhibited the development of *R. solani* (30.7%) and *T. harzianum* (20%) mycelium. BAP protein has not shown any positive or negative effect in *F. oxyporum.* These findings have shown similarity with the results obtained from the studies carried out of Lodha et al (2010) by BBAP1 *(Bougainvillea xbuttiana* antiviral protein). In parallel, the BAP protein derived from *B. spectabilis* plant has inhibited mycelial growth of the *Sclerotium rolfsii* which is a plant pathogen and determined to reduce the growth in 3 days (Abbas 2007). In other study, conducted by Barbieri et al (2006), the RIP gene isolated from *Cucurbita moschata* plant inhibited the growth of two strain of *Phytophthora infestans.* Roberts & Selitrennikoff (1986) reported that the barley RIP inhibited *Trichoderma reesei* growth on agar plates. Besides, the abundant maize kernel ribosome-inactivating protein (RIP1) has been shown to have antifungal activity against *Aspergillus nidulans* (Nielsen et al 2001). In our trials, the BAP antiviral protein has encouraged sporulation of *T. harzianum.*

Interestingly, we have found a unique characteristic of BAP protein that distinguishes it from known types of RIPs. We found that the BAP treatment along with 10% DMSO causes a severe size reduction in squash. It has been hypothesized that once the BAP reaches the plant cells, it may depurinate the host plant ribosomes and arrests the protein synthesis necessary for the growth. In eukaryotes, it has been well documented that the ribosome inactivating trait of RIPs is responsible for inhibition of protein synthesis (Gessner & Irvin 1980; Irvin 1995). To date, the potential size reduction effect of RIPs in plants has not been investigated. To our knowledge, this is the first time that plant size reduction activity has been reported for BAP protein which has been grouped among type I RIPs. The RIPs are known to have the ability to inactivate fungal, bacterial, mammalian and plant ribosomes (Girbés et al 2004). RIPs act on ribosomes to inhibit polypeptide chain elongation (Olsnes & Pihl 1980; Barbieri & Stirpe 1982; Irvin 1983) and thereby arresting protein synthesis and causing cell death (Endo & Tsurugi 1988). For better understanding of the molecular mechanisms of BAP blockage of cellular functions that are needed for growth, would be the focus in the future research.

With the present study, we cloned and expressed the RIP gene from Bougainvillea and tested antiviral and antifungal activities by constructing the prokaryotic expression plasmid encoding BAP gene. Here, we describe the isolation and properties of BAP antiviral protein with a molecular mass of 28 kDa from *B. spectabilis*. Full-length DNA encoding bouganin antiviral protein gene was generated using gene specific forward and reverse primers and cloned into pETDuet-1 expression vector. In conclusion, purified *E. coli*-expressed BAP antiviral protein from *B. spectabilis* has multifunctional activity against ZYMV, *R. solani*, and *T. harzianum*. This is the first report of the plant growth reduction effect of BAP.

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Effects of Kefir Powder Fortification on Yogurt Quality

Burcu BOZOVA[a], Tuğba KÖK TAŞ[b], Zeynep B. GUZEL-SEYDİM[b]

[a]*Burdur Provincial Directorate of Agriculture and Rural Affairs, Burdur, 15200, TURKEY*

[b]*Suleyman Demirel University, Engineering Faculty, Department of Food Engineering, Isparta, 32260, TURKEY*

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Corresponding Author: Zeynep B. GUZEL-SEYDİM, E-mail: zeynepseydim@sdu.edu.tr, Tel: +90 (246) 211 16 81
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ABSTRACT

The aim of this research was to improve functional properties and sensory of plain set type yogurt since yogurt bacteria have low probiotic properties. Total solid standardization (to 15% w v^{-1}) of milks was made with either using natural kefir powder as a treatment group (KTYO) or milk powder as a control group (YKON). Kefir powder was produced with freeze-dried kefir made from kefir grains. Microbial, chemical, sensory and physical properties of yogurts were determined during cold storage. The attributes determined on the yogurts were pH, total solids, titratable acidity, whey separation, sensory properties, color parameters and flavor. Numbers of *Lactobacillus* spp. in yogurt samples contained either kefir powder or milk powder were similar and did not change significantly during cold storage. *L. acidophilus* and *Bifidobacterium* spp. contents of KTYO ranged between 5.79-6.93 log cfu g^{-1} and 4.05-4.83 log cfu g^{-1} during the cold storage, respectively. There was no significant reduction in the number of *L. acidophilus* and *Bifidobacterium* spp. during the storage ($P>0.05$). In general, sensory properties of the YKON and KTYO were similar ($P>0.05$).

Keywords: Yogurt; Kefir powder; Probiotic; Fortification agent

Kefir Tozu Zenginleştirmesinin Yoğurt Kalitesi Üzerine Etkisi

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Zeynep B. GUZEL-SEYDİM, E-posta: zeynepseydim@sdu.edu.tr, Tel: +90 (246) 211 16 81
Geliş Tarihi: 08 Ekim 2014, Düzeltmelerin Gelişi: 18 Kasım 2014, Kabul: 23 Aralık 2014

ÖZET

Bu araştırmanın amacı sade pıhtısı kırılmamış yoğurdun fonksiyonel ve duyusal özelliklerinin iyileştirilmesidir. Sütlerin toplam kuru madde standardizasyonu (% 15 w v^{-1}), uygulama grubunda (KTYO) doğal kefir tozuyla, kontrol grubunda (YKON) süt tozuyla yapılmıştır. Kefir tozu kefir danelerinden üretilen doğal kefirin liyofilize edilmesiyle üretilmiştir. Soğuk depolama süresince yoğurt örneklerinin mikrobiyolojik, kimyasal, duyusal ve fiziksel özellikleri belirlenmiştir. Örneklerin pH, titrasyon asitliği, serum ayrılması, duyusal değerlendirmesi, renk değerleri ve flavor bileşenleri çalışılmıştır. Kefir tozu veya süt tozu içeren yoğurt örneklerinin tümünde *Lactobacillus* spp. içeriği benzer olarak tespit edilmiş ve soğuk depolama sürecinde de önemli bir değişim gözlenmemiştir. KTYO örneğinin *L. acidophilus* ve *Bifidobacterium* spp. içeriği soğuk depolama süresince sırasıyla 5.79-6.93 log cfu g^{-1} ve 4.05-4.83 log cfu g^{-1} olarak

tespit edilmiştir. Soğuk depolama süresince bu bakterilerin içeriğinde azalma tespit edilmemiştir (P>0.05). Kefir tozu ilaveli yoğurt örneklerinin duyusal değerlendirme bulguları süt tozu ilaveli yoğurt örneklerinin duyusal değerlendirmeleri ile benzer tespit edilmiştir (P>0.05).

Anahtar Kelimeler: Yoğurt; Kefir tozu; Probiyotik; Duyusal

1. Introduction

Yogurt has become a basic element of daily nutrition in many parts of the world because of the various health claims, therapeutic values and acceptance of its organoleptic properties (Routray & Mishra 2011). Gel structure, taste and flavor are the important attributes for consumer acceptance. Total solid content is significant for an appropriate gel texture formation of set type yogurt. Total solid adjustment to 15% could be carried out using addition of milk powder, evaporation or membrane filtration techniques. In recent years, it has been reported that yogurt does not have high potential for probiotic activity; therefore, yogurt has increasingly being used as a carrier of added probiotic bacteria for their potential health benefits (Ashraf & Shah 2011).

Kefir is produced by activity of natural starter culture, kefir grains, which contain a diverse range of inherent lactic acid bacteria and yeast in a unique polysaccharide structure (Guzel-Seydim et al 2011); therefore it is a significant source of probiotics and prebiotics. Kefir contains *Lactobacillus kefiranofaciens, Lactobacillus kefirgranum, Lactobacillus helveticus, Lactobacillus casei, Lactobacillus brevis, Lactobacillus acidophilus, Lactococcus lactis, Streptococcus thermophilus and Kluyveromyces marxianus* (Kök Taş et al 2012). Several studies have showed that the health benefits associated with kefir are gastrointestinal proliferation, antibacterial spectrum, anticarcinogenic effect, hypocholesterolemic effect, antidiabetic properties, antimutagenic activity, β-galactosidase activity, scavenging activity, lactic acid content, effect on lipid and blood pressure level, protection against apoptosis, antiallergic properties, anti-inflammatory action, bacterial colonization, and immune system booster (Guzel-Seydim et al 2011; Ahmed et al 2013). The aim of this research was to use freeze-dried natural kefir powder for total solid increment in set type yogurt production to improve its functional and sensory properties.

2. Material and Methods

2.1. Yogurt production

Cow milk was provided by the Unsut Dairy Plant, Suleyman Demirel University, Isparta, Turkey. Kefir powder was kindly provided by the Danem Co., Technopark at Suleyman Demirel University, Isparta, Turkey.

In this study, yogurts were made with using addition of natural kefir powder (treatment group was abbreviated as KTYO) and nonfat milk powder (control group was abbreviated as YKON) into milk for 15% total solid standardization.

Control yogurt (YKON) was produced as followed; raw milk was standardized to 15% (w w^{-1}) total solids with skimmed milk powder at 30 °C, homogenized with warring blender for 2 min, heated to 85 °C for 15 min and cooled to 46 °C and inoculated with 2% (w w^{-1}) commercial yogurt starter culture (w w^{-1}) (YC-350, Yo-Flex; thermophilic yogurt culture, DVS Chr. Hansen). After transferring into 200 mL plastic cups yogurts incubated at 42 °C until pH 4.6 and stored at 4 °C.

Yogurt enriched with kefir powder (KTYO) was made as followed: 12% solid content of raw milk heated to 85 °C for 15 min and cooled to 46 °C and standardized to 15% (w w^{-1}) total solids with kefir powder than immediately inoculated with 2% (w w^{-1}) commercial yogurt starter culture (YC-350, Yo-Flex; thermophilic yogurt culture, DVS Chr. Hansen). After transferring into 200 mL plastic cups yogurts incubated at 42 °C until pH 4.6 and stored at 4 °C. Yogurt samples were analyzed during cold storage (4 °C) at days 1^{st}, 7^{th}, 14^{th} and, 21^{st}.

2.2. Microbiological analyses

Yogurt samples during storage were analyzed to determine changes in their microbial content. The lactobacilli counts were determined by using de Man, Rogosa and Sharpe (MRS) medium (Accumedia 7543, East Lansing, MI, USA) after incubation at 37 °C under 6% CO_2 at 37 °C for 48 h. The *Lactococcus* spp. and *Streptococcus thermophilus* were plated on M17 medium (Oxoid, Basingstoke, UK) and incubated under 6% CO_2 at 37 °C for 48 h. Yeasts were grown on Potato Dextrose Agar (Merck, Darmstadt, Germany) with 0.14% added lactic acid at 25 °C for 5 days (Mossel et al 1995). *L. acidophilus* and *Bifidobacterium* spp. were cultured on MRS with sorbitol (10%) and on MRS with NNLP (20%) containing neomycin sulfate (100 mg L^{-1}), nalidixic acid (50 100 mg L^{-1}), lithium chloride (3000 100 mg L^{-1}) and paramycin sulfate (200 100 mg L^{-1}), respectively (Özer et al 2008).

2.3. Chemical analyses

The pH of yogurt was measured using digital pH meter (WTW, Measurement System, FL, USA). Titratable acidity and total solids were determined according to AOAC International (1992) methods. The extent of syneresis was measured according to Atamer & Sezgin (1986).

2.4. Color measurement

L* (whiteness to blackness), a* (redness to greenness) and b* (yellowness to blueness) values of the yogurts were determined by Minolta (Minolta Corp, Ramsey, NJ, USA).

2.5. Flavor compounds

Agilent 7697A Headspace and Agilent 7890A Gas Chromatography with 5975C MS were used for determination of acetaldehyde, ethanol, acetone and diacetyl (Yılmazer & Seçilmiş 2006).

2.6. Sensory analyses

Sensory evaluation of plain yogurt samples was conducted by 12 taste panelists. Panelists were selected from volunteer undergraduate/graduate students and academic staff of the Department of Food Engineering. The panelists (n= 12: 8 women, 4 men, aged 19-43 years old) received a 30-h training session including basic tastes and flavor identification, and using a 5-point product specific scale with references (Meilgaard et al 1999). The samples were presented to the panelists for every week (1st, 7th, 14th and, 21st). Yogurts were presented in 30 g sample cups with plastic lids with three digit codes. The panelists were asked to evaluate the color, appearance, odor, taste, texture (hand), texture (mouth) and overall acceptability, based on a 5 point scale; between like extremely = 5 point and dislike extremely = 1 point.

2.7. Statistical analyses

Data analyses were performed using SPSS statistical software Version 22 (SPSS Inc., Chicago, IL). Microbial and physico-chemical data were analyzed using repeated measurement ANOVA. A factorial arrangement was set up to study the influence of two treatment and four storage time using 3 replicates. Tukey A test was performed for group means comparison. P value < 0.05 was considered statistically significant for all analysis.

3. Results and Discussion

The changes in the viable counts of *Lactococcus* spp., *Streptococcus thermophilus* and *Lactobacillus* spp. during storage of yogurt were presented in Table 1. *Lactobacillus* spp. contents of yogurt samples contained either kefir powder or milk powder were similar and did not have any significant changes during cold storage (P>0.05).

YKON sample had higher numbers of *Lactococcus* spp., *Streptococcus thermophilus* (9.12 log cfu g^{-1}) than the KTYO sample (8.83 log cfu g^{-1}) at first day, respectively (P<0.05). Cocci numbers were higher than *Lactobacillus* spp. in both of the samples. It is important that *Lactobacillus acidophilus*, *Bifidobacterium* spp. and yeasts were detected only in the KTYO product due to natural kefir powder inoculation (Table 2). The cell counts of *L. acidophilus*, *Bifidobacterium* spp. and yeasts ranged between 5.79-6.93, 4.05-4.83 and 3.64-4.95 log cfu g^{-1} during cold storage of KTYO product,

Table 1- *Lactococcus* spp., *S. thermophilus* and *Lactobacillus* spp. content of yogurt samples during cold storage at 4 °C

Samples	Storage time (day)			
	1st	*7th*	*14th*	*21st*
	Lactococcus spp. and *Streptococcus thermophilus* (log cfu g^{-1})			
YKON*	9.12±0.05[a]	9.13±0.05[a]	9.11±0.05[a]	9.16±0.03[a]
KTYO	8.83±0.02[a]	8.74±0.08[b]	8.69±0.07[b]	8.80±0.08[b]
	Lactobacillus spp. (log cfu g^{-1})			
YKON	8.01±0.10[a]	8.19±0.13[a]	8.15±0.14[a]	7.88±0.29[a]
KTYO	7.92±0.04[a]	8.22±0.18[a]	8.18±0.22[a]	8.10±0.29[a]

[a,b], statistical differences (P<0.05) for treatments are indicated by different superscripts in the same column; treatment group has been abbreviated as KTYO; control group has been abbreviated as YKON

Table 2- *L. acidophilus, Bifidobacterium* spp. and yeast content (viability) of KTYO sample during cold storage at 4 °C

Microorganism	Storage time (day)			
	1st	*7th*	*14th*	*21st*
L. acidophilus (log cfu g^{-1})	6.93±0.54[a]	6.40±0.54[b]	6.13±0.80[c]	5.79±0.80[d]
*Bifidobacterium s*pp. (log cfu g^{-1})	4.83±1.77[a]	4.39±1.47[b]	4.25±1.42[b]	4.05±1.58[c]
Yeast (log cfu g^{-1})	3.64±0.77[c]	4.19±1.17[b]	4.36±1.56[b]	4.95±1.04[a]

[a,b], statistical differences (P<0.05) during the storage are indicated by different superscripts in the same row

respectively. There were significant differences in the cell counts of *L. acidophilus*, *Bifidobacterium* spp. and yeasts during storage (P<0.05). It is difficult to keep Bifidobacteria active in food during storage due to oxygen sensitivity and low acid tolerance. However, high oxygen utilization ability of *S. thermophilus* prevents reduction of Bifidobacteria (Lourens-Hattingh & Viljoen 2001). Inclusion of freeze dried kefir with probiotics affected the microbiota of yogurt and the functional properties of yogurt were improved.

Total solid contents of YKON and KTYO were 15.90% and 15.51%, respectively (P>0.05). Chemical properties of yogurt samples were shown in Table 3. The pH level of YKON and KTYO sample ranged between 4.10-4.38 and 4.03-4.41

Table 3- Physical and chemical properties of yogurts during cold storage at 4 °C

Samples	Storage time (day)			
	1st	*7th*	*14th*	*21st*
	pH			
YKON	4.38±0.04[a]	4.34±0.07[a]	4.17±0.10[ab]	4.10±0.01[b]
KTYO	4.41±0.04[a]	4.22±0.06[ab]	4.16±0.13[bc]	4.03±0.03[c]
	Titratable acidity (%)			
YKON	1.12±0.03[a]	1.28±0.02[a]	1.32±0.08[b]	1.35±0.04[b]
KTYO	1.06±0.02[a]	1.22±0.08[a]	1.25±0.15[a]	1.35±0.08[b]
	Whey separation (mL)			
YKON	3.44±1.11[a]	2.07±0.20[ab]	1.64±0.14[b]	2.03±0.05[ab]
KTYO	3.47±0.18[a]	2.31±0.44[ab]	2.52±0.22[ab]	1.68±0.24[b]
	Total solids (%)			
YKON	15.85±0.21[a]	15.92±0.03[a]	15.82±0.35[a]	16.02±0.24[a]
KTYO	15.74±0.43[a]	15.59±0.45[a]	15.28±0.61[a]	15.43±0.46[a]

[a,b], statistical differences (P<0.05) during the storage are indicated by different superscripts in the same row; treatment group has been abbreviated as KTYO; control group has been abbreviated as YKON

during the cold storage, respectively. A gradual decrease in pH through the storage was noted both of the samples at the 14th day (P<0.05). There were no significant differences among the pH values of all yogurt samples during the storage period (P>0.05). Acidity is one of the most critical parameters affecting the viability of the probiotics in yogurt (Dave & Shah 1997; Ranadheera et al 2012). The titratable acidities of YKON and KTYO samples were ranged between 1.12-1.35% and 1.06-1.35% during the storage. The titratable acidities of yogurt samples increased at during storage (P<0.05).

The volumes of serum separation from the YKON and KTYO samples were 1.64-3.44 mL and 1.68-3.47 mL during the cold storage, respectively. There was no significant difference in the amount of syneresis between control yogurt and the yogurt enriched with kefir powder (P>0.05). However, syneresis was significantly affected by the storage time (Table 3). There was significant decrease in syneresis amount of yogurt samples between 1st day and 21st day (P<0.05).

The L*, a* and b* values were presented in Table 4. L*, a* and b* values are important factors for the appearance of the food products. The L* and a* values of KTYO sample was significantly higher than those of YKON sample (P<0.05). The b* value of YKON sample was significantly higher than that of KTYO sample (P<0.05). Cais-Sokolinska & Pikul (2006) determined high correlation coefficient between the L*, a* and b* color parameters and syneresis and titratable acidities. It was observed that the L*, a* and b* color parameters decreased during the storage time (Cais-Sokolinska & Pikul 2006).

Yogurt flavor consists of more than 90 different flavor compounds. The significant flavor compounds are acetaldehyde, acetone, acetoin, and diacetyl in yogurt (Guzel-Seydim et al 2005). *L. bulgaricus* and *S. thermophilus* both are required for typical flavor of yogurt (Yalçın 1985); flavor production of starter culture is very important in plain yogurts. In this study, it was found that YKON had significantly higher contents of acetaldehyde and acetone than KTYO samples (P<0.05) (Table 5). The typical aroma of yogurt resulted when especially acetaldehyde was greater than 8 mg kg^{-1} (Routray & Mishra 2011). Both KTYO and YKON samples had contained acetaldehyde more than 8 mg kg^{-1}. Certain amount of acetaldehyde in KTYO was possibly metabolized to ethanol not only during fermentation but also during storage. Ethanol content of KTYO sample was significantly higher than YKON since yeast content (Table 2) of kefir powder affected the microbial metabolism (P<0.05). It was noted that natural kefir microflora results in lactic acid and ethanol fermentation together (Guzel-Seydim et al 2011). Diacetyl contents of yogurt samples contained either kefir powder or milk powder were similar at the first storage day (P>0.05).

Table 4- CIE L*, a* and b* values of yogurt samples during the storage (n= 3)

Samples	*Storage time (day)*			
	1st	*7th*	*14th*	*21st*
	CIE L*			
YKON	84.01±0.04[a]	83.96±0.08[a]	83.86±0.07[a]	83.92±0.06[a]
KTYO	84.32±0.05[a]	84.44±0.04[b]	84.37±0.03[b]	84.45±0.11[b]
	CIE a*			
YKON	-2.93±0.01[b]	-2.99±0.04[b]	-3.02±0.01[b]	-3.03±0.06[b]
KTYO	-2.62±0.07[a]	-2.68±0.02[a]	-2.67±0.04[a]	-2.60±0.06[a]
	CIE b*			
YKON	7.29±0.02[a]	7.35±0.11	7.42±0.15	7.45±0.19
KTYO	6.58±0.16[a]	6.96±0.10	6.96±0.16	6.90±0.16

[a,b], statistical differences (P<0.05) for treatments are indicated by different superscripts in the same column; treatment group has been abbreviated as KTYO; control group has been abbreviated as YKON

Table 5- Changes in flavor compounds contents of yogurt samples during cold storage at 4 °C

Samples	*Storage time (day)*	
	1st	*21st*
	Acetaldehyde (mg kg^{-1})	
YKON	27.38±2.41^{a}	22.36±0.37^{a}
KTYO	12.23±7.86^{b}	12.14±1.25^{b}
	Acetone (mg kg^{-1})	
YKON	0.95±0.02^{a}	1.09±0.02^{a}
KTYO	0.54±0.02^{b}	0.66±0.09^{b}
	Ethanol (mg kg^{-1})	
YKON	5.55±0.77^{a}	9.13±2.71^{a}
KTYO	36.56±15.66^{b}	138.16±61.77^{b}
	Diacetyl (mg kg^{-1})	
YKON	1.58±0.21^{a}	1.98±0.23^{a}
KTYO	1.49±0.69^{a}	1.08±0.25^{b}

^{a,b}, statistical differences (P<0.05) for treatments are indicated by different superscripts in the same column; treatment group has been abbreviated as KTYO; control group has been abbreviated as YKON

The sensory scores of the yogurt samples for taste, color, odor, appearance, texture with spoon and texture with mouth were detailed in Figure1.

Color, odor, appearance, texture with spoon, texture with mouth and taste scores of YKON samples ranged between 4.33-4.46, 3.96-4.17, 4.08-4.17, 4.38-4.50, 4.08-4.25 and 3.83-4.58 during the storage, respectively. Color, odor, appearance, texture with spoon, texture with mouth and taste scores of KTYO samples ranged between 4.00-4.29, 3.58-3.83, 3.38-4.21, 3.75-4.13 and 3.38-4.00 during the storage, respectively.

There were no significant differences in the appearance scores of the YKON sample during the storage (P>0.05). The appearance scores of the KTYO sample decreased significantly at the 21st day (P<0.05). Foamy surface (1-2 mm thickness) of the KYTO was noticed due to CO_2 production of yeasts in kefir. Texture with spoon scores of YKON sample

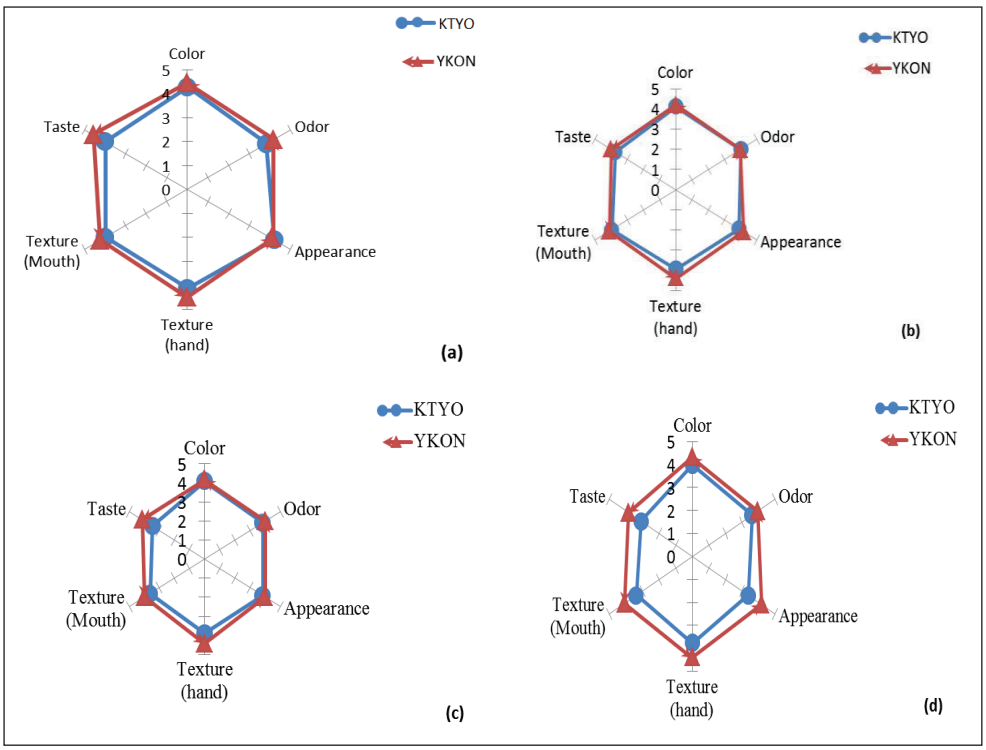

Figure 1- Sensory evaluation of yogurt samples during cold storage (n= 12) at 4 °C_((a)1, (b)7, (c)14, (d)21)

were significantly higher than that of KTYO sample (P<0.05). There were no significant differences between texture with mouth scores of both samples (P>0.05). The texture (mouth) scores of the samples decreased significantly at the 21st day (P<0.05). Taste scores of the YKON sample were significantly higher than KTYO sample (P<0.05) possibly due to less amount of acetaldehyde and higher amount of ethanol contents in KTYO. There were no significant differences in the color and odor scores of control yogurt and the yogurt enriched with kefir powder (P>0.05). The general sensory evaluation scores of the YKON and KTYO were similar (P>0.05).

4. Conclusions

This study showed that a combination of kefir powder and yogurt starter cultures would be used to renovate the traditional yogurt production. Kefir powder was included for mainly total solid standardization. Furthermore, it was concluded that kefir powder also had a significant role during fermentation. Probiotic properties of traditional yogurt improved with the kefir powder. The refreshing taste and health benefits of kefir could be transferred to commercial yogurt by this application. This study formed a new product for consumers in terms of acceptable sensory properties. In order for consumers to gain health benefits from yogurt, kefir powder could be a significant fortification agent for yogurt. It is not only increased total solids but also enhanced probiotic content.

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Effects of Ozone Treatment on the Degradation and Toxicity of Several Pesticides in Different Groups

Yakup Sedat VELIOGLU[a], Şeyda FİKİRDEŞİCİ-ERGEN[b], Pelin AKSU[c], Ahmet ALTINDAĞ[b]

[a]*Ankara University, Faculty of Engineering, Department of Food Engineering, Gölbaşı, Ankara, TURKEY*

[b]*Ankara University, Faculty of Science, Department of Biology, Tandoğan, Ankara, TURKEY*

[c]*Plant Protection Central Research Institute, Yenimahalle, Ankara, TURKEY*

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Corresponding Author: Yakup Sedat VELIOGLU, E-mail: velioglu@ankara.edu.tr, Tel: +90 (312) 203 33 00/3619
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ABSTRACT

The effects of ozone treatment on the degradation and toxicity of nine pesticides were determined with different chromatographic techniques, using bubbled ozone and distilled water and two different buffer solutions as test media. The toxicity experiments were performed using *Daphnia magna,* a cladoceran fresh water flea. The results revealed that thiacloprid and acetamiprid can only be degraded by ozonation to a limited extent (max 2.6%). The other seven pesticides were successfully degraded by ozone. The degradation rates (%) were found to be 93, 99, 95, 99, 87, 98, and 85 for fenazaquin, lambda cyhalothrin, azoxystrobin, chlorpyrifos, spiromesifen, clothianidin and thiamethoxam, respectively, after 5 minutes of ozone treatment in distilled water. The ozone treatment yielded reduced toxicity in fenazaquin, lambda cyhalothrin, azoxystrobin, chlorpyrifos and spiromesifen. However, the degradation products of clothianidin and thiamethoxam were found to be more toxic than the pesticide itself. In general, the use of buffer solutions has no significant effect on pesticide degradation compared to water as an ozonation medium.

Keywords: Ozonation; *Daphnia magna;* Insecticide; Fungicide; Transformation; Toxic

Ozonlama İşleminin Farklı Gruplardaki Pestisitlerin Parçalanma ve Toksisitesi Üzerine Etkileri

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Yakup Sedat VELIOGLU, E-posta: velioglu@ankara.edu.tr, Tel: +90 (312) 203 33 00/3619
Geliş Tarihi: 04 Mart 2016, Düzeltmelerin Gelişi: 26 Ekim 2017, Kabul: 04 Kasım 2017

ÖZET

Ozonlama işleminin dokuz farklı pestisitin parçalanma ve toksisitesi üzerine etkileri farklı kromatografik tekniklerle incelenmiştir. Çalışmada ozon gazı baloncuk yöntemiyle su ve iki farklı tampon çözelti ortamında uygulanmıştır. Toksisite denemelerinde *Cladocera* takımında yer alan su piresi (*Daphnia magna)* kullanılmıştır. Bulgulara göre, ozonlama ile thiacloprid ve acetamiprid çok sınırlı bir şekilde (en çok % 2.6) parçalanabilirken diğer yedi pestisit

çok iyi parçalanmıştır. Saf suda 5 dakikalık ozonlama uygulamasıyla fenazaquin, lambda cyhalothrin, azoxystrobin, chlorpyrifos, spiromesifen, clothianidin ve thiamethoxamın parçalanma oranları (%) sırasıyla 93, 99, 95, 99, 87, 98 ve 85 olarak bulunmuştur. Ozonlama işlemi fenazaquin, lambda cyhalothrin, azoxystrobin, chlorpyrifos ve spiromesifende toksisitenin azalmasını sağlarken clothianidin ve thiamethoxamın parçalanma ürünlerinin toksisitesi başlangıç bileşiğinden daha fazla bulunmuştur. Genel olarak tampon çözelti kullanımı pestisitlerin parçalanmasında suya göre önemli bir farklılık oluşturmamıştır.

Anahtar Kelimeler: Ozon; *Daphnia magna;* İnsektisit; Fungusit; Bozunma; Toksik

1. Introduction

Pesticides are widely used pre-and post-harvest to meet the nutritional needs of a growing population, thus reducing product losses due to diseases, pests and weeds. Although pesticides have many benefits, their unconscious or intensive use during cultivation or storage has resulted in the presence of residual compounds or its degradation products in food products. In addition to contaminating food products, pesticide residues also create serious problems in the soil, on the ground, in surface water and in the air, therefore creating public and regulatory concern (Albanis et al 1998; Horvitz & Cantalejo 2014).

Pesticide residues on food products can be reduced through various methods, including washing with water or soaking in several chemical solutions, e.g. chlorine, ozone, hydrogen peroxide, salts, and detergents. The degradation mechanism of pesticides relies on oxidation, hydrolysis, reduction, photolysis, metabolism, temperature and pH (Bajwa & Sandhu 2014). Chemical oxidation is one of the most promising applications for destroying pesticides, chemical residues, and mycotoxins, converting non-biodegradable organic materials into biodegradable forms and reducing the microbial load of food products or water. Ozonation is considered to be one of the best variations of chemical oxidation. Ozone can be combined with hydrogen peroxide and UV radiation, which are other hydroxyl radical-based advanced oxidation techniques (Karaca & Velioglu 2007; Karaca et al 2010). The advantages of ozone as an oxidant are that it provides oxygen to the oxidizing medium and that no harmful substances are formed in this environment. Ozone is also used efficiently for industrial, domestic and drinking water purification (Wu et al 2007).

A number of studies indicate that some pesticides are successfully degraded with ozone in aqueous solutions. Azinphos-methyl, captan and formenate can be degraded by a combination of ozone and chlorine. Dipping apples into 0.25 mg kg^{-1} ozonated water reduced the contents of these three pesticides by 75%, 72% and 46%, respectively. The maximum degradation rate of azinphos-methyl was 83% in a model system (Ong et al 1996). Hwang et al (2001) reported that mancozeb and ethylene-thiourea (ETU) in apples can be decreased by 56-97% with 1-10 mg kg^{-1} ozone, and the ETU was completely removed with 1 and 3 mg kg^{-1} ozone. In another study it was shown that an ozone wash with 3 mg kg^{-1} ozone was the most effective treatment for mancozeb and ETU removal. The authors also indicated that the degradation by-products of some organophosphate pesticides might be more toxic than the initial compound (Hwang et al 2002). Wu et al (2007) reported that 1.4 mg kg^{-1} ozone was effective to degrade 60-99% of 0.1 mg kg^{-1} diazinon, parathion, methyl-parathion and cypermethrin within 30 min. The degradation rate was highly dependent on the dissolved ozone, and the maximum removal was detected at 15-20 °C. Kim et al (2000) treated soybeans with 0.3 mg kg^{-1} ozonated water for 30 min and determined the changes on the carbendazim, captan, diazinon, fenthim, dichlorvos and chlorpyriphos residues. The ozone treatments destroyed residues better than water itself. A treatment temperature of 30 °C was found to be more effective than other tested

temperatures on the fenitrothion degradation by ozone in lettuce and cherry tomatoes, with relatively little effect on crop quality (Ikeura et al 2013). The ozone/UV/TiO_2 combination in tea leaves reduced the cypermethrin and malathion residues by 80% and 78%, respectively (Lin et al 2012). Five minutes of ozonation reduced the tetradifon residue 98.6% in lemons and 94.2% in grapefruits (Kusvuran et al 2012). The ozone flow at 500 mg kg^{-1} reduced chlorfluazuron and chlorothalonil residues 75% and 77%, respectively, in vegetables (Chen et al 2013). The storage of table grapes in an ozone atmosphere accelerated fenhexamid, cyprodinil and pyrimethanilin degradation (Karaca et al 2012). In olives ozonated water wash for 5 min reduced chlorpyrifos, beta-cyfluthrin, alpha-cypermethrin and imidacloprid contents by 38%, 50%, 55% and 61%, respectively (Kırış & Velioglu 2016).

Daphnia magna, a cladoceran fresh water flea, is an important bio indicator (Martins et al 2007) used in the evaluation of the toxicity levels of pesticides and pesticide degradation products in aquatic ecosystems (Sanchez-Bayo & Goka 2006). During the ozonation process, degradation products are formed but chemical compositions cannot be identified. In vivo and in vitro toxicity tests are important to specify the effects of degradation products on human and animal health.

Previous studies have demonstrated that most pesticides can be degraded with ozone treatment. However, ozonation may produce by-products caused by the reaction between ozone and the pesticide and, as shown in this paper, degradation products are formed but their chemical structure cannot be easily identified and this product can be more toxic than the initial product. The aim of this study is to reveal the effects of ozone treatment on the degradation and toxicity of nine pesticides belonging to six different groups.

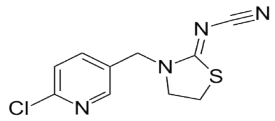
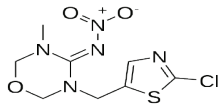
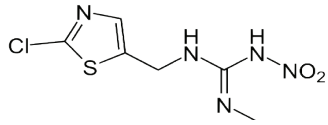
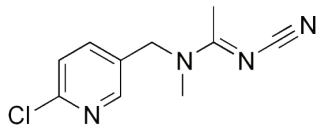
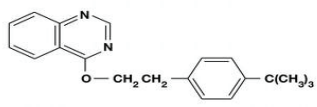
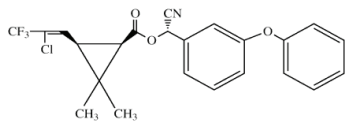
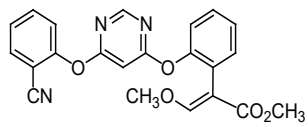
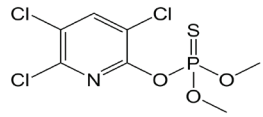
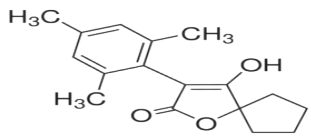
2. Material and Methods

Names, sources, functions, sub-groups, purities, stock solution concentrations (approx. 10000 mg kg^{-1}), open structures and the chromatographic method of tested pesticides were given in Table 1. Test solutions were freshly prepared by dissolving the pesticides with a few drops of dimethylsulfoxide (fenazaquin, clothianidin, thiacloprid and acetamiprid) or acetonitrile (lambda cyhalothrin, azoxystrobin, chlorpyrifos, spiromesifen and thiamethoxam) and then diluting the solution to its final volume using ASTM Type 2 high-purity water (TKA Scientific, Niederelbert, Germany) and citrate-buffer solutions (10^{-3} M at pH 5.5 and pH 6.5). Because of the high dilution rate and the use of control solutions in the toxicity experiments, the use of acetonitrile did not result in any problems. Each test solution of pesticides was diluted to concentration of 10 mg kg^{-1} from the stock solutions.

2.1. Ozonation apparatus and procedure

Ozone was produced from air using a corona discharge ozone generator (OG-20, Opal, Turkey), with a production capacity of 20 g h^{-1} of ozone. The pesticide solutions were ozonated in 30 mL Falcon test tubes; alternatively, in cases where large volumes of solvent were needed because of a pesticide's low toxicity, 250 mL glass bottles (Isolab, Boro 3.3, Wertheim, Germany) were used for the pesticide degradation and toxicity tests. Tube (or bottle) caps had a hole in the centre, allowing a tube to be passed through. The tubing was connected to a stainless steel solvent inlet filter (10 µm pore size) (Fisher Scientific, Schwerte, Germany). The filter was kept at the bottom of the tube (or bottle) during the ozonation process, allowing efficient ozone diffusion in the liquid phase. The caps also had 6-8 other small holes around the centre hole to permit the release of ozone. The ozone flow was adjusted to be 600 mL min^{-1} with a "Riteflow" flowmeter, size 2, (Bel-Art Products, Pequannock, USA). All ozonation processes were performed at 15 °C, using a cooling water bath (Polyscience, USA). After the ozonation was complete, the reaction was stopped using 10 µL of 5.2 g L^{-1} Difco neutralizing buffer (Cat. No. 236210, Becton, Dickinson and Sparks, USA). All experiments were conducted in triplicate in a fume hood.

Table 1- Chemical structures and properties of the studied pesticides

Active substance/ sources	*Function/ sub-group*	*Purity (%)/ stock solution used (mg kg^{-1})*	*Open structure*	*Analysis method*
Thiacloprid/ BAYER	Insecticide/ neonicotinoid	99.7/ 10000		HPLC
Thiamethoxam/ SYNGENTA	Insecticide/ neonicotinoid	99.7/ 20000		HPLC
Clothianidin/ BAYER	Insecticide/ neonicotinoid	99.7/ 10000		HPLC
Acetamiprid/ AGRO-BEST	Insecticide neonicotinoid	99.0/ 10000		HPLC
Fenazaquin/ AGRO-BEST	Acaricide/METİ	96.0/ 10000		HPLC
Lambda cyhalothrin/ DR. EHRENS-TORFER	Insecticide/ Pyrethroid	98.0/ 10000		GC/µECD GC/MS
Azoxystrobin/ SYNGENTA	Fungicide/ methoxyacrylate	98.0/ 10000		HPLC
Chlorpyrifos/ DR. EHRENS-TORFER	Insecticide/ organophosphate	98,5/ 10000		GC/µECD GC/MS
Spiromesifen/ BAYER	Insecticide & acaricide/tetronic acid derivative	99.9/ 10000		LC-MS/MS

2.2. Analysis of pesticides

The concentration of thiacloprid, acetamiprid, clothianidin and thiamethoxam measured with HPLC (Shimadzu, Japan) consisted of a LCX-20AD pump, SPD-M20A diode array detector (DAD), DGU 20A5-E degasser, and a CTO-10ASVP column oven. The working temperature and injection volume were 25 ºC and 20 µL, respectively, and the detection wavelength was 242 nm for thiacloprid and acetamiprid, 267 nm for clothianidin and 252 nm for thiamethoxam. A mixture of methanol water (50:50 v v^{-1} for thiacloprid and acetamiprid; 60:40 v v^{-1} for clothianidin and thiamethoxam) at a flow rate of 0.5 mL min^{-1} was used as the mobile phase under isocratic conditions. Samples were filtered through a 0.45 µm PTFE filter (Millipore, Millex-LCR) and injected directly. The concentrations of fenazaquin and azoxystrobin were measured using an Agilent (1100) HPLC system that consisted of a gradient elution pump and a DAD detector. The working temperature, injection volume and detection wavelengths were 25 ºC, 20 µL, and 216 nm (fenazaquin) and 254 nm (azoxystrobin), respectively. A mixture of acetonitrile water (90:10 v v^{-1} for fenazaquin; 37:63 v v^{-1} for azoxystrobin) at a flow rate of 1.0 mL min^{-1} was used as the mobile phase under isocratic conditions. A Nucleosil (Phenomenex, Torrance, CA, USA) or Inertsil (GL Sciences, Japan) column (C18, 5 µm; 250 mm × 4.6 mm) was used in the HPLC analyses. The reductions in the pesticide contents were calculated from the reduction of the peak areas after ozone treatments.

The concentrations of lambda cyhalothrin and chlorpyrifos were measured using a GC/µECD. The Agilent (6890N GC) system consisted of an Agilent 6890 series auto sampler and a fused-silica capillary column HP-5 (30 m x 0.25 mm ID and 0.25 µm film thickness) (Chrom Tech., Apple Valley, MN, USA). The split ratio was 50:1 and the carrier gas was 99.999% helium at 1 mL min^{-1}. The working temperature, detector temperature and injection volume were 250 ºC, 300 ºC and 1 µL, respectively. The column program was 70 ºC (2 min) to 280 ºC at 25 ºC min^{-1} (7 min). The detector's make-up gas was nitrogen (99.999%) at 59 mL min^{-1}. The total analysis time was 17.40 min. For the GC analyses, samples were treated as follows: 10 g of sample was weighed into 50 mL centrifuge tube and 10 mL of acetonitrile, 4 g of anhydrous $MgSO_4$ and 0.5 g of NaCl were added. Tube was shaken immediately for 2 min. The extract was than centrifuged at 5000 rpm for 5 min. A 1 µL aliquot of supernatant was then injected into the GC/µECD. The concentration of the spiromesifen was measured under isocratic conditions using LC-MS/MS equipment (Waters, TQD Triple Quadrupole Mass Spectrometer) and an Acquity UPLC BEH column (C18, 2.1 × 100 mm × 1.7 µm) (Waters, USA). The working conditions are shown in Table 2.

2.3. Toxicity tests

A total of 10 neonates (age <24 h) attained from the original culture were exposed to pesticide. There was no feeding during the test. The toxicity was expressed by the median lethal concentration, that is, the dose required to kill half of the daphnid members of LC_{50} (median lethal concentration) exposure. After 24h-48h the live *D. magna* were counted. Exposure to the different concentrations was carried out in triplicate. LC_{50} values were calculated using the regression line obtained by plotting the concentration against the death percentage on a probit scale, and the results were assessed with probit analysis (SPSS 22.0v.). (Fikirdeşici et al 2012). A total of 10 neonates (age <24 h) obtained from the original culture were exposed to five different concentrations of clothianidin (120000, 125000, 130000, 135000, 140000 µg L^{-1}); thiamethoxam (70000, 110000, 150000, 190000, 230000 µg L^{-1}); fenazaquin (1, 5, 9, 13, 17 µg L^{-1}); lambda cyhalothrin (0.001, 0.005, 0.009, 0.013, 0.017 µg L^{-1}); azoxystrobin (50, 100, 150, 200, 250 µg L^{-1}); chlorpyrifos (0.3, 0.6, 0.9, 1.2, 1.5 µg L^{-1}); and spiromesifen (1, 1.2, 1.4, 1.6, 1.8 µg L^{-1}).

2.4. Statistical analysis

Experimental results were expressed as the means±standard errors. Two-way ANOVA was performed using SPSS for Windows (ver. 10.1, USA). Significant differences between the means

Table 2- LS-MS/MS working conditions for the determination of spiromesifen

Equipment/ Column	Water, Model TQD Triple Quadrupole Mass Spectrometer Acquity UPLC BEH C18 2.1 x 100 mm x 1.7 µm					
Mobil phases	A1: 2 mM ammonium format containing MeOH: water (10:90) B1: 2 mM ammonium format containing MeOH: water (95:5) A2: MeOH: water (50:50) B2: MeOH: acetonitrile (50:50) Weak needle wash and seal wash: water: MeOH (95:5) Strong needle wash: MeOH					
Inlet programme:	Gradient Time Flow %A %B - 0.45 90 10 1.00 0.45 90 10 12.00 0.45 5 95 13.00 0.45 5 95 13.05 0.45 90 10 16.00 0.45 90 10 Seal wash: 5 min, run time: 15 min, max pressure: 1500 psi					
Autosampler	Full loop Wash solvents: 600 µL weak, 300 µL strong Column temp.: 50 ºC Sample temp.: 10 ºC					
Injection volume:	20 µL					
Tune parameters:	Polarity: ES+ or ES-; capillary (kV): 1.00; cone (V): compound dependent; extractor (V): 3; RF lens (V): 0.1; source temp.: 130 ºC Desolvation temp.: 400 ºC; cone gas flow (L h^{-1}): 50; desolvation gas flow (L h^{-1}): 900; LM1 resolution: 14; HM1 resolution: 14; ion energy 1: 0,5; entrance: 50; collision: compound dependent; exit: 50 LM2 resolution: 14; HM2 resolution: 14; ion energy 2: 0.8; gain: 1.0; multiplier (V): 650					
Spiromesifen MRM parameters						
Spiromesifen (ESI+)	Parent (m/z)	Daughter1 (m/z)	Daughter2 (m/z)	Cone (V)	Collision1 (V)	Collision2 (V)
	371.20	255.15	273.15	23	24	8

were determined using Duncan's multiple range test. Differences were considered significant at $P<0.05$. All of the experiments were performed in triplicate.

3. Results and Discussion

3.1. Thiacloprid and acetamiprid degradation by ozone treatment

The results revealed that thiacloprid degradation with ozone was quite difficult and the degradation rate was never more than 2.6% at 10 minutes of treatment time (Table 3). To determine the effects of unpractically longer treatment times, the samples were ozonated for 20 minutes and the degradation rate only reached 5% (not shown in the table). Buffer use yielded a non-significant degradation rate ($P>0.05$) as compared to treatment in water. Because the thiacloprid degradation was much lower than the expected level (20-25%) it was, therefore, not subjected to toxicity tests.

The acetamiprid degradation was found to be even more difficult, and a very long treatment time (20 minutes) yielded only 0.22% degradation in

Table 3- Effects of ozonation time and media on the degradation (%) of pesticides[a] (initial pesticide concentration and volume in ozonation media 10 mg kg^{-1} and 20 mL, respectively)

Pesticides	*Ozonation time (min)*	*Ozonation media*		
		Water	*Buffer (pH 5.5)*	*Buffer (pH 6.5)*
Fenazaquin	2	91.26±0.61^{Aa}	94.56±0.18^{Aa}	93.67±0.12^{Aa}
	5	93.38±0.21^{Aa}	96.46±0.29^{Aa}	95.70±0.32^{Aa}
Lambda cyhalothrin	1	79.21±1.74^{Ab}	82.22±0.03^{Ab}	73.21±2.40^{Aa}
	2	97.00±0.39^{Ba}	95.80±0.37^{Ba}	94.93±0.05^{Ba}
	5	98.62±0.62^{Ba}	99.19±0.16^{Ba}	98.61±0.1^{Ca}
Azoxystrobin	2	93.71±0.67^{Ac}	89.76±0.48^{Ab}	95.43±0.21^{Aa}
	5	94.68±0.17^{Ab}	95.02±0.33^{Bb}	97.76±0.15^{Ba}
	10	98.82±0.14^{Ba}	98.19±0.28^{Ca}	99.24±0.04^{Ca}
Chlorpyrifos	2	93.25±0.15^{Ac}	67.05±1.63^{Ab}	24.90±1.84^{Aa}
	5	98.96±0.04^{Bc}	72.14±0.67^{Bb}	96.85±0.32^{Ba}
	10	99.00±0.09^{Bb}	86.99±0.29^{Ca}	98.70±0.24^{Bb}
Spiromesifen	2	76.70±0.13^{Aa}	94.05±0.02^{Ab}	78.49±0.20^{Aa}
	5	86.93±0.26^{Ba}	99.00±0.03^{Bb}	82.05±0.08^{Ba}
	10	99.07±0.02^{Cb}	99.72±0.05^{Bb}	90.98±0.15^{Ca}
Clothianidin	2	88.17±0.06^{Ab}	67.24±6.92^{Ab}	66.32±2.87^{Aa}
	5	98.45±0.02^{Ba}	96.91±0.58^{Ba}	93.12±0.86^{Ba}
	10	99.08±0.02^{Ba}	99.16±0.04^{Ba}	99.16±0.46^{Ba}
Thiamethoxam	2	71.15±1.85^{Aa}	61.46±1.06^{Aa}	61.44±0.40^{Aa}
	5	85.36±0.23^{Ba}	81.44±0.02^{Ba}	79.00±0.03^{Ba}
	10	97.50±0.07^{Ca}	92.76±0.03^{Ca}	92.79±0.12^{Ca}
Thiacloprid	2	1.4±1.07^{Aa}	1.6±1.03^{Aa}	0.80±0.23^{Aa}
	6	2.6±1.14^{Ba}	0.43±0.13^{Aa}	1.41±1.85^{Aa}
	10	2.6±0.20^{Ba}	0.47±0.37^{Aa}	2.07±0.12^{Aa}
Acetamiprid	20	0.22±0.01^{a}	nd	1.28±0.18^{b}

[a], data expressed as the means ±SE of triplicate experiments. For a specific pesticide, different letters in a column are shown in uppercase (A-C) or in a row in lower case (a-c), indicating a statistically significant difference ($P<0.05$); nd, not detected.

water and 1.28% in pH 6.5 buffer; toxicity tests were also not completed for acetamiprid.

Thiacloprid and acetamiprid pesticides are not persistent in the environment because of a high water solubility (the water solubility and log octanol-water partition coefficient (log K_{OW}) of the active ingredient at 20 °C is 185 mg kg^{-1} and 1.26, respectively), resulting in the potential contamination of surface water following rainfall (EPA 2003). Krohn & Hellpointner (2002) reported that thiacloprid was stable in water between pH 5.0 and 9.0 for a relatively long period of time. Acetamiprid is relatively non-persistent and although it is mobile, rapid degradation will reduce its potential to leach to groundwater. Pitam et al (2013) reported that acetamiprid is stable in acidic and neutral conditions compared to alkaline conditions. The stability of thiacloprid and

acetamiprid in water was also indicated elsewhere (EPA 2002; 2003; Cernigoj 2007), so it is important to develop a successful method for thiacloprid and acetamiprid degradation.

3.2. Clothianidin and thiamethoxam degradation by ozone treatment

Two and 5 minutes of ozone treatment reduced the clothianidin content 88% and 98%, respectively. Extending the treatment time from 5 min to 10 min had no effect on clothianidin degradation in water. The degradation rates were 71 and 85% for thiamethoxam at 5 and 10 min, respectively (Table 3) (Figure 1). Therefore, 2 minutes of ozone treatment yielded a significant reduction for both of these two pesticides and no extended treatment times were needed. However, ozone treatment increased the toxicity for these two pesticides (Table 4) because of the formation of new compounds. The degradation products of thiamethoxam were considerably (approximately 2.7 times) more toxic than the original compound. The toxicity experiments on these two pesticides were repeated numerous times and the same results were always obtained. As indicated by Hwang et al (2002), the degradation products may have a higher toxicity than the active ingredients themselves. Thus, "the risk from pesticides in the diet cannot be completely removed by ozonation" unless the breakdown products are proven safe (Karaca & Velioglu, 2007). Degradation of clothianidin and thiamethoxam by ozone cannot be recommended because of the higher toxicity of the degradation products.

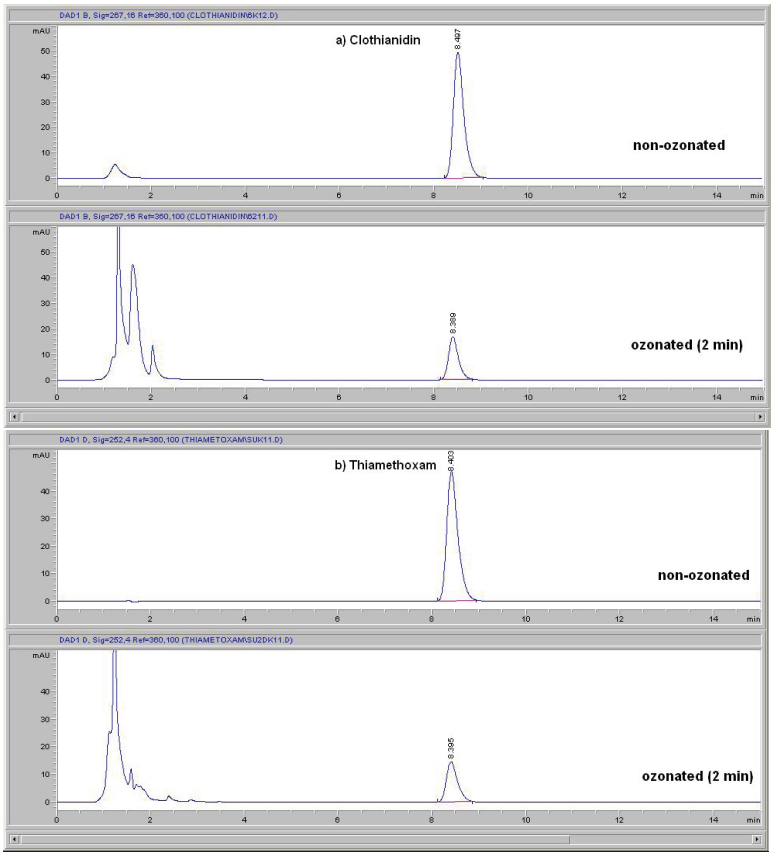


Figure 1- Effects of ozone treatment on the degradation of clothianidin (a) and thiamethoxam (b)

Table 4- The effects of ozonation on the acute pesticide toxicity on *Daphnia magna* (48 hours, 20 mL)

	LC_{50} (µg L^{-1})		*Toxicity reduction coefficient by ozonation*	*Ozonation time (sec)*	*Ozonated pesticide concentration (mg L^{-1})*
Pesticides	*Non-ozonated*	*Ozonated*			
Fenazaquin	1623	14662	9.03	60	10
Lambda cyhalothrin	0.003	0.010	3.33	120	10
Azoxystrobin	88317	197258	2.23	120	80
Chlorpyrifos	0.220	1.037	4.70	30	10
Spiromesifen	1338	1954	1.46	1200	50
Clothianidin	132403	120000	increased toxicity	120	100
Thiamethoxam	213316	77868	increased toxicity	300	100
Thiacloprid	Very low degradation by ozone. Toxicity experiments were not performed.				
Acetamiprid	Very low degradation by ozone. Toxicity experiments were not performed.				

3.3. Fenazaquin, lambda cyhalothrin, azoxystrobin, chlorpyrifos and spiromesifen degradation by ozone treatment

Fenazaquin, lambda cyhalothrin, azoxystrobin, chlorpyrifos and spiromesifen (Figure 2) were perfectly degraded with the application of ozone (Table 3). Degradation rates in 5-min ozonated samples in water solutions were between 87 and 99% for all of the five pesticides. Even 2 minutes of treatment time yielded > 90% degradation of all pesticides, except for spiromesifen (approximately 77%).

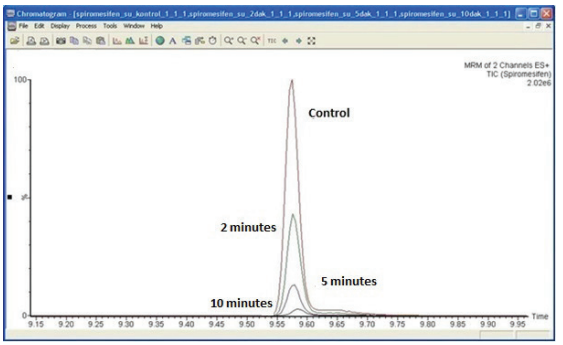


Figure 2- A sample LC-MS/MS chromatogram for spiromesifen degradation using ozone

When considering 5 min of treatment time, changing the ozonation media from water to a buffer solution did not significantly change the degradation rates of fenazaquin and lambda cyhalothrin. The use of a buffer revealed some changes on the other three pesticides; however, in all samples, the degradation rates were more than 80% (except in chlorpyrifos, which was 72% at a pH 5.5 buffer). This finding indicates that these pesticides can be successfully degraded by ozone without the need of buffer use. Ozone treatment also significantly reduced the toxicity. Reduction rates varied between 1.46 and 9.03 times depending on the pesticide tested. The degradation mechanism of azoxystrobin was probably based on a hydroxyl radical attack (Calza et al 2006; Lofrano et al 2010). This study indicated that azoxystrobin was more prone to degradation under ozone conditions than other methods (UV, photo catalytic process, etc.) (Calza et al 2006). In contrast to our study, Lozowicka et al (2014) reported that only 48.9% of azoxystrobin decomposed in 5 min in their ozonation study.

4. Conclusions

Ozone is a strong oxidizing agent and can degrade some pesticides successfully. Among the nine pesticides tested in this study, thiacloprid and acetamiprid can only be degraded to an insignificant extent (max 2.6%). Clothianidin and thiamethoxam can be easily degraded by ozone; however, their degradation products were found to be more toxic than the initial products. Other pesticides, namely,

fenazaquin, lambda cyhalothrin, azoxystrobin, chlorpyrifos and spiromesifen, were easily degraded by ozone, and their toxicities were significantly reduced. It seems that chemical composition is a more significant factor than the chemical group on the degradation rate and toxicity. Thiacloprid, acetamiprid, clothianidin and thiamethoxam all belong to the same chemical group (neonicotinoids); however, the first two pesticides were not degraded by ozone, and the latter two were degraded easily. When investigating pesticide degradation by ozone, the toxicity of some degradation products must be seriously considered because of their higher toxicity, as shown in this study.

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Effects of Soil Application of Neem on Some Biological Characteristics of *Myzus persicae* (Sulzer) (Hemiptera: Aphididae)

Ali Kemal BİRGÜCÜ[a], Şenay ÖZGER[a], Daniel POHL[a], İsmail KARACA[a]

[a]*Süleyman Demirel University, Faculty of Agriculture, Department of Plant Protection, 32260, Isparta, TURKEY*

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Corresponding Author: Ali Kemal BİRGÜCÜ, E-mail: alibirgucu@sdu.edu.tr, Tel: +90 (246) 211 81 69
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ABSTRACT

The study investigated the effect of soil application of neem at different concentrations on lifetable parameters of *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). After pepper seedlings were transplanted to pots of 1.5 L, the pepper plants had been divided into five different groups to be watered with only irrigation water (as control) and irrigation water containing 250, 500, 750 and 1000 mg L^{-1} of neem.

Based on the results, intrinsic rate of increase (r_m), net production rate (R_0) and mean generation time (T_0) ranged from 0.039 to 0.352 female/female/day, 1.700 to 57.295 female/female and 11.503 to 15.086 days respectively. Doubling time (T_2) and finite rate of increment (λ) ranged from 1.970 to 17.915 days and 1.039 to 1.422 individual/female/day, respectively. Consequently, the effect of neem on biological characters of the pest was increased in response to the increase in neem concentration applied systemically through plant root.

Keywords: Pepper; Neem; Green peach aphid; Lifetable; Weibull; Enkegaard

Topraktan Neem Uygulamasının *Myzus persicae* (Sulzer) (Hemiptera: Aphididae)'nin Bazı Biyolojik Özellikleri Üzerine Etkisi

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Ali Kemal BİRGÜCÜ, E-posta: alibirgucu@sdu.edu.tr, Tel: +90 (246) 211 81 69
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ÖZET

Bu çalışmada biber bitkilerinin kök bölgesine uygulanmış neem ekstraktının *Myzus persicae* (Sulzer) (Hemiptera: Aphididae)'nin yaşam çizelgesi parametreleri üzerine etkileri incelenmiştir. Biber fideleri 1.5 L'lik saksılara şaşırtıldıktan sonra, 250, 500, 750 ve 1000 mg L^{-1} neem içeren sulama suları ile sulanmak üzere dört farklı gruba ayrılmıştır.

Yapılan analiz sonuçlarına göre kalıtsal üreme yeteneği (r_m), net üreme gücü (R_0) ve ortalama döl süresi (T_0) değerleri sırasıyla 0,039 ile 0,352 dişi/dişi/gün, 1,700 ile 57,295 dişi/dişi ve 11,503 ile 15,086 gün arasında değişiklik göstermiştir.

Popülasyonun ikiye katlanma süresi (T_2) ve üreme gücü sınırı (λ) değerleri ise sırasıyla 1,970 ile 17,915 gün ve 1,039 ile 1,422 birey/dişi/gün arasında değişmiştir. Sonuç olarak kökten uygulanan neem konsantrasyonu arttıkça, neemin zararlının biyolojik özellikleri üzerine etkisi de artmıştır.

Anahtar Kelimeler: Biber; Neem; Yeşil şeftali yaprakbiti; Yaşam çizelgesi; Weibull; Enkegaard

1. Introduction

The green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) which is a polyphagous pest has more than 400 host plants (Cloyd & Sadof 1998). This pest causes damage on different stone fruit species, especially peach, and on many vegetable species such as tomatoes, pepper, potato, cabbage, pumpkin, and okra. It also feeds on lots of weeds as well (Uygun et al 2013).

M. persicae causes curling downwardly of the end portions of leaves, deceleration of the host plat growth and dark-colored sooty mold, known as fumagine, by honeydew secreted during feeding on the host plant. It is also know that this insect is a vector of more than 86 plant virus diseases (Cloquemin et al 1990).

Chemical control is primarily preferred in controlling the pest because of its easy application and rapid responding. These chemicals have many adverse effects on human health and environment, which led producers to use alternative control methods (Bajwa & Ahmad 2012). In this context, one of the alternative control methods against pests is also the use of plant-based preparations.

Natural products with pesticidal effects have been in the control of weeds, plant diseases and pests since ancient times (Isman 1997; Ujváry 2001). One of these plant-based insecticides is the compound derived from various parts of *Azadirachta indica* A. Juss (Meliaceae), also known as neem.

Neem-containing products are effective against more than 400 species of insects. Neem has antifeedant and lethal effects, in addition to its effect on metamorphosis of insects. Also, it can affect eggs and fecundity of the pests (Isman 1997; Waghmare et al 2007). Neem-containing products may have slightly phytotoxic effect on some plants (Walter 1999; Girish & Shankara Bhat 2008; Saha et al 2011). It is known that more than 550 pest species have become resistant to synthetic pesticides. The control of these pests may be possible with neem-containing products (Ascher 1993; Bajwa & Ahmad 2012).

This study examined the effect of soil application of neem at 250, 500, 750 and 1000 mg L^{-1} concentrations on some biological characteristics, such as the time of development, preoviposition, oviposition, postoviposition and adult life, and the rates of survival and fecundity of *M. persicae*. Additionally, age-specific survival and fecundity rates of individuals on plants exposed to neem at different concentrations were described by the Weibull frequency distribution and the Enkegaard regression models.

2. Material and Methods

2.1. Insect and plant breeding

Pepper seedlings were transplanted to pots of 1.5 L, containing a mixture of peat and sterilized soil in ratio of 1:1. Afterwards, the pots with pepper seedlings were divided into five different groups to be watered with only irrigation water (as control) and irrigation water containing 250, 500, 750 and 1000 mg L^{-1} of neem (NeemAzal®-T/S). All plant groups were simultaneously watered every 3-4 days. None of fertilizers and other pesticides was applied for the plants. When the plants reached the 5-6 cm height, three plants randomly selected from each plant groups were infected separately with 10 apterous female aphids by a fine paintbrush. The first offspring of these female aphids were the mother individuals, of which life table parameters were determined.

The studies of insect and plant breeding were conducted in controlled climate room conditions at 25±1 ºC temperature, 60±5% relative humidity and 16:8 h photoperiod. All plant groups exposed to neem at different concentrations were arranged in a completely randomized pot design.

2.2. Experimental establishment

Fifteen individuals randomly and separately selected from the first offspring born within 24 hours on each plant groups were used in the experiments on life table. Each of these 15 individuals was separately put on pepper leaves in petri dishes at the base of which located a thin damp sponge and blotting paper, using a fine paintbrush. Thus the experiment was set, so that 15 replications for each plant groups exposed to neem at different concentrations. Pepper leaves in petri dishes were renovated when considered necessary. Parameters of development time, survival rate, times of preoviposition, oviposition and postoviposition, and longevity during the experiment were observed. Also, after numbers of offspring given by adult females were recorded daily, offspring individuals were removed from the petri dishes. The experiment which continued until death of all individuals was conducted in a climate chamber at 25 ºC temperature, 60% relative humidity and 16:8 h photoperiod (light:dark).

2.3. Life table and statistical analysis

After recording the data on daily basis, the following parameters of the age-specific life table of *M. persicae* reared on plants exposed to neem at different concentrations were calculated by using RmStat-3 (Özgökçe & Karaca 2010).

The age-specific survival rate (l_x) and fecundity rate (m_x, offspring/female/day) which is computed by multiplying the mean number of offspring by the sexual ratio (Birch 1948),

Net reproductive rate ($R_0 = \sum l_x.m_x$) (female/female), i.e. the mean number of offspring which are laid by a female in her lifetime (Birch 1948), (1)

Intrinsic rate of increase (r_m, female/female/day) by taking advantage from Euler-Lotka equation ($\sum e^{(-r_m.x)}\, l_x.m_x = 1$) (Birch 1948),

Mean generation time (day), $T_o = \frac{\ln R_0}{r_m}$ (Birch 1948), (2)

Gross reproduction rate, $GRR = \sum m_x$ (Birch 1948), (3)

Finite rate of increase (individual/female/day), $\lambda = e^{r_m}$ (Birch 1948), (4)

Theoretical population-doubling time (day), $T_2 = \frac{\ln 2}{r_m}$ (Kairo & Murphy 1995), (5)

Reproductive value (female/female), $V_x = \frac{\sum_{y=x} \left(e^{r_m.y}.l_y.m_y\right)}{l_x.e^{-r_m.x}}$ (Imura 1987) (6)

Where; x is the female's age in days; e is Euler's number which is a mathematical constant (approximately equal to 2.71828).

Pseudo-r_{mj} values of the intrinsic rate of increase (r_m) values computed on the data obtained from these populations, in order to be used in comparison test, were calculated according to the jackknife resampling method (Meyer et al 1986; Özgökçe & Atlıhan 2004) and then, Tukey multiple comparison test (Tukey 1949) was applied after One-Way

ANOVA for these pseudo-r_{mj} values of the intrinsic rates. Statistical analyses were performed by using IBM® SPSS® Statistics (Version 20.0, August 2011, SPSS Inc., Chicago, IL, USA) and MS Excel 2010 (Version 14.0) package programs.

Two-parameter Weibull distribution model was used to describe age-specific survival rate (l_x) of individuals on plants exposed to neem at 250, 500, 750 and 1000 mg L^{-1} concentrations (Deevey 1947; Pinder et al 1978; Tingle & Copland 1989; Wang et al 2000). The parameters of this distribution model were calculated according to the following formula:

$$S_p(x) = e^{\left[-\left(\frac{x}{b}\right)^c\right]} \qquad x, b, c > 0 \qquad (7)$$

Where; $S_p(x)$ is the probability of survival at x age; x is the female's age in days; b is a scale parameter and c is a shape parameter. The shape parameter of the curve belonging to the age-specific survival rate c>1, c=1 or c<1 correspond to Deevey's (1947) type I, II or III survivorship curves, respectively (Pinder et al 1978).

Description of the age-specific fecundity rate (m_x) of apterous adult females on plants exposed to neem at 250, 500, 750 and 1000 mg L^{-1} concentrations was performed by Enkegaard regression model (Enkegaard 1993; Hansen et al 1999).

$$F_{(x)} = a.x.e^{(-bx)} \qquad (8)$$

Where; $F(x)$ is the probability of fecundity at x age (female/female/day); x is the female's age in days; a and b are constant parameters; e, Euler's number which is mathematical constant (approximately equal to 2.71828).

The parameters and the coefficients of determination (R^2) in both models were obtained using SigmaPlot® (Version 11.0, Systat Software, Inc., San Jose California, USA) package program.

3. Results and Discussion

3.1. Development and adult longevity

The durations of development and lifespan of *Myzus persicae* on plants exposed to 250, 500, 750 and 1000 mg L^{-1} neem concentrations are given in Table 1. According to these results, duration of all immature stages increased in response to the rise of exposure neem concentration. However, the adult lifespan decreased inversely with increasing concentrations (Table 1).

Neem increased significantly the development period of *Aphis glycines* (Hemiptera: Aphididae) in a study by Kraiss & Cullen (2008) investigating the effect of neem-based insecticides on *A. glycines* and one of its predators. Silva et al (2013) studied the sublethal effects of the extracts derived from *Azadirachta indica* to *Ceratitis capitata* (Diptera: Tephritidae). The results of the study showed that the adult lifespan was decreased by the extracts. In addition, preoviposition time was not affected; however, a reduction in the number of eggs laid per day occurred.

3.2. Age-specific survival rate

The age-specific survival rate (l_x), fecundity rate (m_x), and reproductive value (V_x) of *M. persicae* exposed to neem at different concentrations are given in Figure 1.

The individuals exposed to 250 mg L^{-1} showed higher reproductive value, such as those in the control group, compared to the individuals exposed to higher concentrations of neem. 46.7% of the individuals in the control group, 60.0% of the individuals exposed to 250 mg L^{-1} and all individuals exposed to higher concentrations of neem died up to the 30th day. Kraiss & Cullen (2008) suggested that the mortality rate of *A. glycines* nymphs exposed to neem-based insecticide reached up to 77-80% and the development time of surviving individuals was also elongated.

Di Ilio et al (1999) established 5 different experimental groups, with liquid rearing diet only (as positive control), no food (as negative control), neem only (20 µL mL^{-1}), neem:food (1:1 mixture) and neem:food (2:1 mixture) researching the efficacy of neem compounds on longevity and fecundity of *Ceratitis capitata* adults. The study revealed that longevities of adults were 22.9 days in the positive control group, 15.9 days in the neem:food (2:1 mixture) group, 15.2 days in the neem:food (1:1

Table 1- Development duration and lifespan of *Myzus persicae* on plants exposed to different neem concentrations

Biological stages	*Concentrations*	*N*	*Duration±SE (day)*
First larval stage	Control	15	1.00±0.00 c
	250 mg L^{-1}	15	1.00±0.00 c
	500 mg L^{-1}	15	1.67±0.13 b
	750 mg L^{-1}	15	1.93±0.07 ab
	1000 mg L^{-1}	15	2.13±0.19 a
Second larval stage	Control	15	1.67±0.13 a
	250 mg L^{-1}	15	1.73±0.12 a
	500 mg L^{-1}	15	1.93±0.12 a
	750 mg L^{-1}	15	1.87±0.10 a
	1000 mg L^{-1}	15	2.00±0.20 a
Third larval stage	Control	15	1.40±0.13 b
	250 mg L^{-1}	15	1.53±0.13 b
	500 mg L^{-1}	15	1.87±0.13 ab
	750 mg L^{-1}	15	2.00±0.10 ab
	1000 mg L^{-1}	15	2.33±0.28 a
Fourth larval stage	Control	15	1.80±0.11 a
	250 mg L^{-1}	15	2.07±0.21 a
	500 mg L^{-1}	15	2.33±0.19 a
	750 mg L^{-1}	15	2.27±0.21 a
	1000 mg L^{-1}	15	2.56±0.30 a
Total development time	Control	15	5.87±0.13 b
	250 mg L^{-1}	15	6.33±0.23 b
	500 mg L^{-1}	15	7.80±0.18 a
	750 mg L^{-1}	15	8.07±0.23 a
	1000 mg L^{-1}	15	7.53±0.50 a
Lifespan	Control	15	29.00±1.44 a
	250 mg L^{-1}	15	28.47±1.43 ab
	500 mg L^{-1}	15	23.27±0.94 bc
	750 mg L^{-1}	15	17.93±1.60 cd
	1000 mg L^{-1}	15	13.44±1.07 d

*, within same biological stages, the values (±standard error) means sharing a letter are not significantly different from each other (Tukey's at $P<0.05$)

mixture) group, 6.5 days in the negative control group and 4.4 days in the group where only neem (20 µL mL^{-1}) was used.

Di Ilio et al (1999) has also explored the effect of 1:1 ratio mixtures of neem solutions at 1, 5, 10 µL mL^{-1} concentrations with liquid rearing diet. In this experiment, 1:1 ratio mixture of distilled water with liquid rearing diet was used as control. According to the data obtained from this experiment, the longevities of adults were 24.7 days in the control group, 20.1 days in the group used 1 µL neem, 9.4 days in the group used 5 µL neem and 4.0 days in the group used 10 µL neem.

The best survival curves for apterous adult females on plants exposed to neem at different concentrations were described by the two-parameter Weibull distribution according to the age-specific survival rates (l_x) (Figure 2).

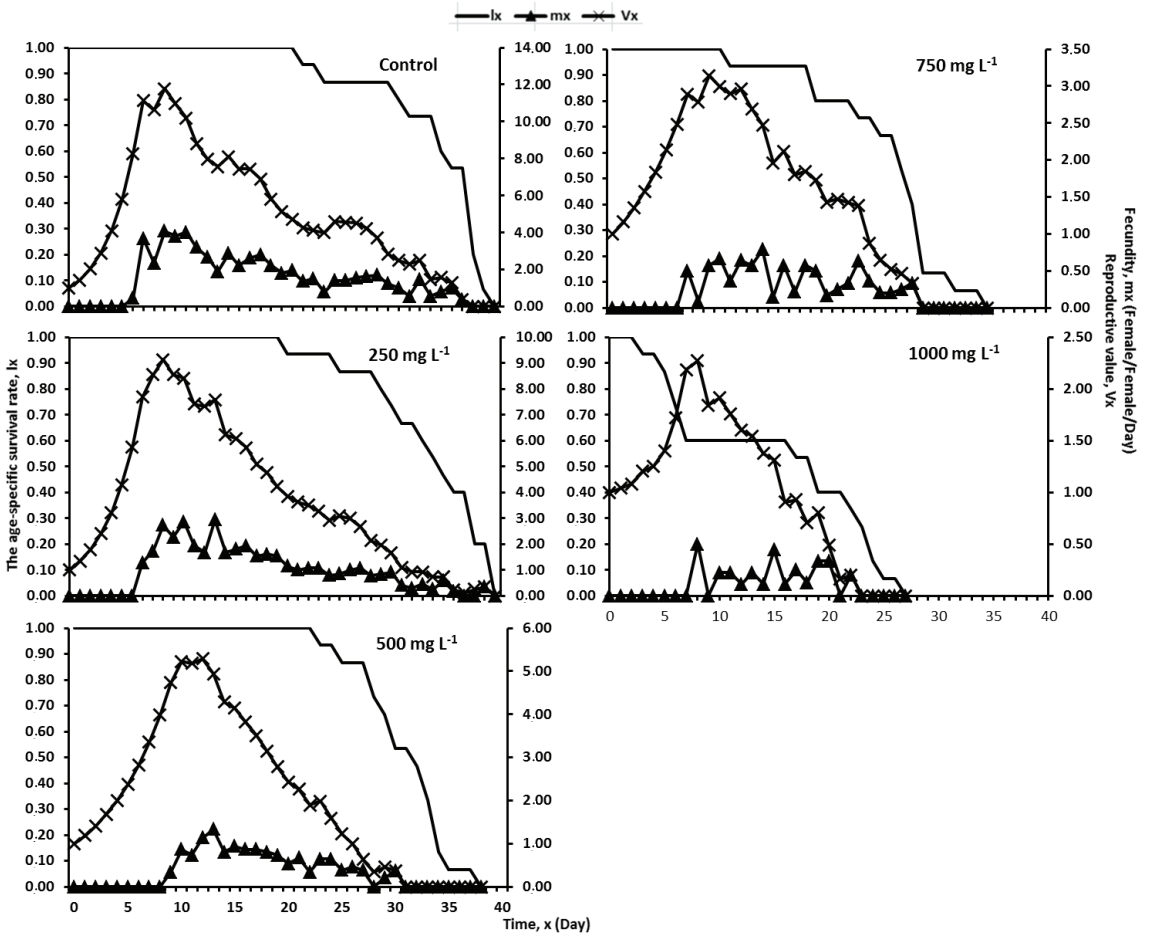

Figure 1- The age-specific survival rate (l_x), fecundity rate (m_x), and reproductive value (V_x) of *Myzus persicae* exposed to neem at different concentrations

Based on the Weibull distribution models' parameters b (scale) and c (shape), and coefficients of determination (R^2), it is possible to say that the individuals exposed to 1000 mg L^{-1} neem have the type 3 life curve, that is the type of decreasing population, and the age-specific survival rates (l_x) of the individuals from other groups fit the type 1 life curve, that is the type of increasing population. Moreover, the Weibull distribution model for the age-specific survival rates (l_x) did not show any compatibility to the data obtained from the individuals exposed to 1000 mg L^{-1}. However, the Weibull distribution model was also found to be highly compatible with the data obtained from individuals in other groups (Figure 2). The very low coefficient of determination (R^2= 0.4601) in the Weibull distribution model for the individuals exposed to 1000 mg L^{-1} is thought to be due to the high mortality rate and very short lifespan of adults in this group.

3.3. Fecundity

Mean daily and total number of offspring laid by females exposed to 250, 500, 750 and 1000 mg L^{-1}

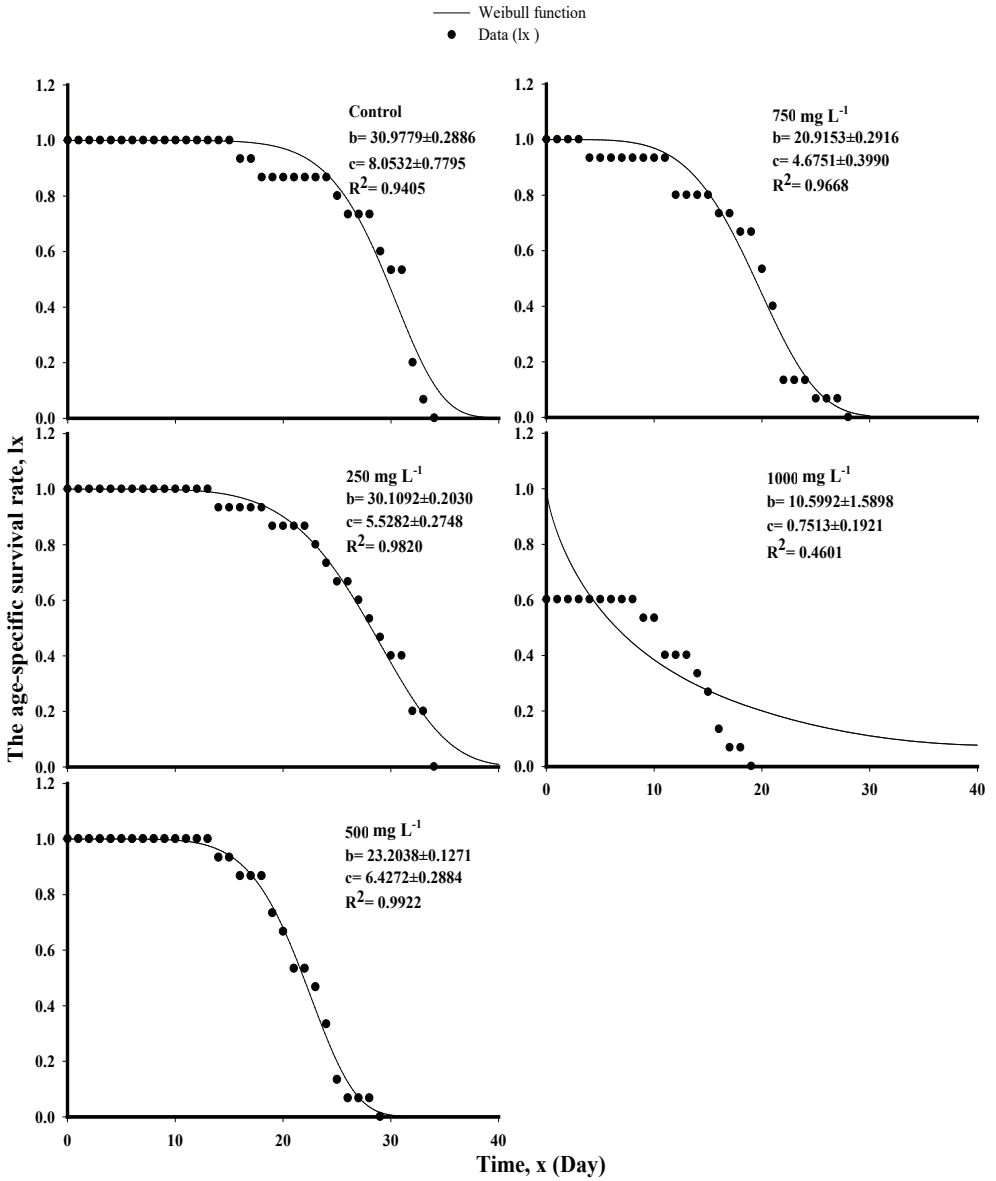

Figure 2- The Weibull distribution model for age-specific survival rates (l_x) of individuals of *Myzus persicae* exposed to neem at different concentrations

neem concentrations are shown in Table 2. According to the obtained results, mean daily and total number of offspring laid by females of the pest decreased with the increasing concentrations of neem (Table 2).

Kraiss & Cullen (2008) reported that the decrease of the fertility in *A. glycines* individuals exposed to neem-based insecticides was observed. Bhardwaj & Ansari (2015) pointed out that neem, compared to the control, has a significant impact

Table 2- Mean daily and total fecundity of Myzus persicae exposed to neem at different concentrations

Concentrations	*N*	*Mean daily fecundity (±SE) (Offspring/female/day)*	*Mean total fecundity (±SE) (offspring/female)*
Control	15	2.00±0.10 a	57.27±3.43 a
250 mg L^{-1}	15	1.37±0.08 b	37.93±1.98 b
500 mg L^{-1}	15	0.58±0.04 c	13.53±1.08 c
750 mg L^{-1}	15	0.40±0.03 d	7.71±0.72 d
1000 mg L^{-1}	15	0.24±0.02 e	3.67±0.50 e

*, within same columns, the values (±standard error) means sharing a letter are not significantly different from each other (Tukey's at $P<0.05$)

on the fertility, hatchability and adult emergence rate of *Earias vittella* F. (Lepidoptera: Noctuidae). According to the results of this study, egg production of a female lepidopteran in its life was 300 eggs in the control group. The fecundity rate in the individuals exposed to neem at 0.70, 1.10, 1.50 and 1.90 mg/individual was 229, 188, 166 and 148 eggs/female, respectively. Hatching rate of these eggs, as compared to that of the control, were 88%, 75%, 63% and 46%, respectively. The adult emergency rates were calculated as 80%, 70%, 61% and 37%, respectively in those studies. Besides that, *A. indica* was reported in a study by Silva et al (2013) to have an impact on the fecundity of *C. capitata*, too. It is announced in this study that the number of eggs laid by females at the 8th day after the application of the extract derived from branches of *A. indica* in dichloromethane was reduced in proportion of approximately 80%, and the hatching rate was decreased up to 30%. The mean numbers of eggs oviposited daily by female of *C. capitata* were 12.7 eggs/female/day in the females exposed to the extract derived from branches of *A. indica* in dichloromethane and also, 21.5 eggs/female/day in the females exposed to the extract derived from leaves of *A. indica* in methanol. Di Ilio et al (1999), in a study using 3 pairs of *C. capitata* adults per each replication, stated that the fecundity rate was 461.4 eggs/replicate in the control group, 279.7 eggs/replicate in the group applying 1 µL neem, 98.2 eggs/replicate in the group applying 5 µL neem and 1.2 eggs/replicate in the group applying 10 µL neem. Khan et al (2007) applied a commercial preparation and leaf dust of neem on the adults of *Bactrocera cucurbitae* (Coquillett) and *B. dorsalis* (Hendel) (Diptera: Tephritidae) to determine the effect on the longevity, fecundity and ovarian development of these pests. The results of this study showed that the degradation in the fecundity rates was due to the prevention of ovarian development and lifespans were decreased in a high proportion according to the control in both species.

The age-specific fecundity rates (m_x) of apterous adult females on plants exposed to neem at different concentrations were described by the Enkegaard regression model (Figure 3).

The coefficient of determination (R^2) value was used as the suitability criteria of the Enkegaard regression model to obtained data (Kontodimas et al 2004). Enkegaard regression model of the data obtained from the individuals exposed to neem at 1000 mg L^{-1} concentration (R^2= 0.2402) set out of the age-specific fecundity rate (m_x) did not show coherence. It is observed that Enkegaard regression models were more compatibility for the data obtained from the other groups (Figure 3).

It is thought that this situation was due to low and variable fecundity rate and short lifespan of the individuals exposed to neem at 1000 mg L^{-1} concentration, as compared to that of other groups (Kontodimas et al 2007). In addition to this, the Enkegaard regression models showed that the fecundity rates decreased with the increasing application concentrations and times after applications. The highest fecundity rates in all experimental groups were observed in the first 7 days (Figure 3).

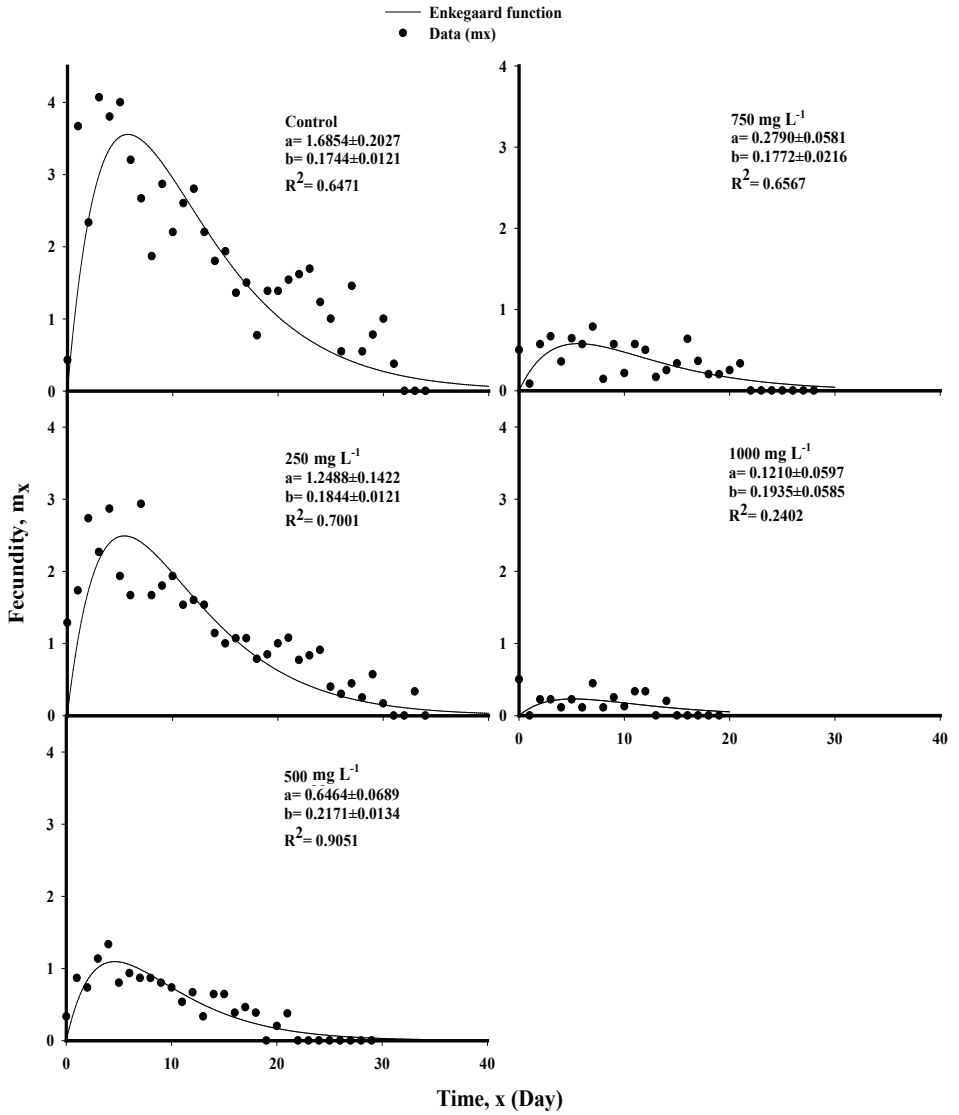

Figure 3- The Enkegaard regression models set out of the age-specific fecundity rate (m_x) of the individuals of Myzus persicae exposed to neem at different concentrations

3.4. Life table parameters

Intrinsic rate of increase (r_m), net reproductive rate (R_0), mean generation time (T_0), theoretical population-doubling time (T_2), gross reproduction rate (GRR) and finite rate of increase (λ) values are seen in Table 3. Based on the results, it is possible to say that the individuals of *M. persicae* showed less breeding with increasing concentration of neem.

In a study performed by Barati et al (2013) on the effect of some botanical insecticides on *Bemisia tabaci*, net reproductive rate (R_0), intrinsic rate of increase (r_m), mean generation time (T_0), theoretical

Table 3- Life table parameters of *Myzus persicae* exposed to neem at different concentrations

Parameters	*Control*	*250 mg L^{-1}*	*500 mg L^{-1}*	*750 mg L^{-1}*	*1000 mg L^{-1}*
Intrinsic rate of increase, r_m	0.352	0.291	0.173	0.152	0.039
Net reproductive rate, R_0	57.295	38.019	13.533	7.688	1.700
Mean generation time, T_0	11.503	12.249	15.086	13.446	13.715
Gross reproduction rate, GRR	60.60	40.46	14.02	8.91	3.19
Theoretical population-doubling time, T_2	1.970	2.381	4.014	4.569	17.915
Finite rate of increase, λ	1.422	1.338	1.188	1.164	1.039
n	15	15	15	15	15

population-doubling time (T_2) and finite rate of increase (λ) values were determined as 8.23 female/female, 0.090 female/female/day, 23.50 day, 7.56 day and 1.094 individual/female/day, respectively. Also it was declared that neem extracts have a high impact on the life table parameters of *B. tabaci* and they can be used in the scope of integrated pest management against this pest.

The statistical analysis practiced on the pseudo-r_{mj} values of the intrinsic rate of increases (r_m) computed on the data obtained from the individuals of *Myzus persicae* exposed to different neem concentrations shows that the intrinsic rate of increases (r_m) are located in the different statistical groups and the intrinsic rate of increases (r_m) of the individuals were decreased with the increase in exposed neem concentration (Figure 4).

Meanwhile, a high correlation [$R^2_{(r_{m,\ dose})}$= 0.97] was also found between the amount of concentration and the intrinsic rate of increase (r_m) in consequence of the linear regression analysis performed on the data of the individuals exposed to different neem concentrations. As for that the formula [r_m = -0.0766*Concentration+0.431] given by this correlation (P<0.01), the increase of neem concentration applied to the root of pepper plants has led to a decrease in the intrinsic rate (r_m) of *M. persicae* individuals (Figure 4).

A study by dos Santos et al (2004) exhibited the effect of neem at 23.8, 122.0, 410.0 and 1410.0 mg.100 mL^{-1} concentrations on the survival rate and fecundity of *Aphis gossypii* Glover (Hemiptera: Aphididae). The results of this study indicated that nymphal mortalities at the highest two concentrations of neem reached up to 60% and 100%. Net reproductive rate (R_0) of the individuals in the control group was defined as 35 female/female/offspring. Also, this value was decreased with the increase of neem concentration and reduced down to zero.

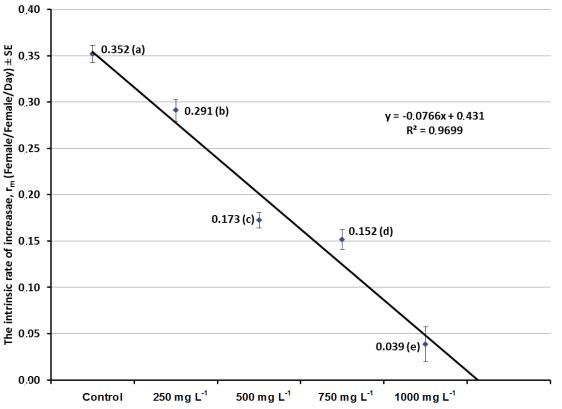

Figure 4- The intrinsic rate of increases (r_m) of *Myzus persicae* exposed to neem at different concentrations and the correlation function between them (P<0.01). The values (±standard error) means sharing a letter are not significantly different from each other (Tukey's at P<0.05)

3.5. Root uptake of neem

Neem taken up by pepper root can be transported in the vascular system systemic and translaminar (Pavela et al 2004; Souza & Vendramim 2005; Hossain et al

2008; Coelho Junior & Deschamps 2014). Pavela et al (2004) performed soil application of neem at low concentrations to the root system of rape plant (*Brassica napus* subsp. *napus*) and explored its systemic effect on the mortality, development time, longevity and fecundity of *Brevicoryne brassicae* L. (Hemiptera: Aphididae). According to the results of the study, nymphal mortality of the pest, especially during the molting periods, was raised significantly with increasing concentrations. Neem has not any effect on the development time of nymphs. Longevity of adults and wasted time for feeding were decreased with increasing concentrations. It is also observed that fecundity rate of the aphid was reduced after the neem applications. Hossain et al (2008) examined the impact of neem applied to the root system of tomato plants on *Liriomyza sativae* (Diptera: Agromyzidae). In the study, 0.75, 1.50, 2.25 and 3.00 g L^{-1} concentrations of NeemAzal®-U preparation were used. Fecundity and hatching rates were influenced slightly at all concentrations of neem, but larval mortality was affected significantly systemically by neem at 3.00 g L^{-1} concentration.

Meanwhile, it is know that neem has a phytotoxic effect on some plants (Walter 1999; Xuan et al 2004; Girish & Shankara Bhat 2008; Soto et al 2010; Esparza-Díaz et al 2010; Saha et al 2011). Also in this present study, phytotoxic symptoms in the form of necrotic spots were seen on the leaves of plants exposed to 750 and 1000 mg L^{-1} concentrations of neem.

4. Conclusions

Based on the results of the present study, neem can be transported systemically, and soil application thereof has an effect on some biological characteristics of the aphid, *Myzus persicae*. Development times in the pre-adult stages were extended as exposed neem concentration was increased. Longevity, however, was decreased inversely with increasing concentration. Additionally, a decrease in the mean daily and total number of offspring laid by females of the pest was observed. On the other hand, azadirachtin-A as the most important compound of neem is highly sensitive to sunlight (Bajwa & Ahmad 2012) and as well as neem was identified to have adverse effect on some parasitoids (Lowery & Isman 1995; Simmonds et al 2002; Lyons et al 2003; Saber et al 2004; Cóndor-Golec 2007; Abedi et al 2014). When considered from this point of view, applying of neem to the plant root zone was understood to provide also an advantage. This last point should also receive attention because it is important to have data in saving parasitoids against applications of neem. Finally, it can be said that applying of neem extracts systemically through plant root systems will provide an important advantage in controlling the sucking pests, like *M. persicae*, in agroecosystems performing integrated pest management.

Abbreviations and Symbols	
a, b, c	Model coefficients
l_x	The age-specific survival rate
m_x	Fecundity rate
V_x	Reproductive value
r_m	Intrinsic rate of increase
R_0	Net reproductive rate
T_0	Mean generation time
T_2	Theoretical population-doubling time
GRR	Gross reproduction rate
λ	Finite rate of increase
$S_p(x)$	The probability of survival at x age
$F_{(x)}$	The probability of fecundity at x age

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Proteolysis in the Beyaz (White) Cheese Produced From Various Milk

Zübeyde ÖNER[a], A. Mine SARIDAĞ[a]

[a]*Suleyman Demirel University, Engineering Faculty, Food Engineering Department Isparta, TURKEY*

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Corresponding Author: Zübeyde ÖNER, E-mail: zubeydeoner@sdu.edu.tr, Tel: +90 (535) 615 60 26
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ABSTRACT

The aim of this study was to investigate proteolysis development and peptide changes during the storage period of Beyaz (White) cheese which is produced from various types of milk, such as sheep, goat and cow milk. Three types of cheese were produced using goat, sheep and cow milk, and all analyses were performed in duplicate. Proteolytic changes were observed in cheese on the 1st day, 3rd, 6th, and 9th month. The total protein was investigated through using water-soluble nitrogen, soluble nitrogen in 12% Trichloroacetic acid (TCA), soluble nitrogen in 5% Phosphotungstic acid (PTA), and free amino acids. Proteolytic changes and peptide formations were observed during maturation by HPLC. While the total protein was 14.33% in sheep Beyaz cheese on the 1st day, this value decreased during the maturation period and reduced to 6.9% in the 9th month. These values were 13.55 and 7.95 for cow cheese and 16.30% and 7.95% for goat cheese, respectively. The water-soluble protein value increased during the maturation period. The value was 1.41% in sheep cheese on the 1st day, and increased to 6.24% in the 9th month. These values were 2.16% and 4.92% for goat cheese, and 1.79% and 8.53% for cow cheese, respectively. 12% TCA soluble nitrogen had been 0.211% at the beginning and was 0.51% in the 9th month. The ripening value based on 12% TCA changed between 9.41%-47.22%. 5% PTA soluble nitrogen changed between 0.075%- 0.25 %. The ripening value based on 5% PTA changed between 3.34-23.14.

Changes in concentration of total free amino acids during cheese ripening were tracked. In water soluble extracts of cheese, the presence of free amino groups in all ripening stages was detected. It is observed that amino acids and smaller peptides-have concentration significantly ($P<0.05$) increased during ripening. The total free amino acid was found as 0.24 mg lysine g^{-1} in sheep cheese, 0.215 mg lysine g^{-1} in goat cheese and 0.208 mg lysine g^{-1} in cow cheese at the end of ripening period.

Keywords: Proteolysis; Sheep milk cheese; Goat milk cheese; Cow milk cheese

Farklı Sütlerden Üretilen Peynirlerde Proteoliz

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Zübeyde ÖNER, E-posta: zubeydeoner@sdu.edu.tr, Tel: +90 (535) 615 60 26
Geliş Tarihi: 19 Ağustos 2016, Düzeltmelerin Gelişi: 07 Kasım 2017, Kabul: 18 Ocak 2018

ÖZET

Bu çalışmada; koyun, keçi ve inek sütlerinden Beyaz peynirler üretilmiş ve depolama süresince farklı sütlerden üretilen peynirlerde proteoliz gelişimi ve peptid değişimlerinin belirlenmesi amaçlanmıştır.

Bu amaçla; koyun, keçi ve inek çiğ sütlerinden geleneksel olarak Beyaz peynir üretilmiştir. Peynirler 3 tekerrür, analizler 2 paralel halinde yapılmıştır. Peynirlerde 1. gün, 3., 6., 9 ay'da oluşan proteolitik değişimler izlenmiştir. Bu amaçla toplam protein, suda çözünen azot, % 12 TCA da çözünen azot, % 5 PTA da çözünen azot, serbest amino asit tayinleri yapılmıştır. Olgunlaşma boyunca görülen proteolitik değişim ve peptid oluşumları RP-HPLC cihazı ile tespit edilmiştir. Koyun Beyaz peynirde 1. gün toplam protein % 14.33 iken olgunlaşma boyunca bu değerde azalma meydana gelmiş toplam protein 9. ayda % 6.9'a düşmüştür. İnek peynirinde toplam protein % 13.55'den % 7.95'e keçi peynirinde ise % 16.30'dan % 7.95'e düşmüştür. Suda çözünen protein değerinde ise olgunlaşma süresince artış izlenmiştir. Koyun peynirinin suda çözünen protein değeri 1. günde % 1.41 iken bu değer 9. ayda % 6.24'e, keçi peynirinde % 2.16'dan % 4.92'ye inek peynirinde ise % 1.79'dan % 8.53'e çıkmıştır. % 12 TCA'da çözünen azot başlangıçta % 0.211 iken 9. ayda % 0.51 olarak belirlenmiştir. % 12 TCA'ya göre olgunlaşma değeri 9.41-47.22 arasında değişmiştir. % 5 PTA çözünen azot birinci günde 0.075 iken 9. ayda 0.25 bulunmuştur. % 5 PTA'ya göre olgunlaşma değeri 3.34-23.14 arasında değişmiştir.

Olgunlaşma süresince toplam serbest amino asit değerleri izlenmiştir. Olgunlaşma süresince peynirin suda çözünen maddeler kısmında serbest amino grupları belirlenmiştir, bu değerler peptit ve amino asitlerin göstergesidir. Olgunlaşma süresince meydana gelen değişim istatiksel açıdan önemli bulunmuştur ($P<0.05$). Olgunlaşma sonunda toplam serbest amino asit koyun peynirlerinde 0.24 mg lysine g^{-1}, keçi peynirlerinde 0.215 mg lysine g^{-1} inek peynirlerinde ise 0.208 mg lysine g^{-1} peynir olarak saptanmıştır.

Anahtar Kelimeler: Proteoliz; Koyun sütü peyniri; Keçi sütü peyniri; İnek sütü peyniri

1. Introduction

Beyaz cheese is highly consumed in our country. Various types of milk (sheep, goat, cow) are used to produce Beyaz cheese. Beyaz cheese has various structure, taste and appearance. To involve these features, Beyaz cheese must mature for a certain period of time.

The biochemical reaction is the most important process that shows the maturation of cheese. It occurs as a result of casein hydrolisation by means of plasmins in addition to the effect of rennin enzyme (Fox et al 2000). As a result of hydrolisation, degradation into low-molecular-weight peptides and amino acids occurs through large-and medium-molecular-weight peptides, enzymes and enzymes synthesized by lactic acid bacteria. Proteolysis in cheese is observed through tracking the changes in total nitrogen and nitrogen fractions. During maturation period, proteins which are present in the structure of cheese degrade into polypeptides and amino acids; and depending on the characteristics of amino acids, taste and flavor of cheese vary (Fox et al 1993).

This study aims to analyse the proteolysis rates in cheese which is produced using different types of milk, and in addition to these analyses, to determine peptides formed in the cheese using the HPLC method.

2. Material and Methods

2.1. Material

Sheep, goat, and cow milk were purchased from different farmers in Isparta region.

2.2. Cheese-making

Beyaz cheese was manufactured according to traditional procedures. Beyaz cheese was produced using with sheep, goat and cow milk. Cheese samples were taken on 0^{th}, 3^{th}, 6^{th}, 9^{th} month of ripening. Cheese manufacturing was performed in triplicate and chemical analyses were made duplicate (Öner et al 2006).

Raw milk was clarified and heated to 25-26 ºC, and then it was transferred to cheese vats. The milk was coagulated by adding calf rennet in 150 min. The coagulum was cut into cubes (2 cm^3) and the curds were allowed to rest in the whey for 5-10 min. The curds were pressed until whey drainage has stopped or decreased to a low level. The weights were removed, cheese were cut 7x7x7 cm^3. The blocks were placed in brine (14 g 100 mL^{-1} NaCl)

for 2 h. The brined blocks were placed in tin can. The cheeses were ripened in the cans at 5-6 ºC for 9 month.

2.3. Chemical analysis

Total protein, nitrogen fractions and total free amino acids in the cheese were analyzed. The protein content was measured by Kjeldahl (Gerhardt Vapotest VAP 30 distillation systems, Gerhardt Turbo therm and Turbo digestion systems) according to AOAC method (AOAC 1990).

Water-soluble nitrogenous compounds were separated with the method presented by Kuchroo & Fox (1982), and water soluble nitrogen ratio was determined by the micro-Kjeldahl method (IDF 1993).

12% Trichloroacetic acid soluble nitrogen (TCA-SN) was determined by the standard micro-Kjeldahl method (Polychroniadou et al 1999).

5% Phosphotungstic acid soluble nitrogen (PTA-SN) content of the obtained filtrate was detected by the micro-Kjeldahl method (Polychroniadou et al 1999).

Total amount of free amino acids were performed through the method presented by Folkertsma & Fox (1992). Absorbance of the sample at 507 nm which was prepared with Cd-ninhydrin reagent was observed.

2.4. Water soluble peptides

The analysis of water-soluble peptides in cheese was carried out with reversed-phase (Shimadzu LC-20 AT series) HPLC and a Zorbax 300 SB-C8 monomeric column (250×9.4 mm i.d., 6.5 µm particle size and 300 A^0 pore diameter, Agilent, Waldbronn, Germany). The samples were dissolved in 0.1% TFA at the rate of 0.2 g 5 mL^{-1}, and 750 µL were injected into the HPLC column by filtering it through an 0.45 µm diameter filter. The peptides were eluted over a linear gradient from 100 to 0% solvent A (0.1% trifluoroacetic acid in deionized water) in solvent B (0.1% trifluoroacetic acid in 90% (v v^{-1}) acetonitrile in deionized water) over 80 minutes (Combes et al 2002). The level 214 nm was detected. Peaks with retention times from 0-40 min were considered to correspond to hydrophilic peptides and peaks with retention times from 40 to 70 min to hydrophobic peptides (Hayaloğlu et al 2004).

2.5. Statistical analysis

Data for experimental cheese varieties were analysed by Anova test through using the general linear model. Differences between means were analysed by Duncan's multiple range test at level of 0.05 using SPSS-software, version 18. This was used in order to comprehend the evaluation of proteolytic changes during storage of each cheese type

3. Results and Discussion

3.1. Total protein

The total protein contents in cheeses varied depending on the amount of casein in milk and cheese production techniques. The protein rate decreased during ripening period (Table 1). The total nitrogen content, which had been 13.55% at the beginning of maturation (0 days), reduced to 7.93% at the end of the 9th month. The change in total nitrogen amount in cheese composition is related to protein degradation. It can be explained by the reduction of nitrogenous substances to other compounds (amine, acid, thiol, etc.) (Fox et al 1996). The ripening index value of cow milk cheese reached 107.62 in 9 months. Melting, rancidity, and decomposition were observed in cow cheese during 9 months.

Protein level in the cheese produced from goat milk was between 16.3-7.95%. This value, which had been 13.28% at the beginning of ripening index, reached 61.87% in the 9th month.

Protein level (Table 1) in the cheese produced from sheep milk was between 14.33-6.92. Ripening index values reached 9.78-90.12%. Depending on the metabolic activity in the storage period, the total protein content reduced while the low-molecular-weight fractions of nitrogen increased. Some of the nitrogen fractions pass to the pickle, and an increase

occurs in the concentration of nitrogen fraction dissolved in the brine (Hayaloğlu et al 2002). For the cheese ripened in the brine, water-soluble nitrogen fractions pass to the brine during the storage period until a balance is reached. Proteolysis level in the cheese produced from raw milk varies depending on the rich content of microorganisms. That the ripening index was 90 in the 9th month showed that it increased depending on the microbial flora in raw milk and its proteolytic activity. As a result of the statistical assessment, the change in the protein content during the maturation period was found to be significant ($P<0.05$).

Series of biochemical events occur during the ripening period of the cheese. Therefore, the taste, flavor and structure formation occur according to the type of cheese.

In the ripening process, enzyme used for maturation as well as the enzyme in milk, starter cultures, and non-starter bacteria play a role (Fox 1989; Fox et al 1996). In the maturation process, the degradation of lactose, as well as lipolysis and proteolysis events, occur (Mc Sweeney & Fox 1997). Although the characteristics of Beyaz cheese depend on the acidity and the salt content, final products of lipolysis and proteolysis are effective in the formation of these characteristics (Güler & Uraz 2004). Many changes were observed in the cheese maturated in the brine within 3 months of production (Hayaloğlu et al 2002). Table 1 reveals the ripening index of the cheese produced from cow milk reached 68% in 3 months. Cow cheese had the highest proteolysis, and goat cheese had lower proteolysis.

Table 1- Protein levels of the Cow, Goat and Sheep cheese

	Months	*TP %*	*WSN%*	*RI*
Cow	0	13.55±0.22^{a}	1.79±0.02^{d}	13.24
	3	8.55±0.11^{b}	5.82±0.07^{c}	68.05
	6	7.29±0.45^{c}	7.10±0.15^{b}	97.50
	9	7.93±0.16^{bc}	8.53±0.00^{a}	107.62
Goat	0	16.30±0.19^{a}	2.16±0.01^{d}	13.28
	3	11.06±0.08^{b}	2.76±0.11^{c}	25.02
	6	9.44±0.04^{c}	3.75±0.13^{b}	39.69
	9	7.95±0.08^{d}	4.92±0.15^{a}	61.87
Sheep	0	14.33±0.25^{a}	1.41±0.12^{d}	9.78
	3	13.86±0.43^{a}	1.97±0.07^{c}	14.23
	6	8.76±0.87^{b}	5.17±0.18^{b}	59.00
	9	6.92±0.39^{c}	6.24±0.0^{a}	90.12

TP, total protein; WSN, water soluble nitrogen; RI, ripening index. The values in the same column having a different letters differ significantly ($P<0.05$). Data were reported as mean±standard deviation (n= 3)

3.2. Water-soluble protein

Depending on the proteolysis, water-soluble protein rate increases in a continuous manner during ripening period. This process gives the cheese mature taste and aroma. A part of casein and para-casein in the cheese is degraded with the effect of the enzyme and microorganisms used in the production, and turned into water-soluble substances such as protease-peptone and amino acids. The degradation of protein continues during the maturation process, and increases the amount of the free amino acids and peptides.

In this study, an increase in the water-soluble protein level was observed in parallel to the decrease in the total protein value (Table 1). These values represent the change in the maturation during proteolysis. Özer et al (2002) stated that water-soluble nitrogen values in the Urfa cheese increased during the maturation period. They also stated that these values in the cheese produced from sheep milk were higher than those of the other types of milk at every stage of storage.

3.3. 12% TCA soluble nitrogen

The level of 12% TCA SN increased during the ripening period (Table 2). This increase indicates the formation of peptides. The difference of peptides from WSN is that the structure of peptides is smaller (peptides consisting of 2-22 amino acids). Immediately after the cheese production, peptide bonds between Phe23 and Phe24 of α_{s1}-casein are hydrolyzed and formed f1-23 and f24-199 fractions. α_{s1}-casein (f-23) fraction, which has a small chain length, is hydrolyzed quickly by microbial proteinases (peptide

bonds between Gln9-Gly10 and Gln 13-GLn14), and free amino acids and low-molecular-weight peptides are formed (Fox et al 1996). These elements formed are dissolved in 12% TCA and are important for the evaluation of proteolysis. Ardo & Polychroniadou (1999) reported that TCA soluble nitrogenous substances consisted of peptides with 600-15000 Da molecular weight, and these peptides were hydrolysis products of α_{s1}-casein. Ripening index on the basis of TCA soluble nitrogenous substance provides information about proteolysis at an advanced level. The nitrogen values in 12% TCA and ripening index changing during the ripening period were given in Table 2. The 12% (v v^{-1}) TCA-SN contents had the highest levels in the sheep cheese during ripening (Table 2). The increase in the TCA soluble nitrogen levels in the cheese during the maturation period was found to be significant ($P<0.05$). These results are similar to the results of Pavia et al (2000b) and Guven et al (2006).

Table 2- Soluble nitrogen fractions and total amino acid concentrations of the cheeses

	Months	*TCA-SN*	*5% PTA-SN*	*Total FAA (mg Lysine g^{-1})*
Cow	0	0.279±0.009^{d}	0.022±0.002^{d}	0.062
	3	0.358±0.004^{c}	0.048±0.001^{c}	0.116
	6	0.460±0.003^{b}	0.064±0.001^{b}	0.200
	9	0.5139±0.004^{a}	0.070±0.001^{a}	0.208
Goat	0	0.186±0.005^{d}	0.070±0.002^{d}	0.070
	3	0.320±0.006^{c}	0.137±0.003^{c}	0.136
	6	0.441±0.008^{b}	0.197±0.004^{b}	0.174
	9	0.484±0.009^{a}	0.233±0.007^{a}	0.215
Sheep	0	0.211±0.117^{d}	0.075±0.003^{d}	0.107
	3	0.354±0.007^{c}	0.090±0.002^{c}	0.126
	6	0.468±0.008^{b}	0.211±0.004^{b}	0.200
	9	0.518±0.001^{a}	0.250±0.002^{a}	0.240

TCA-SN, 12% trichloroacetic acid-soluble nitrogen; PTA-SN, 5% phosphotungstic acid-soluble nitrogen; FAA, free amino acid. The values in the same column having a different letters differ significantly ($P<0.05$). Data were reported as mean±standard deviation (n= 3)

3.4. 5% PTA-SN

It was reported that the fractions of nitrogen soluble in 5% PTA in the cheese included peptides smaller than 600-700 Dalton (di-, tri- and tetra- peptides) and amino acids (Mc Sweeney & Fox 1997; Hannon et al 2003).

The level of nitrogen soluble in phosphotungstic acid in the cheese increased during the ripening period. The 5% (v v^{-1}) PTA-SN contents had the highest levels in the sheep cheese. It was observed that the ripening time was changed significantly ($P<0.05$), according to maturation value 5% PTA (Table 2). Feeney et al (2001) found that the nitrogen solubility in 5% PTA in mozzarella cheese increased during the maturation period (70 days). Hayaloğlu (2003) reported that the nitrogen solubility increase in 5% PTA level during the ripening period of the cheese was due to the solubility in 5% PTA of the small-molecule peptides and amino acids that emerge during the ripening period.

3.5. Total free amino acids

During the ripening of Beyaz cheese, the concentration of total free amino acids increased, no matter whether cheeses were produced from different types of milk. It was observed that this difference observed during the storage period increased the formation of free amino acids in cheese depending on the amount of protease. The

concentration of total free amino acids on the day 0 was not significant (P>0.05). It is known that starter bacteria and non starter bacteria are important factors for proteolysis (Hickey et al 2007). At the end of ripening, statistically significant differences were determined in the concentration of total free amino acids between the 0th and 9th month. As a result, during the ripening of Beyaz cheese, the concentration of total free amino acids significantly increased (P<0.05). The free amino acid ratio in the Beyaz sheep cheese had the total free amino acids at the highest level (Table 2).

It was reported in many studies that additional culture increased the formation of free amino acids (Oneca et al 2007; Wishah 2007; Radeljević et al 2013; Bezerra et al 2016). Similar results were found in Picante cheese and Manchego cheese by using Cd-ninhydrin method for monitoring the proteolysis (Freitas et al 1997; Pavia et al 2000b).

3.6. Water soluble peptides

The peptide concentrations of cheese show a sequence according to different molecular weights (Mc Sweeney & Fox 1997). The mechanism of separation of the peptides released during the ripening period of cheese analysed with the RP-HPLC technique. Compatible to this, a fewer hydrophobic molecules give a peak at first, and then the peaks of hydrophobic molecules are observed (Pavia et al 2000a; Hayaloğlu et al 2004; Hesari et al 2006).

The samples of sheep Beyaz cheese in month 0, 3rd, 6th and 9th were injected into HPLC, the images of peptide profiles at different wavelengths are given in Figure 1. In the study carried out to choose the appropriate wavelength, it was found that the peaks were detected better at 214 nm.

When the peaks in the RP-HPLC analysis performed to observe peptide formation in sheep Beyaz cheese during the ripening period, hydrophilic

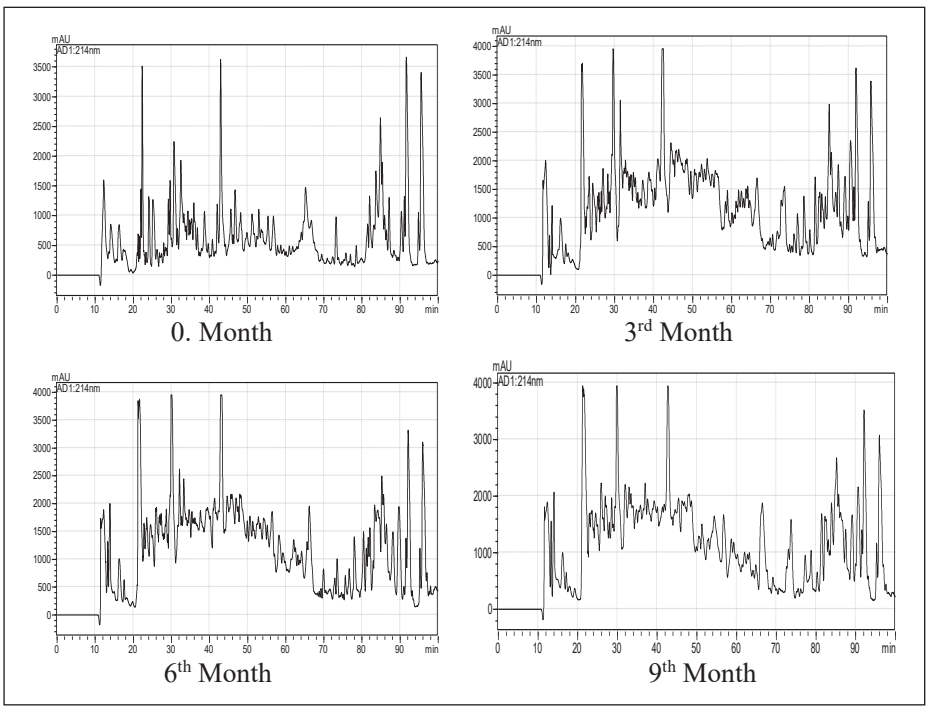

Figure 1- The comparison of chromatogram in the sheep Beyaz cheese during the ripening period (214 nm)

peptides were observed to be more in 0 month while hydrophilic peptides (with high retention time) and hydrophilic peptides (peaks at early retention time) in the 3rd, 6th and 9th months were close to each other. Similar results were found for cow and goat cheese (Figure 2-3).

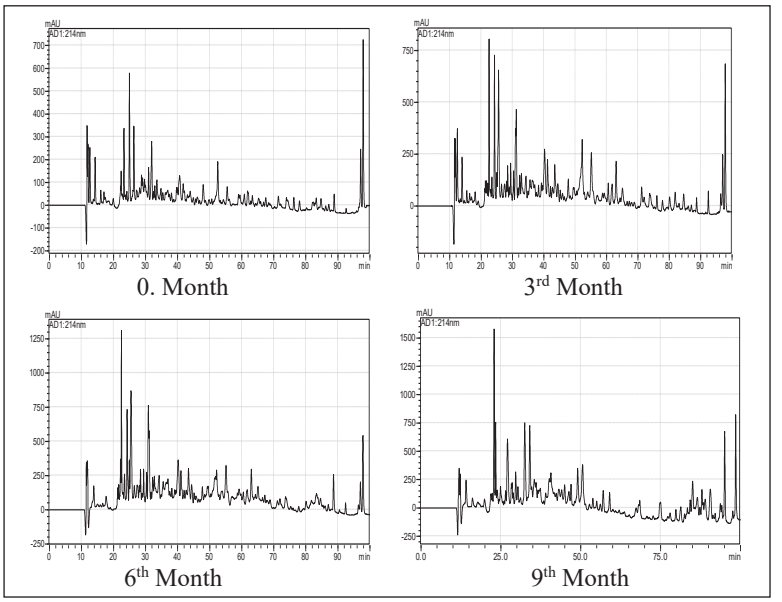

Figure 2- The comparison of chromatogram in the cow Beyaz cheese during the ripening period (214 nm)

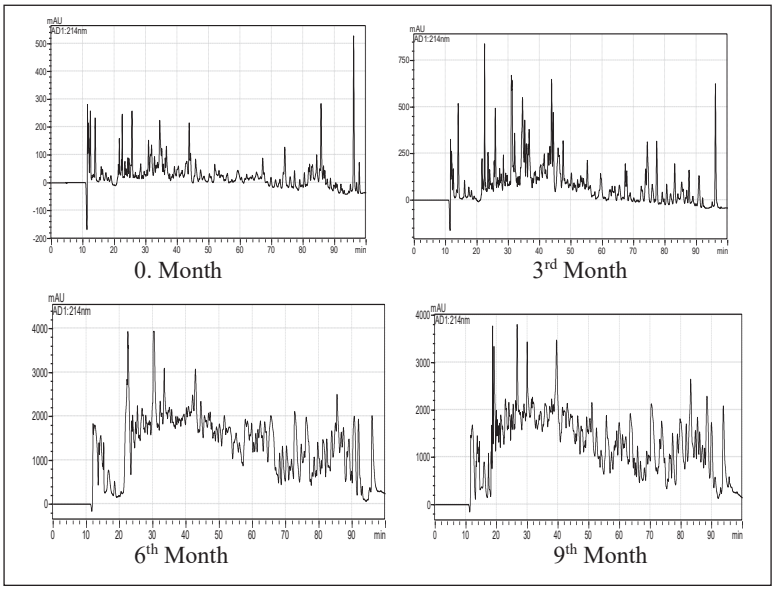

Figure 3- The comparison of chromatogram in the goat Beyaz cheese during the ripening period (214 nm)

Hayaloğlu & Karabulut (2013) explored peptides in Civil, Canak, Ezine and Dil cheeses; and the chromatograms of the water-soluble fractions of the cheeses showed that many peptides with early or late retention times. Major qualitative and quantitative differences were observed between the peptide profiles of each cheese variety. Civil cheese had high concentrations of peaks and high levels of soluble nitrogen fractions and both early retention time (hydrophilic) and late retention time (hydrophobic) peptides were determined. However high concentrations of peptides were not detected in Ezine cheese even though this cheese is ripened for at least 6 months. Hayaloglu & Karabulut (2014) had interpreted the disintegration of peptides probably into amino acids and volatile compounds, such as acids, aldehydes, and alcohols.

Gomez et al (1997) found out that both hydrophilic and hydrophobic peptide concentrations were the same in water soluble nitrogen fractions in Hispanico and semi-hard goat cheese produced from both raw milk and pasteurized milk. Since the sheep Beyaz cheese used in our experiment was produced from raw milk. The concentrations of peptides were affected due to the microflora of raw milk and aminopeptidase activity of non-starter lactic acid bacteria. Feta cheese is similar to Beyaz cheese. Sousa et al (2001) showed that in Feta cheese, most of the peptides from the WSF was originated from α_{s1}-casein 2 peptides originated from the C-terminal of β-casein and 1 peptide from κ-casein. Most of the peptides could be explained with the basis of known specificity of chymosin.

4. Conclusions

The total protein, water-soluble nitrogen, ripening index, 12% TCA soluble nitrogen, 5% PTA soluble nitrogen, and free amino acids analyses were performed in the cheese in order to observe the proteolysis event allowing the formation of peptides. Melting and rancidity occurred in cow and goat cheese in the 9th month. This situation can be seen as normal taking the long ripening period of the cheese into consideration. During the ripening period, the amino acid ratio was observed to increase in all cheese types. It was found out that the shelf life of the cheese produced from cow milk was shorter, rancidity occurred in this type of cheese during the extended storage, and goat and sheep cheeses were more appropriate for storage for longer periods.

Consequently, the cheese made from various types of milk exhibited different protein ratio. Also, it was observed that during the ripening for each cheese has an effect on their proteolysis.

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Physicochemical, Functional and Microbiological Properties of Hardaliye Beverages Produced from Different Grapes and Collected from Different Households

Fatma COSKUN[a], Muhammet ARICI[b], Mehmet GULCU[c], Gulnaz CELIKYURT[d], Mustafa MIRIK[e]

[a]*Namık Kemal University, Agricultural of Faculty, Food Engineering Department, 59030 Suleymanpasa, Tekirdag, TURKEY*

[b]*Yıldız Technical University, Faculty of Chemical and Metallurgical Engineering Faculty, Food Engineering Department, 34210 Istanbul, TURKEY*

[c]*Tekirdag Viticultural Research Station, 59100 Suleymanpasa, Tekirdag, TURKEY*

[d]*Balikesir Metropolitan Municipality, 10010 Karesi, Balıkesir, TURKEY*

[e]*Namık Kemal University, Agricultural of Faculty, Plant Protection Department, 59030 Sulaymanpasa, Tekirdag, TURKEY*

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Corresponding Author: Fatma COSKUN, E-mail: fcoskun@nku.edu.tr, Tel: +90 (282) 250 21 62
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ABSTRACT

Twenty-three hardaliye beverage samples were collected from different regions of Kirklareli city in Turkey and five hardaliye samples were produced under laboratory conditions according to traditional methods. Total phenolic compounds, monomeric anthocyanins (malvidin-3-glucoside), volatile acid, ethyl alcohol, total sugar contents, total acidity (lactic acid), pH, color values (*L* (brightness), *a* (red-green), *b* (yellow-blue)), total mesophilic aerobic bacteria, lactic acid bacteria, yeast and mold and coliform counts were determined. Acidity between 0.38 and 0.91%, pH from 3.54 and 4.33, color (*L.a.b.*) values from 7.57 to 13.74, from 0.71 to 7.68, from 0.73 to 4.50, respectively were determined. Total phenolics from 368.8 to 2647.5 mg L^{-1}, ethanol between 0.4 and 6.0% in 7 out of 23 examples, TMAB number $3x10^{1}$-$3.2x10^{6}$ cfu mL^{-1}, LAB $1x10^{2}$-$3x10^{5}$ cfu mL^{-1} in 16 out of 23 samples, yeast and mold count $1x10^{1}$-$2,3x10^{4}$ cfu mL^{-1} in 18 out of 23 samples were found. According to the results obtained, coliforms and *Escherichia coli* were not found in the analyzed samples. Statistical analysis results revealed that differences between samples were significant ($P \leq 0.01$). Given its bioactive characteristics, hardaliye beverage should be produced at large-scale and be recognized by large communities.

Keywords: Fermentation; Grape juice; Hardaliye; Mustard seeds; Traditional beverage

Farklı Evlerden Toplanan ve Farklı Üzümlerden Üretilen Hardaliye Örneklerinin Fizikokimyasal, Fonksiyonel ve Mikrobiyolojik Özellikleri

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Fatma COSKUN, E-posta: fcoskun@nku.edu.tr, Tel: +90 (282) 250 21 62
Geliş Tarihi: 17 Kasım 2014, Düzeltmelerin Gelişi: 31 Mayıs 2017, Kabul: 07 Haziran 2017

ÖZET

Bu araştırmada, Kırklareli'nin farklı bölgelerinden yirmi üç hardaliye örneği toplanmış ve laboratuvar şartlarında geleneksel metotlara göre beş adet hardaliye üretilmiştir. Bu örneklerde toplam fenolik madde, monomerik antosiyanin (malvidin-3-glikozit), uçucu asit, etil alkol, invert şeker, toplam asitlik (laktik asit cinsinden), pH, renk değerleri (*L* (parlaklık), *a* (kırmızı-yeşil), *b* (sarı-mavi)), toplam aerobik mezofil bakteri, laktik asit bakterileri, maya-küf ve koliform bakteri sayıları belirlenmiştir. Asitlik % 0.38-0.91 arasında, pH 3.54-4.33 arasında, renk (*L.a.b.*) değerleri sırasıyla 7.57-13.74; 0.71-7.68; 0.73-4.50 olarak tespit edilmiştir. Toplam fenolik madde 368.8-2647.5 mg L^{-1} arasında, etanol 23 örneğin 7'sinde % 0.4-6.0 arasında, TMAB sayısı $3x10^{1}$-$3.2x10^{6}$ kob mL^{-1} arasında, LAB sayısı 23 örneğin 16'sında (16 out of 23 samples) $1x10^{2}$-$3x10^{5}$ kob mL^{-1} arasında, maya-küf sayısı 23 örneğin 18'inde (18 out of 23 samples) $1x10^{1}$-$2.3x10^{4}$ kob mL^{-1} arasında bulunmuştur. Elde edilen sonuçlara göre, analiz edilen hiç bir örnekte koliform bakteri ve *Escherichia coli* tespit edilmemiştir. Analizler sonuçlarına göre örnekler arasındaki farklılıklar istatistiksel olarak önemli bulunmuştur ($P \leq 0.01$). Biyoaktif özellikleri dikkate alındığında, hardaliyenin büyük ölçekli üretiminin yapılması ve toplumun tanıması sağlanmalıdır.

Anahtar Kelimeler: Fermantasyon; Üzüm suyu; Hardaliye; Hardal tohumu; Geleneksel içecek

1. Introduction

Hardaliye is a lactic acid fermented beverage that is produced from red grape or grape juice with the addition of crushed mustard seeds and benzoic acid and it is widely consumed in the Thrace region of Turkey (Arici & Coskun 2001). As well known, grape is rich fruit interms of phenolic compounds (Shahidi & Naczk 1995). In vitro experimental systems also showed that flavonoids possess antiinflammatory, antiallergic, antiviral, and anticarcinogenic properties (Middleton 1998). Kanemaru & Miyamoto (1990) detected that 0.8% mustard extract showed bacteriostatic action against *Staphylococcus aureus* and *Escherichia coli* in culture medium. When essential oil of mustard was used, total mesophilic aerobic bacteria and lactic acid bacteria were significantly lower than the control after 2 days of storage (Lemay et al 2002). In a research conducted by Nielsen & Rios (2000), allyl isothiocyanate, a bioactive substance of mustard, was shown to have an inhibitory effect against *Penicillium commune, P. roqueforti, Aspergillus flavus* and *Endomyces fibuliger*. They also determined minimum inhibitory concentration to range between 1.8 and 3.5 mg mL^{-1}. Therefore, it is possible to say that the beneficial effects of hardaliye are not only related with grape which includes bioactive components but also with mustard which gained antibacterial and antifungal effects to hardaliye beverage.

The present study is composed of two parts. In the first part, 23 hardaliye beverage samples collected from local producers were analyzed in terms of their physicochemical, functional and microbiological properties. In the second part, fresh hardaliye beverage samples were produced from different grape types and their physicochemical and microbiological properties were investigated.

2. Material and Methods

2.1. Material

In this research, twenty-three hardaliye samples were collected from different households of Kirklareli city in Turkey. In the second stage, hardaliye samples were produced in the laboratory using different types of grapes. These grape types were Dingil Kara, Kara Uzum, Carbernet Sauvignon, Yerli Izabella and Siyah Uzum and procured from Tekirdag Viticulture Research Institute.

2.2. Methods

Grapes are washed and pressed in oak barrels which have a tap at the position of 10 cm above the bottom of the barrel. Then 0.2% of crushed raw mustard

seed and 0.1% of sodium benzoate were added and the solution was left to fermentation at room temperature (22 °C) for 7 days.

The pH of the samples was measured using WTW 330 model pH meter with composite electrodes. Total acidity was determined according to the titration method reported by Cemeroğlu (2007). Total sugar contents of the samples were determined using Lane Eynon method (Cemeroğlu 2007). In order to determine total phenolic content of the samples, Folin-Ciocalteu spectrophotometric method was used (Singleton & Rossi 1965). Monomeric anthocyanins were determined using the method reported by Cemeroğlu (2007). Alcohol contents of the samples were determined using ebulliometer (Jacobson 2006). Total volatile acid contents were determined using the method reported by Fidan (1975). The color of the samples was measured using a Hunter Lab colorimeter (Model D25 LT).

Standard methods defined by FDA/Bacteriological Analytical Manual for microbiological analysis were used for preparation of the samples for analysis. 25 mL hardaliye sample was added into Maximum Recovery Diluent (MRD) and it was homogenization. Other serial dilutions (10^{-2}, 10^{-3},10^{-4} and 10^{-5}) were prepared using the initial dilution. These dilutions prepared were used for all microbiological analysis. Total bacteria were enumerated using plate count agar (PCA). For counting coliform bacteria, violet red bile agar (VRBA) and for counting *E. coli,* eosin methylene blue (EMB) agar were used. For counting yeast-mold, potato dextrose agar (PDA), was used. Numbers of total mesophilic aerobic bacteria, coliform bacteria, *E. coli* and yeast and mold were determined by providing the appropriate incubation temperature and duration (Anonymous 2001a; 2001b; 2001c; 2002). De Man Rogosa Sharpe (MRS) agar was used for determination of lactic acid bacteria (Baumgart 1993).

In order to determine differences between samples, one-way ANOVA was performed using SPSS 18.0 statistical package. All statistical analyses were performed according to completely randomized design (SPSS 2009).

3. Results and Discussion

Some physicochemical properties of hardaliye samples collected from different households are shown in Table 1. Some chemical properties of hardaliye samples collected from different households are shown in Table 2. As can be seen from the tables, remarkable differences between the values of total phenolic, monomeric anthocyanins and total sugar contents were observed. Differences between the samples were found to be significant ($P<0.01$). Some microbiological properties of hardaliye samples collected from different households are shown in Table 3.

In the second part of the study, the fresh hardaliye samples were produced from different grape types according to the traditional production method. Their physicochemical and chemical properties are presented in Tables 4 and 5. Differences between the samples were found to be significant ($P<0.01$). Total bacteria, lactic acid bacteria and yeast-mold counts of the samples are shown in Table 6. Coliforms and *E. coli* were not observed.

In this study, pH values of hardaliye samples collected from producers are between 3.54 and 4.33. Arici & Coskun (2001) collected twenty-six hardaliye samples from different spots of the Kirklareli city and the pH values determined in this study was similar to those determined in their study. pH values of samples produced in the laboratory are between 3.33 and 3.73. These values were lower than pH value (4.42) reported by Coşkun et al (2009). Total acidity values obtained in these two studies are similar. Volatile acid values of all samples were similar or slightly higher than value (0.25 g L^{-1}) reported by Coşkun et al (2009).

Total phenolics of anthocyanins in hardaliye samples collected from houses are generally higher than those produced in the laboratory. Diversity of grape varieties used in the production might have caused different anthocyanin and total phenolic contents in the samples. Unsuitable storage conditions, time and temperature causes

Table 1- Some physicochemical properties of the hardaliye samples collected from different households

Sample no	*Acidity (%)*	*pH*	*Color*		
			L	*a*	*b*
1	0.84±0.02	3.62±0.02	7.78±0.02	2.88±0.03	2.59±0.01
2	0.49±0.01	4.11±0.01	7.79±0.04	1.62±0.02	2.10±0.00
3	0.63±0.02	4.02±0.00	7.86±0.01	1.78±0.02	0.47±0.00
4	0.59±0.02	4.01±0.01	8.46±0.01	2.21±0.01	1.27±0.02
5	0.49±0.06	3.80±0.02	8.67±0.02	3.48±0.02	1.79±0.01
6	0.42±0.01	3.90±0.01	8.57±0.02	2.65±0.05	1.44±0.04
7	0.56±0.04	3.81±0.01	7.57±0.02	1.11±0.01	0.85±0.05
8	0.70±0.03	3.56±0.02	9.80±0.02	4.49±0.01	1.86±0.01
9	0.56±0.01	4.02±0.02	12.09±0.01	3.45±0.02	4.33±0.01
10	0.42±0.02	4.25±0.02	10.77±0.02	4.71±0.01	2.36±0.01
11	0.35±0.00	4.12±0.02	11.45±0.02	2.09±0.01	1.96±0.01
12	0.38±0.02	4.03±0.03	11.81±0.01	2.46±0.01	3.77±0.02
13	0.42±0.01	4.33±0.03	12.49±0.02	5.09±0.01	4.50±0.05
14	0.49±0.02	3.88±0.02	13.74±0.02	4.19±0.02	4.48±0.03
15	0.70±0.01	3.99±0.01	8.35±0.02	0.71±0.01	0.73±0.01
16	0.45±0.02	4.13±0.03	7.99±0.01	1.76±0.01	0.99±0.01
17	0.56±0.01	3.87±0.02	11.06±0.01	3.82±0.02	2.45±0.05
18	0.56±0.02	4.00±0.01	11.82±0.02	1.81±0.01	1.91±0.01
19	0.88±0.01	3.60±0.02	10.42±0.02	3.55±0.05	3.82±0.02
20	0.77±0.03	3.54±0.02	13.25±0.05	5.23±0.03	1.30±0.02
21	0.56±0.01	3.97±0.02	7.91±0.01	4.07±0.02	1.99±0.01
22	0.38±0.00	3.80±0.01	13.69±0.04	7.68±0.03	1.53±0.03
23	0.91±0.01	4.01±0.01	9.23±0.03	3.95±0.05	0.92±0.02
Mean	0.58±0.018	3.93±0.017	10.11±0.020	3.25±0.021	2.15±0.020

Table 2- Chemical properties of the hardaliye samples collected from different households

Sample no	*Total phenolic content (mg L^{-1})*	*Monomeric anthocyanin^{+} (mg L^{-1})*	*Volatile acids (g L^{-1})*	*Ethanol (%)*	*Total sugar (%)*
1	1067.5±0.50	54.4±0.03	0.30±0.01	n.d^{-}	18.0±0.10
2	1102.5±1.50	44.6±0.10	0.24±0.00	n.d	17.6±0.10
3	2647.5±2.02	118.4±0.11	0.30±0.01	1.6	15.9±0.02
4	2727.5±1.50	109.1±0.10	0.38±0.01	1.3	15.8±0.07
5	1129.6±0.10	29.0±0.01	0.43±0.00	0.9	17.7±0.01
6	861.8±0.08	65.4±0.03	0.26±0.01	n.d	24.8±0.05
7	2318.9±1.53	198.0±0.00	0.24±0.00	n.d	24.5±0.10
8	1693.9±1.00	37.8±0.02	0.50±0.01	n.d	21.3±0.01
9	1111.8±0.10	15.5±0.10	0.42±0.01	n.d	22.3±0.02
10	611.8±0.15	55.0±0.53	0.30±0.01	n.d	24.1±0.02
11	922.50±1.50	12.1±0.02	0.54±0.01	0.4	19.3±0.03
12	1336.8±0.40	6.5±0.00	0.36±0.00	n.d	19.4±0.07
13	870.9±0.10	39.5±0.20	0.60±0.01	n.d	24.3±0.10
14	583.1±0.02	27.1±0.01	0.36±0.01	n.d	15.2±0.02
15	1636.8±0.03	147.2±0.14	0.50±0.01	6.0	13.7±0.01
16	1129.6±0.04	124.6±0.02	0.24±0.01	n.d	25.4±0.06
17	813.1±0.10	4.5±0.03	0.61±0.01	n.d	20.8±0.05
18	576.60±0.18	12.7±0.10	0.48±0.00	n.d	19.50±0.02
19	512.4±0.40	11.6±0.05	0.55±0.01	n.d	23.1±0.03
20	908.8±0.10	27.9±0.10	0.72±0.01	2.1	7.2±0.01
21	1311.8±0.09	71.9±0.04	0.38±0.00	n.d	18.6±0.04
22	368.8±0.10	23.4±0.01	0.30±0.01	n.d	24.4±0.09
23	890.4±0.30	103.2±0.08	0.54±0.01	1.0	12.9±0.02
Mean	1179.7±0.515	55.5±0.080	0.41±0.007		19.4±0.046

$^{+}$, Monomeric anthocyanidin (malvidin-3-glocoside); $^{-}$n.d, not detected. Note: Ethanol values are not included in the statistical analysis

Table 3- Microbiological properties of the hardaliye samples collected from different households (cfu mL^{-1})

Sample no	*T.M.A.B.*	*LAB (Lactobacilli)*	*Yeast-Molds*
1	$4.00x10^3±1x10^2$	$1.00x10^2±0.00$	$<10±0.00$
2	$3.00x10^1±0.6x10^1$	$<10±0.00$	$2.30x10^4±1.5x10^3$
3	$6.00x10^3±1x10^3$	$4.00x10^3±2x10^2$	$3.30x10^3±5.7x10^1$
4	$1.19x10^6±5x10^3$	$1.00x10^2±0.00$	$<10±0.00$
5	$1.15x10^5±1x10^3$	$1.80x10^4±4x10^2$	$1.00x10^4±1x10^2$
6	$3.00x10^5±1x10^4$	$4.10x10^4±1.5x10^3$	$1.00x10^1±0.1x10^1$
7	$3.20x10^6±2x10^5$	$<10±0.00$	$2.00x10^1±0.1x10^1$
8	$3.00x10^3±0.00$	$9.00x10^2±1x10^2$	$1.20x10^3±1x10^2$
9	$2.00x10^3±2x10^2$	$1.60x10^3±0.00$	$1.00x10^4±1x10^2$
10	$4.00x10^3±1x10^2$	$3.00x10^2±1x10^1$	$2.00x10^2±1x10^1$
11	$2.00x10^3±1x10^2$	$<10±0.00$	$2.00x10^2±1x10^1$
12	$2.00x10^5±3x10^3$	$1.00x10^5±1x10^1$	$7.00x10^2±1x10^1$
13	$2.00x10^5±2x10^3$	$1.70x10^5±3x10^3$	$<10±0.00$
14	$5.00x10^5±1x10^4$	$3.00x10^5±5x10^3$	$4.00x10^1±0.1x10^1$
15	$1.00x10^5±0.00$	$3.20x10^3±1x10^2$	$<10±0.00$
16	$1.00x10^5±1x10^3$	$1.00x10^3±1x10^2$	$1.00x10^1±0.1x10^1$
17	$3.00x10^4±3x10^3$	$1.00x10^4±1x10^2$	$6.60x10^2±1x10^1$
18	$2.00x10^4±4x10^2$	$1.20x10^4±1x10^3$	$7.00x10^2±6.1x10^1$
19	$1.00x10^5±2x10^3$	$<10±0.00$	$2.50x10^3±1x10^2$
20	$3.00x10^4±1x10^3$	$<10±0.00$	$3.80x10^3±1x10^2$
21	$3.00x10^4±1x10^3$	$<10±0.00$	$<10±0.00$
22	$4.00x10^4±3x10^3$	$<10±0.00$	$4.00x10^2±1x10^1$
23	$2.60x10^4±1x10^3$	$1.50x10^2±1x10^1$	$1.90x10^4±5x10^2$
Mean	$2.70x10^5±1.07x10^4$	$2.90x10^4±5.46x10^2$	$3.30x10^3±1.17x10^2$

cfu, colony forming units

Table 4- Physicochemical properties of the hardaliye samples produced in laboratory

Trials	*Grape variety*	*Acidity (%)*	*pH*	*Color*		
				L	*a*	*b*
1	Dingil Kara	$0.73±0.02^b$	$3.66±0.02^d$	$9.24±0.02^c$	$2.89±0.04^a$	$1.27±0.01^c$
2	Kara Üzüm	$1.05±0.04^e$	$3.47±0.32^b$	$9.71±0.01^d$	$3.69±0.02^c$	$1.04±0.03^b$
3	Cabernet Sauvignon*	$0.56±0.01^a$	$3.73±0.03^e$	$8.45±0.05^a$	$4.20±0.04^d$	$1.89±0.01^d$
4	Yerli İzabella**	$0.77±0.01^c$	$3.33±0.03^a$	$9.86±0.02^e$	$3.13±0.03^b$	$1.28±0.01^c$
5	Siyah Üzüm	$0.98±0.02^d$	$3.50±0.06^c$	$8.75±0.05^b$	$5.00±0.03^e$	$0.86±0.02^a$

Note: The analyzes were carried out at the end of the fermentation. The different letters in the form of upper indices indicate that the averages are different (P<0.01) according to the Duncan Multiple Comparison Test. *, Cabernet Sauvignon; **, Yerli İzabella

Table 5- Chemical properties of the hardaliye samples produced in laboratory

Trials	*Grape variety*	*Total phenolic content (mg L^{-1})*	*Monomeric anthocyanin$^+$ (mg L^{-1})*	*Volatile acids (g L^{-1})*	*Ethanol (%)*	*Total sugar (%)*
1	Dingil Siyah	$2286.8±2.15^e$	$210.8±1.33^e$	$0.43±0.01^c$	n.d*	$16.5±0.4^b$
2	Kara Üzüm	$1858.2±0.20^d$	$116.3±1.3^a$	$0.55±0.02^d$	n.d	$15.2±0.3^a$
3	Cabernet Sauvignon**	$1165.4±1.40^c$	$200.6±0.3^c$	$0.42±0.02^{bc}$	n.d	$19.7±0.4^d$
4	Yerli İzabella***	$1093.9±0.50^b$	$201.3±1.30^d$	$0.39±0.01^a$	n.d	$21.4±0.3^e$
5	Siyah Üzüm	$961.8±0.40^a$	$161.1±1.00^b$	$0.40±0.02^{ab}$	n.d	$17.9±0.2^c$

$^+$, Monomeric anthocyanin (malvidin-3-glucoside); *, n.d, not detected; **, Cabernet Sauvignon; ***, Yerli İzabella. Note: Ethanol values are not included in the statistical analysis

Table 6- Microbiological properties of the hardaliye samples produced in laboratory (cfu mL^{-1})

Trials	*Grape variety*	*TMAB*$^{+}$	*LAB*$^{-}$ *(Lactobacilli)*	*Yeast-Molds*
1	Dingil Kara	$7.00x10^{6}\pm1x10^{6}$ b	$1.68x10^{6}\pm1x10^{4}$ c	$<10\pm0.00$ a
2	Kara üzüm	$1.60x10^{7}\pm2x10^{6}$ c	$2.20x10^{5}\pm2x10^{4}$ d	$1.00x10^{2}\pm0.1x10^{1}$ c
3	Cabernet Sauvignon*	$2.00x10^{5}\pm1x10^{4}$ a	$1.00x10^{2}\pm0.1x10^{1}$ a	$3.50x10^{1}\pm0.00$ b
4	Yerli İzabella	$8.16x10^{5}\pm6x10^{3}$ a	$2.76x10^{4}\pm3x10^{2}$ c	$<10\pm0.00$ a
5	Siyah üzüm	$1.00x10^{5}\pm1x10^{3}$ a	$3.00x10^{3}\pm3x10^{2}$ b	$<10\pm0.00$ a

$^{+}$TMAB, total mesophilic aerobic bacteria; $^{-}$LAB, Lactic acid bacteria; cfu, colony forming units; *, Cabernet Sauvignon

the amount of these components to decrease. For this reasons, total phenolics of anthocyanins of hardaliye samples collected from producers may be lower than others. Coşkun et al (2012) determined that the amount of monomeric anthocyanins (malvidin-3-glucoside) and total phenolics of hardaliye samples decreased from 114.1 to 54.4 mg L^{-1} and from 1392.5 to 1067.5 mg L^{-1} respectively, during 1 year of storage. Zarfilla et al (2003) produced wine from red grapes grown organically and traditionally in Spain and stored in glass bottles in a dark environment for 7 months at 20 °C. They observed a decrease of approximately 65% over 7 months in the total amount of total phenolics. In the same study, the monomeric anthocyanins (malvidin-3-glucoside) decreased from 248.34 mg L^{-1} to 32.29 mg L^{-1} in the conventional red grape wine, from 228.5 mg L^{-1} to 22.45 mg L^{-1} in the organic red grape wine. Hardaliye was produced from papazkarası blue-black grapes by conventional method by Aşkın & Atik (2016). After having been exposed to lactic acid fermentation, it was bottled and then stored at +4 °C and 20 °C for 60 days. The analyses were carried out in prepared beverage within the 15, 30, 45 and 60 days of storage. The results of color parameters obtained show the highest proportion of red color in the samples at the beginning (dA%= 94.87). As expected, the brown color increased with storage time and the highest value was determined for 60 days depending on the storage temperature. Storage under 4 °C and 20 °C resulted in 60 and 78% losses in anthocyanin content, respectively.

In the present study, ethyl alcohol was found in some hardaliye samples collected from different households. The reason might be that preservatives such as benzoic acid and mustard seeds were not used at the sufficient amount. Moreover, improper storage conditions and prolonged storage time increases formation of alcohol.

In the study conducted by Coşkun et al (2009), hardaliye sample was produced using black grape from Malatya region's grape varieties by applying traditional. Total mesophilic aerobic bacteria count in the hardaliye sample was lower ($4.7x10^{4}$) than those in the samples produced in laboratory in this study. This may be caused by harvesting and fermentation conditions.

The results of this study were also in accordance with the study conducted by Arici & Coskun (2001) in which red colour (measured by Hunter Lab) of hardaliye samples ranged from 1.33 to 9.66 and total mesophilic aerobic bacteria, lactic acid bacteria and yeasts-molds counts ranged from $3.5x10^{2}$ to $8x10^{5}$ cfu mL^{-1}, $1.0x10^{2}$ to $4.0x10^{4}$ cfu mL^{-1} and $1.0x10^{2}$ to $8.1x10^{4}$ cfu mL^{-1}, respectively. Furthermore, coliforms and *E. coli* were not found in none of the samples.

Güven & Aksoy (2009) produced hardaliye using verigo variety of grapes. 2.5 g mustard seeds were used. Hardaliye was fermented for 21 day. On the 7th and 21st days of fermentation, pH and total acidity were detected to range from 4.17 to 3.94 and 3.39 to 10.40%, respectively. They found higher pH value than the present study, however, total acidity was comparable. They did not detect ethyl alcohol which was similar to the hardaliye samples produced by applying traditional method in the present study.

Kılıc & Copur (1988) used Muskule, Razaki and Erenkoy Beyazı as grape varieties in their research. The total sugar content of hardaliye produced from these varieties was determined as 12.92, 14.94 and 11.47 g 100 mL^{-1}, respectively. Only the 3 hardaliye samples from the present study were in accordance with Kılıç & Çopur (1988)'s observation. In this study, the total sugar contents of hardaliye samples were detected to range from 7.2% to 25.4%, which may be attributed to different grape varieties and maturities. The total amount of sugar in the grapes increased towards end of the maturity period (Çelik 1998). The grape harvest for hardaliye production usually starts on October or November. The hardaliye samples with high sugar content may be produced from late harvested grapes. The hardaliye samples with low sugar content may be produced from early harvested grapes. The formation of alcohol due to uncontrolled fermentation or improper storage conditions may have caused to decrease of sugar content.

4. Conclusions

There are very few scientific studies about traditional hardaliye beverage although it has been produced traditionally for years. It brings the opportunity of consuming grapes in winter or spring seasons; thus, preventing the excess grapes from being wasted. Just like any other popular traditional food products, industrial-scale production of hardaliye should be realized and this traditional beverage should enjoy the large number of consumers' preference. In addition, more research is needed in order to its adaptation to industry.

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Examining of Relationships Among Traits Using Correlation, Path and Principal Components Analysis Methods in Turkish Opium Poppy (*Papaver somniferum* L.) Cultivars

Nimet KARA[a], Hasan BAYDAR[a]

[a]*Suleyman Demirel University, Faculty of Agriculture, Department of Field Crops, Cunur Campus, 32260, Isparta, TURKEY*

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Corresponding Author: Nimet KARA, E-mail: nimetkara@sdu.edu.tr, Tel: +90 (246) 211 85 95
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ABSTRACT

Opium poppy is an important medicinal plant because of its very valuable pharmaceutical alkaloids such as morphine, codeine, and papaverine, etc. The research was carried out with the aim to examining the relationships between capsule yield, some other yield and quality traits by means of correlation, path and principal components analysis (PCA) in fifteen opium poppy (*Papaver somniferum* L.) cultivars registered in Turkey. The experiments were conducted designed according to a randomized complete block with three replications during 2012/13 and 2013/14 growing years in Isparta conditions.

In the research, there were significant positive correlations between capsule yield and capsule width, and 1000 seed weight, while insignificant associations between capsule yield (non-seed) and seed yield, plant height, capsule number, capsule length, oil yield, and morphine content were determined. The significant and high negative correlation was determined between capsule yield with oil content. According to the path analysis, the highest positive direct effect on capsule yield was seed yield (48.202%), and the highest positive indirect effect on capsule yield was capsule width (47.877%) via seed yield. The ten PCA components (PC1 to PC10) created 90.09% of the total variation among traits in poppy cultivars. PC1, PC2, PC3, and PC4 with values of 52.0%, 16.9%, 11.8%, and 10.2%, respectively contributed to the total PCA value, and plant height, capsule width, capsule number, and 1000 seed weight were found to be effective components on the yield in PCA analysis. The bi-plot analysis showed that capsule number, capsule length, seed yield, and oil yield created the same group and plant height and capsule width were another group, and these characters showed stability for the cultivars.

The according to the results of the correlation, path, and PCA, capsule width and 1000 seed weight were effective components on capsule and seed yields of the opium poppy.

Keywords: Opium poppy; Correlation; Path analysis; Principal components analysis

Türk Haşhaş Çeşitlerinde (*Papaver somniferum* L.) Korelasyon, Path ve Temel Bileşenler Analiz Metotları Kullanılarak Özellikler Arası İlişkilerin İncelenmesi

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Nimet KARA, E-posta: nimetkara@sdu.edu.tr, Tel: +90 (246) 211 85 95
Geliş Tarihi: 18 Mayıs 2015, Düzeltmelerin Gelişi: 22 Ağustos 2017, Kabul: 19 Eylül 2017

ÖZET

Haşhaş; morfin, kodein, papaverin ve bunun gibi içermiş olduğu çok değerli alkaloidlerden dolayı önemli bir tıbbi bitkidir. Araştırma, Türkiye'de son yıllarda tescil edilmiş on beş haşhaş çeşidinde (*Papaver somniferum* L.) korelasyon, path ve temel bileşenler analizleri (TBA) kullanılarak kapsül verimi, bazı diğer verim ve kalite özellikleri arasındaki ilişkileri incelemek amacıyla yürütülmüştür. Denemeler, Isparta koşularında 2012/13 ve 2013/14 yıllarında tesadüf blokları deneme desenine göre 3 tekerrürlü olarak yürütülmüştür.

Araştırmada, kapsül verimi (tohumsuz) ile tohum verimi, bitki boyu, kapsül sayısı, kapsül boyu, yağ verimi ve morfin içeriği arasında önemsiz ilişkiler belirlenirken, kapsül verimi ile kapsül genişliği ve 1000 tohum ağırlığı arasında önemli pozitif ilişkiler tespit edilmiştir. Kapsül verimi ile yağ içeriği ve yağ verimi arasında önemli ve yüksek negatif ilişki belirlenmiştir. Path analizine göre, kapsül verimi üzerine en yüksek pozitif direk etkiyi tohum verimi (% 48.202) ve en yüksek pozitif indirek etkiyi ise tohum verimi üzerinden kapsül genişliği (% 47.877) yapmıştır. Haşhaş çeşitlerinde özellikler arasındaki toplam % 90.09'luk farkı on temel bileşen (TB1–TB10) oluşturmuştur. Toplam temel bileşenlere TB1, TB2, TB3 ve TB4 sırasıyla, % 52.0, % 16.9, % 11.8 ve % 10.2'lik oranında katkıda yapmıştır ve bitki boyu, kapsül genişliği, kapsül sayısı ve 1000 tohum ağırlığı verim üzerine etkili özellikler olarak bulunmuştur. Bi-plot analizine göre kapsül sayısı, kapsül boyu, tohum verimi ve yağ verimi aynı grupta yer almış, bitki boyu ve kapsül genişliği ise başka bir grubu oluşturmuştur ve bu özellikler tüm çeşitlerde stabil etki yapmıştır.

Korelasyon, path ve temel bileşenler analiz sonuçlarına göre, haşhaşta kapsül ve tohum verimi üzerine en etkili özellikler kapsül genişliği ve 1000 tohum ağırlığıdır.

Anahtar Kelimeler: Haşhaş; Korelasyon; Path analizi; Temel bileşenler analizi

1. Introduction

The opium poppy is mainly evaluated by its oil and morphine content. The poppy cultivars with high alkaloid content are used for medicinal purposes, and the cultivars with low alkaloid content are evaluated for food production (Prajapati et al 2002). It is a plant which has been traditionally cultured since ancient times in Anatolia, and today half of the legal production area in the world under the control of the United Nations is located in Turkey. Turkey is a historically significant opium poppy producer with the Irano-Anatolian region accepted as the center of the origin of this crop (Tetenyi 1997). The Turkish Soil Products Office has undertaken the control of cultivation areas, products purchase, storage, and trade of poppy since 1938. After the establishment of the alkaloid factory in the Afyonkarahisar province in 1976, alkaloids are produced only by extractions from the dry poppy capsule. In Turkey, the poppy is cultivated on an area of 61591 hectares, with an annual production of 30730 tons, and an average seed yield of 500 kg ha^{-1} (Anonymous 2015).

Although the genetic diversity of the Turkish opium poppy germplasm is a good source for improving the straw and seed yields (Gümüşcü & Arslan 1999; Koç et al 2012), it is not a good source for the genes responsible for high morphine content (Çelik et al 2016). Because the morphine content of the cultured varieties is low (usually less than 1%), Turkey is third in world morphine production despite being the first in the legal poppy cultivation areas in the world (Anonymous 2014). For this reason, intensive breeding studies on the development of the poppy varieties with high morphine content have been carried out for many years. In addition, the opium poppy is a crop that not only expresses great economic value for its capsules rich in opium alkaloids, but also for its seeds rich in edible oil. Because it contains no alkaloids in its seeds, the development of the varieties with both high capsule yield for alkaloid production and high seed yield for oil production is an important breeding target in the opium poppy.

The combination of capsule number, capsule weight, capsule size, and seed weight constitute

the most desirable plant type in the opium poppy (Singh et al 2004). However, the strong positive or negative associations between the desirable traits in the opium poppy are major problems in boosting the yield and quality (Sharma et al 1981; Singh & Khanna 1993; Singh et al 2003; Yadav et al 2006; Öztürk & Günlü 2008). If the phenotypic and genotypic relationships among traits are related to the capsule and seed yields, and the morphine and oil contents, they can be considered as the selection criteria for breeding trials to achieve this started goal.

Correlation and path analysis is a significant statistical method to evaluate the yield and quality in plant breeding. The correlation coefficients mainly show relationships among the independent variables and the degree of linear relations among the variables; however, the correlation coefficients cannot sufficiently describe the complex relationship between the variables. Therefore, the direct and indirect effects of the various traits on both yield and quality should be known in breeding programs. For this aim, the path coefficient analysis is used to determine the amounts as the percent of direct and indirect effects of the independent variables on the dependent variable (Harman 1976; Neter et al 1983). A positive association between the seed yield with capsules per plant and capsule size was determined by Singh & Khanna (1993) and Singh et al (2003), while a negative correlation was reported by Sharma et al (1981) in different poppy populations. Singh et al (2004) determined that there was a significant positive association among themselves in capsule size, capsules per plant, seed yield per plant, and branches per plant. The same author pointed out that there were negative direct effects and positive indirect effects on the yield of capsule weight, capsule size, capsule per plant, and plant height. However, Singh et al (2003) noticed that there were positive direct effects on the yield of these characteristics.

Principal components analysis (PCA) is a method showing the similarities and dissimilarities for explaining and defining them by simplifying complex data. PCA allows for data compression and its size reduction without any data loss. Principal components analysis is a suitable method to obtain a smaller number of artificial variables accounting for determining a new variable. This method is also a better predictor of the similarities or dissimilarities among the morphological and physiological characteristics of the plants (Aygün & Olgun 2014). PCA is widely used in breeding programs due to it being able to explain the association among the plant characteristics (Flores et al 1998).

The aim of the study was to investigate the associations among the yield and quality traits including plant height, capsule number, capsule width, capsule length, 1000 seed weight, morphine and oil contents, and capsule and seed yields by means of correlation, and path and principal component analysis methods in the poppy.

2. Material and Methods

This research was conducted in the Faculty of Agriculture at Süleyman Demirel University in the Isparta province of Turkey. Isparta by having a typical continental climate in Southwestern Anatolia, is one of thirteen provinces where poppy farming is allowed by the Turkish Soil Products Office. Eleven poppy cultivars (TMO-1, TMO-2, TMO-3, TMO-T, Ofis-3, Ofis-4, Ofis-8, Ofis-95, Ofis-96, Afyon-95 and Bolvadin-95), which are registered by the General Directorate of Soil Products Office in Ankara and four poppy cultivars (Tınaztepe, Anayurt, Kemerkaya and Zaferyolu) registered by the Western Transitional Zone Agricultural Research Institute in Eskişehir were used for the genetic materials in the research. Generally, these cultivars are new varieties that have been recently years registered in Turkey.

The field experiment was conducted in a randomized complete block design with three replicates in the autumn sowing in 2012/13 and 2013/14. The poppy seeds were sown at about a 0.5-1 cm depth using a dibbler, on 5 and 9 October for the first (2012/13), and the second years (2013/14), respectively. The spacing used was 0.45x0.15 m, and the plot length was 5 m (5x2.7= 13.5 m^2) with

6 rows. In both years, the experiments were non-irrigated at any growing stage. All the necessary cultural practices were applied identically to all the cultivars in both years. 100 kg nitrogen ha^{-1} (two equal doses at the sowing and at plant height 10-15 cm stages) and 30 kg P_2O_5 ha^{-1} (all by sowing) fertilizers were applied to the form ammonium sulfate (21%) and triple super phosphate (43-46%), respectively (Koç et al 2012).

The climatic data during the vegetation period (from October to the end of August) in 2012/13 and in 2013/14, showed there was a total precipitation of 555.0 and 579.9 mm, an average temperature of 12.8 and 12.1 °C, and average humidity of 58.8 and 58.3%, respectively. In the years 2012/13 and 2013/14, the soil at a depth of 60 cm was low in organic matter (1.80% and 1.66%, respectively), slightly alkaline (pH 7.5 and 7.8, respectively), limey (9.67% and 9.89% $CaCO_3$, respectively), and clay-loamy.

The capsules from 4 rows in the center of each plot in the full ripeness period according to the maturity stage of the cultivars were manually harvested. Capsule yield (kg ha^{-1}), seed yield (kg ha^{-1}), plant height (cm), capsule number per plant (capsule), capsule width (mm), capsule length (mm) and 1000 seed weight (g) were determined as described by Karabük (2012). Plant characteristics such as plant height, capsule number per plant, capsule width, and capsule length were measured from 15 plants per parcel. The oil from the poppy seeds was extracted with *n*-hexan for 6 hours using the soxhlet apparatus. For the morphine analysis, the poppy shell was dried for 24 hours at 70 °C and was powered by grinding, and was extracted with 5% acetic acid under sonication. The morphine analyses were conducted at the laboratory of Bolvadin Opium Alkaloids Factory by using the HPLC-MS/MS apparatus.

A matrix of simple correlation coefficients among the yield and quality traits was computed. The direct and indirect effects of traits on capsule yield were made using path coefficient analysis. The principle components analysis (PCA) explained by Harman (1976) involves a mathematical producer that transforms a number of correlated variables into a smaller number of uncorrelated variables called principal components. Correlation, path, and PCA analyses were performed according to means of two years using the SPSS v.16.0 software (Chicago, IL. USA).

3. Results and Discussion

The minimum, maximum values, and standard deviation for all traits (variables) determined in 15 Turkish opium poppy cultivars are presented in Table 1.

Table 1- Minimum and maximum values for the variables of opium poppy cultivars

Variables	*Minimum values±SD*	*Maximum values±SD*
Plant height (cm)	80.08±7.15	122.85±4.47
Capsule number (per plant)	3.58±0.11	6.48±1.09
Capsule width (mm)	34.58±1.66	46.26±3.16
Capsule length (mm)	34.07±1.89	45.05±2.49
1000 seed weight (g)	0.34±0.004	0.43±0.006
Oil content (%)	38.66±0.57	44.00±1.00
Oil yield (kg ha^{-1})	239.39±15.34	558.67±23.77
Morphine content (%)	0.47±0.005	1.00±0.10
Seed yield (kg ha^{-1})	575.20±21.03	1325.56±51.76
Capsule yield (kg ha^{-1})	457.5±22.50	1149.5±38.68

SD, Standard deviation

3.1. Correlation coefficient analysis

The results of correlation analysis among the yield and quality traits of the opium poppy cultivars are shown in Table 2. According to the results of the correlation analysis, while there were significant and high positive correlations between capsule yield with capsule width (r= 0.217*) and 1000 seed weight (r= 0.307**), a significant and negative correlation was determined between capsule yield with oil content (r= -0.290**) in the poppy. The non-significant correlations were determined between capsule yield and seed yield (r= 0.162) and

morphine content (r= -0.116) in the opium poppy (Table 2). The correlation analysis showed that there were significant and high positive correlations between seed yield with plant height, capsule number, capsule width, capsule length, 1000 seed weight, and oil yield in the poppy. The insignificant associations were determined between seed yield and oil content (r= -0.113) and morphine content (r= -0.048). In the research, the significant and high positive correlations were determined between oil yield with seed yield, plant height, capsule number, capsule width, capsule length, and 1000 seed weight. While the significant and negative correlation between morphine content with plant height was shown, the associations between morphine content with capsule number, capsule width and capsule length were not significant (Table 2).

Generally, the bilateral relations between capsule number, capsule width, capsule length, and 1000 seed weight, were positive and significant. Srivastava & Sharma (1987) and Singh et al (2003) noted that capsule yield, seed yield, oil yield, and morphine content were significantly affected by capsule weight, capsule size, and capsule number per plant. Sing et al (2004) reported that there were positively significant correlations between capsule yield and seed yield, capsule weight, plant height and capsule area, and among themselves. The similarity of positive association among seed yield with capsule weight and capsule area was reported by Singh et al (2003). Öztürk & Günlü (2008) reported that positive and significant relationships were found between capsule yield with seed yield, capsule yield and oil yield. Harvest et al (2009) stated that morphine content was correlated with capsule size. The positive correlations between morphine content with size of capsule size/weight were determined by Yadav et al (2006). Positive correlations between seed yield with capsules per plant and capsule size was noticed by Singh & Khanna (1993) and Singh et al (2003), while negative correlation was reported by Sharma et al (1981) in different populations.

3.2. Path coefficient analysis

Partitioning the correlation coefficient in to direct and indirect effects can be carried out through path analysis. The direct and indirect contributions on capsule yields of some of yield and quality traits in the poppy cultivars are given in Table 3. In the research, capsule yield as a dependent variable, and seed yield, plant height, capsule number, capsule width, capsule length, 1000 seed weight, oil content, oil yield and morphine content as determinative variables, were used for the path coefficient analysis. The direct effects of the examined yield characteristics (except for oil yield and morphine content) on capsule yield in the poppy cultivars were positive. The highest positive direct effect on

Table 2- A matrix of simple correlation coefficients (r) for the estimated variables of opium poppy

Variables	*Capsule yield*	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9
Seed yield (X_1)	0.162^{ns}	1.000								
Plant height (X_2)	0.003^{ns}	0.558**	1.000							
Capsule number (X_3)	-0.183^{ns}	0.431**	0.203^{ns}	1.000						
Capsule width (X_4)	0.217*	0.743**	0.714**	0.287**	1.000					
Capsule length (X_5)	-0.025^{ns}	0.657**	0.146^{ns}	0.204^{ns}	0.248*	1.000				
1000 seed weight (X_6)	0.307**	0.701**	0.357**	0.152^{ns}	0.558**	0.539**	1.000			
Oil content (X_7)	-0.290**	-0.113^{ns}	-0.069^{ns}	-0.062^{ns}	-0.245*	0.076^{ns}	-0.142^{ns}	1.000		
Oil yield (X_8)	0.110^{ns}	0.988**	0.545**	0.426**	0.708**	0.670**	0.682**	0.039^{ns}	1.000	
Morphine content (X_9)	-0.116^{ns}	0.048ns	-0.241*	-0.033^{ns}	-0.083^{ns}	0.084^{ns}	0.153^{ns}	-0.180^{ns}	0.027^{ns}	1.000

*, **, significant at P<0.05 and P<0.01 probability levels, respectively; ns, non-significant

capsule yield was seed yield (48.202%), followed by oil content (36.215%), plant height (5.946%), capsule number (5.380%), capsule length (4.301%), 1000 seed weight (3.685%), and capsule width (1.054%), respectively (Table 3). Capsule width (47.877%), 1000 seed weight (47.368%), capsule length (46.913%), capsule number (46.837%), and plant height (46.539%) had the highest indirect contributes on capsule yield via seed yield (Table 3). These results indicate that the direct and indirect effects of plant traits including plant height, capsule number and capsule length on capsule yield were higher than the other yield components. These results were similar to the findings of Singh & Khanna (1993) and Singh et al (2004). Shukla & Khanna (1987) and Singh et al (2003) stated that the direct and indirect contribution on yield of plant height and capsule weight/length were positive. Öztürk & Günlü (2008) determined that the direct and indirect effects on seed yield of capsule yield were both positive and minimal.

3.3. Principal components analysis

The main target in the principal components is determining the maximum amount of variance with the lowest number of components. Principal component analysis assists in explaining the underlying data, and designing a small amount of uncorrelated variables. Besides, it reveals the data of the orthogonal eigenvectors on the correlation matrix in the variables. Each principal component explains its largest percent of the total variation. The first principal component covers the largest percent of the total variation. The data presented in

Table 3- Path coefficient (direct and indirect effects) of the yield components on capsule yield of opium poppy

	Direct effects	*Indirect Effects*								
Variables	*Capsule yield*	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9
Seed yield (X_1)	6.280	-	-0.247	-0.132	0.075	-0.245	0.237	-0.072	-5.648	-0.013
	48.202		1.917	1.029	0.585	1.905	1.841	0.563	43.845	0.100
Plant height (X_2)	0.442	3.464	-	-0.062	0.072	-0.054	0.120	-0.044	-3.116	0.065
	5.946	46.539		0.838	0.974	0.733	1.624	0.593	41.871	0.879
Capsule number (X_3)	0.307	2.677	-0.089	-	0.029	-0.076	0.051	-0.039	-2.436	0.008
	5.380	46.837	1.571		0.509	1.332	0.901	0.697	42.615	0.154
Capsule width (X_4)	0.101	4.609	-0.316	-0.088	-	-0.092	0.188	-0.156	-4.051	0.022
	1.054	47.877	3.284	0.915		0.961	1.961	1.626	42.083	0.233
Capsule length (X_5)	0.373	4.076	-0.064	-0.062	0.025	-	0.182	0.048	-3.832	-0.022
	4.301	46.913	0.743	0.721	0.289		2.099	0.556	44.111	0.262
1000 seed weight (X_6)	0.338	4.349	-0.158	-0.046	0.056	-0.201	-	-0.090	-3.898	-0.041
	3.685	47.368	1.722	0.510	0.616	2.193		0.989	42.461	0.451
Oil content (X_7)	0.640	-0.703	0.030	0.019	-0.024	-0.028	-0.048	-	-0.224	0.048
	36.215	39.791	1.726	1.082	1.404	1.597	2.715		12.694	2.766
Oil yield (X_8)	-5.719	6.131	-0.241	-0.131	0.071	-0.250	0.230	0.025	-	-0.007
	44.652	47.869	1.883	1.022	0.561	1.955	1.801	0.196		0.057
Morphine content (X_9)	-0.271	0.296	0.106	0.010	-0.008	-0.031	0.051	-0.115	-0.154	-
	25.917	28.349	10.211	0.959	0.805	3.009	4.940	11.033	14.774	

The first lines is path coefficient (pc) and the second lines is path percentage (%)

Table 4 demonstrated that an increase in number of components was associated with a decrease in the Eigen values. This trend reached its maximum at four factors or components. With 5.202, the first principal component covers 52.0% of the total variance. With 1.685, 1.175 and 1.024 the second, third, and fourth principal components explain 16.9%, 11.8% and 10.2% of the total variance, and the four principal components explain 90.9% of the total variance (Table 4). In the first principal component, plant height, capsule number, width and length of the capsule, seed yield, 1000 seed weight, and oil yield could be considered to be the effective components on yield, and they have the same sign and their terms were not close to zero. In the second principal component, the terms of capsule length, capsule yield, 1000 seed weight (negative sign) and plant height, and capsule number and oil content (positive sign) were not close to zero. The third PCA showed a positive effect on capsule length, 1000 seed weight, oil content oil yield, and morphine content, and a negative effect on plant height, capsule number, capsule width, and capsule yield were not close to zero. The fourth PCA showed positive effects on capsule number, capsule width, seed yield, and morphine content, and a negative effect on plant height, capsule length, capsule yield, 1000 seed weight, and oil content and oil yield were not close to zero (except for in seed yield, 1000 seed weight and oil yield). Özdamar (1999) demonstrated that the main component describing 67.0% of the data is sufficient to determine the main characteristics in the principal component analysis. In a study conducted on wheat by Hailegiorgis et al (2011), Beheshtizadeh et al (2013), Olgun et al (2014) and Priya et al (2014) it was stated that the principal component was explained in 80.0%, 76.0%, 90.8%, and 75.0%, respectively, and rates with an association among the quality characteristics as cumulative. The highest effective components were plant height, width and length of capsule, capsule number, and 1000 seed weight on yield in the PCA.

The bi-plot analysis showing the stability of the cultivars and yield components are given in Figure 1. The higher performance and stability; similarity/ dissimilarity in the cultivars and yield components

Table 4- Eigen values of the correlation matrix and principal component analysis for the variables in opium poppy

Variables	*PC1*	*PC2*	*PC3*	*PC4*	*PC5*	*PC6*	*PC7*	*PC8*	*PC9*	*PC10*
Seed yield (X_1)	0.430	-0.074	0.070	0.040	0.027	0.088	0.313	0.297	-0.283	-0.727
Plant height (X_2)	0.327	0.375	-0.167	-0.095	0.573	-0.111	-0.512	-0.131	-0.309	-0.011
Capsule number (X_3)	0.295	0.247	-0.370	0.313	-0.641	0.116	-0.391	0.193	0.049	0.013
Capsule width (X_4)	0.398	0.050	-0.155	0.193	0.203	0.444	0.260	-0.476	0.495	0.012
Capsule length (X_5)	0.380	-0.114	0.365	-0.135	0.110	-0.292	-0.223	0.416	0.605	0.071
1000 seed weight(X_6)	0.329	-0.339	0.344	-0.042	-0.342	-0.290	-0.181	-0.616	-0.203	-0.038
Oil content (X_7)	-0.037	0.456	0.366	-0.622	-0.254	0.429	-0.073	-0.069	0.035	-0.098
Oil yield (X_8)	0.428	-0.008	0.125	-0.036	-0.021	0.154	0.338	0.232	-0.389	0.675
Morphine content (X_9)	-0.159	0.030	0.602	0.608	0.131	0.369	-0.270	0.061	-0.108	-0.005
Capsule yield (Y)	0.004	-0.674	-0.207	-0.275	0.084	0.506	-0.381	0.123	-0.063	0.212
Eigen value	5.202	1.685	1.175	1.024	0.388	0.338	0.115	0.058	0.009	0.000
Proportion	0.520	0.169	0.118	0.102	0.039	0.034	0.012	0.006	0.001	0.000
Cumulative (%)	0.521	0.689	0.806	0.909	0.948	0.993	0.999	1.000	1.000	1.000

were explained in detail. The PC1 shows a high value and performance, the PC2 assigns the stability in the cultivars and yield components. In the cultivars, varieties 6, 7, and 13 have a higher capacity and stability for yield components. Moreover, the cultivars 3, 6 and 7, cultivars 11 and 13, the cultivars 1 and 15, and the cultivars 2, 4, 8, 9, and 10 joined the same groups. In yield components, plant height and capsule width created the same group, and capsule number, capsule length, seed yield and oil yield were in another similar group, and they both showed stability for the cultivars, the others were placed in a separate group. In a study conducted on white clover by Aygün & Olgun (2014), it was explained that the plant characteristics and genotypes were classified in five specific groups on plant height using the PCA. In studies conducted on wheat by Çiftçi & Yağdı (2012) and Priya et al (2014), it was determined that the plant genotypes were classified in five groups.

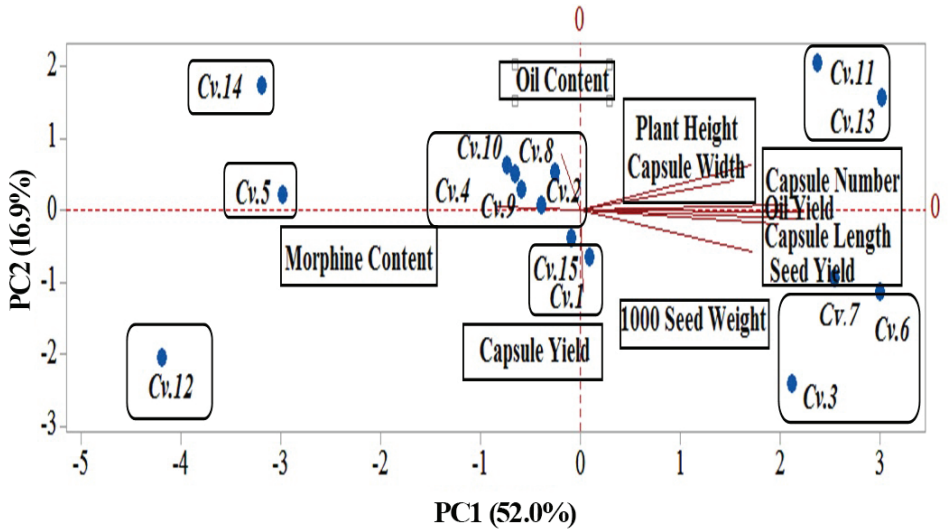

Figure 1- Bi-plot analysis showing stability and the variables in opium poppy cultivars (Codes of cultivars: Cv1.TMO-1, Cv2.TMO-2, Cv3.TMO-3, Cv4.TMO-T, Cv5.Ofis-3, Cv6.Ofis-4, Cv7.Ofis-8, Cv8.Ofis-95, Cv9. Ofis-96, Cv10.Afyon-95, Cv11.Bolvadin-95, Cv12.Tınaztepe, Cv13.Anayurt, Cv14.Kemerkaya and Cv15. Zaferyolu)

4. Conclusions

As a result, high positive and significant associations were determined between capsule yield with capsule width and 1000 seed weight. The associations between seed yield with plant height, capsule number, capsule width, capsule length, 1000 seed weight and oil yield were highly positive and significant. There were insignificant and negative correlations between capsule yield and capsule number, capsule length and morphine content, but the association between capsule yield with oil content was negative and significant.

While the effects on capsule yield of plant height, capsule number, capsule length and capsule width were positive but low, the direct effects on capsule yield of seed yield and oil content were positive and high. The indirect effects of capsule width, 1000 seed weigh, capsule number and plant height, respectively, on capsule yield via seed yield were high and positive.

The PCA analysis showed that ten components accounted for about 90.9% of the total variation among traits in the poppy. The PC1 contributed the most to the total variation, followed by PC2, PC3,

and PC4. According to the bi-plot analysis, capsule number, capsule length, seed yield, and oil yield created the same group, and they showed stability in the cultivars. Different statistical techniques have been used in modeling crop yields, including correlation, path analysis, and PCA. Since different traits have had an important role according to the statistical techniques of opium poppy cultivars, different criteria can be used for the improvement of capsule and seed yields. Thus, the results showed that capsule width and 1000 seed weight were effective components on capsule and seed yields.

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

Books

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

Book Chapter

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

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

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

Conference Proceedings (Full papers)

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

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