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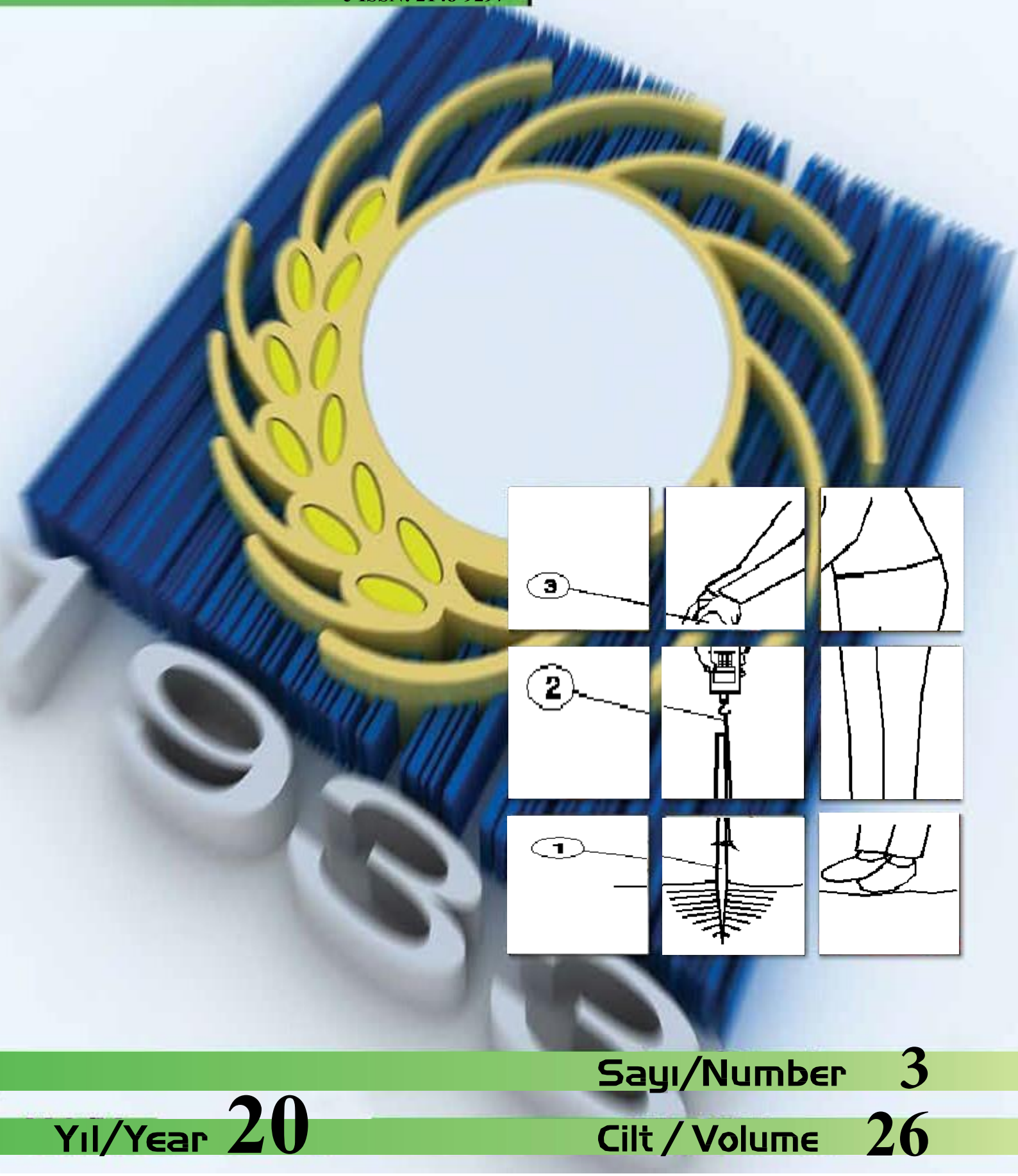

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A Multivariate Analysis in Relation to Edaphic and Environmental Factors of Rangelands Vegetation of Mugla Province

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ABSTRACT
This study was carried out in order to compare in 20 different rangeland sample areas that determined in order to environmental variables, vegetation and soil properties by multivariate ordination analysis in Mugla province. Cluster analysis was made to determine the similarity and species compositions of sample areas, and as a result of this analysis, three different groups have occurred. Additionally, detrended correspondence analysis (DCA) was made after the indicator species analysis. The interaction between environmental and soil-borne factors as altitude, distance to village, soil depth, pH in saturated soil with water, lime and surface stoniness were found to be significant and this significance was expressed by graphs. Moreover, it was indicated that relationship with species in the vegetation of the variables that were determined as significant by tables and figures. The relationship with the species in the vegetation of the variables that were determined as significant was also indicated. The result of the study showed that environmental variables as soil depth, soil pH saturated with water, stony surface, altitude and distance to villages had a significant effect on the species diversity and distribution in the samples areas.

Keywords: Clustering analysis; Multivariate analysis; Rangeland

1. Introduction

The best way to learn about habitat, niche, and vegetation beside the different interaction between plants in an ecosystem is an investigation of plant biodiversity (Khan et al 2016). Biodiversity is defined as species richness that consisting of the influence of different environmental characters (Khan et al 2018). It is important that examination of the relationship between environmental factors and plants in these ecosystems for better understanding and management rangeland ecosystems, which are among the areas with the richest vegetative biodiversity in the world. The impact of environmental factors on plant communities has been the subject of many ecological studies in recent years (Amiri & Saadatfar 2009; Altın et al 2011; Ispirli et al 2016). Determination of the interaction between different biotic and abiotic components of an ecosystem is an important part of ecological studies (Rahman et al 2016; Khan et al 2017). The species composition, which is one of the main components of rangeland ecosystems, is highly controlled by environmental factors. Climate,

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topography, and soil characteristics are the main environmental factors (Hoveizeh 1997; Clark & Mann 1999; Escudero et al 2000; Solon et al 2007; Altın et al 2011; Surmen & Kara 2018). When the relationship between environmental factors and vegetation is investigated, it is thought that the location of plants is not a coincidence. Therefore, researchers investigate various factors (Abiotic and Biotic) that interact in the formation of plant community structure. These factors contribute to understanding the distribution, composition, and diversity of plant species and communities (Brown 1984). It is difficult to state which factors actually cause changes in vegetation when a large number of environmental factors are extremely effective (Partridge & Wilson 1989). Researchers have stated that each of the ecological factors had many effects on plant distribution (Clark & Mann 1999). The most important factors that cause the vegetation to dissipate due to the soil characteristics of different species in ecosystems are topographic parameters such as slope and aspect. Additionally, variations in the composition of the plant species throughout the altitude and latitude are one of the most effective environmental variables in the classification of plant species in these regions (Kitayama 1992; Altın et al 2011). Quantitative classification and ordination analysis methods are used to understand the basic relationship between environmental characteristics and plant communities. These methods help to identify ecological similarities between different vegetations and finding the environmental factors that are important in ecological structure determination (Zhang et al 2006; Amiri & Saadatfar 2009). Scientists, who are interested in ecology, use a multivariate approach to investigate and summarize the ecological data set related to environmental variables. Statistical analysis of these data helps to find the actual position of plant species in rangelands (Curtis & McIntosh 1950). Multivariate analysis methods are commonly used to qualitative and quantitative relationships with botanical composition and environmental factors (Villers-Ruiz et al 2003; Kargar-Chigani et al 2017). One of these methods, the ordination analysis, is a commonly used method to study the vegetation-environment relationships (Jin-Tun & Oxley 1994; Siefert et al 2012). Multivariate statistical analytical programs contribute to ecologists to analyze the effects of environmental variables on all species and to know the structure in the data set (Anderson et al 2006). Mugla province, where the study was conducted, is located in the southwestern of Turkey, has rich biotope and biota, endemic species, and it is one of the few provinces in terms of natural resources and environmental-conservation areas. For this reason, the effects of different environmental variables on rangeland botanical composition were investigated by multivariate analysis methods in 20 different sample areas in Mugla province that has rich biodiversity.

2. Material and Methods

The experiment was carried out in 20 different rangeland areas within the borders of Mugla province. The general characteristics of environmental factors that are important in the ordination analysis are given in Table 1. In the vegetation study, wheel point method (Gokkus et al 1995) and adapted loop method were used (Koc & Cakal 2004). In this study; soil texture properties were determined according to the hydrometer method by Gee & Bauder (1986), pH-saturated soil in the water was determined according to the pH meter method by McLean (1982), $CaCO_3$ content was determined that according to the Scheibler calcimeter by Nelson & Sommers (1982), soil P_2O_5 content was determined that according to the molybdosophosphoric blue color method by Olsen & Sommers (1982), the K_2O content was determined that according to the flame photometry by Thomas (1982) and the organic matter content was determined that according to the Smith-Weldon method (Nelson & Sommers 1982). In the result of the vegetation study, 116 species in 20 sample areas were determined, however, the species which were below 5% frequency according to coverage ratio were removed before analysis (Table 2). Cluster analysis was performed to determine the similarity characteristics of the sample areas using the PC-ORD package program in the study. Before the analysis, the remaining 5% of the species detected in the sample areas were eliminated and the analysis was continued with the remaining species. The Cluster Euclidean Ward's method was used for the analysis and distinction groups were determined by MRPP (Multi-Response Permutation Procedures) test (McCune & Mefford 1999). After the separation groups, DCA was preferred for ordination analysis (Hill & Gauch 1980).

Table 1- General characteristics of pasture areas that are important in the ordinate analysis in 20 different pasture areas of Mugla Province

Locations	*Altitude*	*pH in soil saturated with water*	*Lime (%)*	*Distance to the Village (km)*	*Stoniness*	*Soil depth*	*County*	*Village*	*Site*	*Latitude*	*Longitude*
MUG001	1384	6.10	0.72	5.0	1	4	Seydikemer		Seki	35S0741063	UTM4079330
MUG002	1420	5.94	0.72	5.0	2	3	Seydikemer		Seki	35S0741213	UTM4079257
MUG003	2	6.40	0.97	2.0	4	2	Bodrum		Kudur	36S0648581	UTM3018040
MUG004	9	6.71	1.08	2.0	2	3	Ortaca		Tepearası	35S0648780	UTM4082848
MUG005	10	7.43	0.97	2.0	1	4	Koycegiz	Donusbeleni	Donusbeleni	35S0641815	UTM4095054
MUG006	17	7.97	17.58	2.5	1	4	Ortaca		Tepearası	35S0649092	UTM4083032
MUG007	12	5.46	0.84	1.0	1	4	Milas		Koru	35S0563867	UTM4122029
MUG008	20	7.57	11.59	2.0	1	4	Ortaca	Tepearası	Tepearası	35S0648947	UTM4083120
MUG009	6	7.39	7.10	1.0	1	4	Milas	Gurcamlar	Alagun	35S0544972	UTM4126429
MUG010	13	6.82	1.04	1.0	1	4	Ortaca	Ortakoy	Tepearası	35S0649005	UTM4083015
MUG011	10	7.43	8.72	1.0	1	4	Milas		Koru	35S0563635	UTM4122702
MUG012	1400	5.61	0.07	5.0	1	3	Seydikemer	Seki	Seki	35S0741086	UTM4079742
MUG013	9	6.89	0.91	1.0	4	4	Milas		Koru	35S0563565	UTM4122805
MUG014	12	7.61	10.91	1.0	4	2	Milas	Gurcamlar	Alagun	35S0544824	UTM4126451
MUG015	9	7.98	13.45	1.0	4	4	Milas		Koru	35S0563565	UTM4122805
MUG016	9	7.36	1.29	2.0	4	4	Koycegiz	Donusbeleni	Donusbeleni	35S0641716	UTM4094888
MUG017	0	6.16	1.11	2.0	3	2	Bodrum	Kudur	Yalıkavak	35S0523593	UTM4108970
MUG018	14	7.35	30.17	1.0	1	2	Milas	Gurcamlar	Gurcamlar	35S0544989	UTM4126614
MUG019	12	5.48	0.91	1.0	1	3	Milas		Koru	35S0563737	UTM4122000
MUG020	30	8.07	13.45	2.0	3	2	Ortaca	Ortakoy	Ortakoy	35S0648668	UTM4082804

Table 2- Correlation values of Detrended Correspondence Analysis (DCA) depending on the species of matrix axis

Axis	*1*			*2*			*3*		
Species	r	r-sq	*tau*	*r*	*r-sq*	*tau*	*r*	*r-sq*	*tau*
Aegilops neglecta	-0.384	0.147	-0.069	0.536	0.287	0.382	-0.149	0.022	-0.260
Avena sativa	-0.017	0.000	-0.030	0.516	0.266	0.500	-0.123	0.015	-0.114
Bromus danthoniae	-0.558	0.312	-0.562	-0.275	0.075	-0.069	0.096	0.009	-0.082
Bromus tectorum	0.177	0.031	0.307	-0.199	0.039	-0.244	-0.073	0.005	-0.244
Cantaurea iberica	0.041	0.002	-0.036	0.239	0.057	0.226	-0.184	0.034	-0.139
Cardopatium corymbosum	0.193	0.037	0.168	0.355	0.126	0.343	0.102	0.010	0.139
Carex acuta	-0.175	0.031	-0.065	0.217	0.047	0.182	0.025	0.001	0.000
Carex atrata	0.466	0.218	0.434	-0.156	0.024	-0.217	0.606	0.368	0.375
Cynodon dactylon	0.356	0.127	0.251	-0.110	0.012	-0.195	0.622	0.387	0.463
Festuca ovina	0.718	0.516	0.560	0.301	0.091	0.248	-0.337	0.114	-0.248
Hordeum bulbosum	0.087	0.008	-0.006	0.417	0.174	0.536	-0.145	0.021	-0.211
Hordeum marinum	0.190	0.036	0.206	-0.089	0.008	-0.039	0.366	0.134	0.318
Juncus acutus	0.291	0.085	0.302	0.287	0.082	0.315	0.153	0.023	0.178
Lolium multiflorum	-0.383	0.147	-0.314	-0.560	0.313	-0.284	-0.103	0.011	-0.080
Lolium perenne	-0.539	0.291	-0.386	-0.361	0.131	-0.168	0.206	0.043	0.314
Notobasis syriaca	0.073	0.005	0.134	0.004	0.000	0.134	-0.065	0.004	-0.055
Pistacia terebinthus	-0.324	0.105	-0.276	-0.693	0.480	-0.474	-0.452	0.204	-0.375
Plantago atrata	-0.189	0.036	-0.143	0.271	0.073	0.195	0.224	0.050	0.235
Poa bulbosa	0.628	0.395	0.494	0.059	0.003	-0.020	-0.297	0.088	-0.276
Polypogon monspeliensis	-0.422	0.178	-0.194	-0.212	0.045	-0.081	0.302	0.091	0.293
Trifolium campestre	0.634	0.402	0.261	-0.075	0.006	-0.274	-0.281	0.079	-0.117
Trifolium hirtum	-0.284	0.081	-0.178	0.105	0.011	0.138	0.096	0.009	0.020

3. Results and Discussion

According to the results of cluster analysis, similar 3 groups occurred. Among these groups, the first group consisted of MUG001, MUG012, MUG005, MUG016, second group consisted of MUG003, MUG013, MUG017, MUG018, MUG004, MUG020, MUG014, MUG006, MUG010 and third group consisted of MUG007, MUG009, MUG011, MUG015, MUG008, MUG019 (Figure 1). Indicator species in segregation groups were determined by indicator species analysis in PC-ORD program. According to the results of the indicator species analysis, *Carex atrata* (60.0), *Poa bulbosa*

(60.0) and *Trifolium campestre* (73.1) were the indicator species of the first group, while *Bromus danthoniae* (57.9) was the inductor species of the second group and finally, the indicators of the third group were *Avena sativa*, *Cardopatium corymbosum*, *Hordeum bulbosum* (70.7), *Juncus acutus* (87.4), *Notobasis syriaca* (57.5). After Multi-Response Permutation Procedures (MRPP) analysis, detrended correspondence analysis was preferred for the ordination analysis and the correlation of both variables with the axes was calculated (Tables 3-4). As a result of the analysis, it was found that 1st axis explanation of the percentage 0.54110, 2nd axis explanation of the percentage of 0.37308 and 3rd axis explanation 0.14921 (Table 4). When the correlation between vegetation data matrix and environmental data matrix was investigated, it was seen that environmental variables such as pH in water-saturated soil, lime, surface stoniness, altitude, usable potassium, distance to village, soil depth are important. It is seen that the species of *Bromus tectorum*, *Cardopitum corymbesum*, *Carex atrata*, *Cynodon dactylon*, *Festuca ovina*, *Juncus acutus*, *Poa bulbosa*, and *Trifolium campestre* have an increase and growth potential with increasing elevation, distance to village and soil depth variables. Among these characteristics, the correlation between altitude (r= 0.624) and *Festuca ovina* (r= 0.718), *Poa bulbosa* (r= 0.628) and *Trifolium campestre* (r= 0.634) was determined as the closest positive relationship. Furthermore, the species that the highest negative relationship with altitude was determined as *Bromus danthoniae* (r= -0.558) and *Lolium perenne* (r= -0.539). *Carex atrata* (r= 0.466) was observed that closest positive relationship with village distance (r= 0.541) and soil depth (r= 0.532) variables. Considering other variables; when pH in saturated with water, lime and surface stoniness increase *Aegilops neclecta*, *Bromus danthoniae*, *Lolium multiflorum*, *Lolium perenne*, *Pistacia terebinthus*, *Polygonum monspeliensis*, and *Trifolium hirtum* species also show an increase. While lime variable parameter was significantly positive correlated to negative position with *Aegilops neclecta* (r= -0.384), *Bromus danthoniae* (r= -0.558), *Lolium multiflorum* (r= -0.383), *Lolium perenne* (r= -0.539) and *Polygonum monspeliensis* (r= -0.422) species, pH in saturated with water (r= -0.350) parameter significantly positive correlated to negative position with *Aegilops neclecta* (r= -0.384), *Lolium multiflorum* (r= -0.383) and *Pistacia terebinthus* (r= -0.324) species. It was observed that surface stoniness (r= -0.241) and *Trifolium hirtum* (r= -0.284) showed the highest positive correlation (Tables 3-4, Figure 2). When the groups were examined together, while the altitude, distance to village and depth of soil were determined as a high in the sample areas belonging to the first group, soil depth was found to be high in the second group of sample areas. Finally, in the third group in the sample areas, the pH in the water-saturated soil, lime and surface stoniness variables were found to be high (Figure 3). According to the results, it was observed that soil factors were closely related to vegetation. It has been determined that soil factors with soil chemistry changes have positive or negative effects on the species in vegetation. Similar results were reported by other researchers (Kumar 1996; Ridolfi et al 2008; Amiri & Saadatfar 2009; Kabir et al 2010; Kirkpatrick et al 2014). It has been determined that different species have different effects depending on the change in altitude, which is one of the environmental factors and has a close relationship with vegetation change. This result was in accordance with Ispirli et al (2016). Many researchers also have investigated the effects of environmental factors on vegetation change (Zhang & Dong 2009; Mofidi et al 2012; Surmen et al 2013; Zhengchao et al 2016).

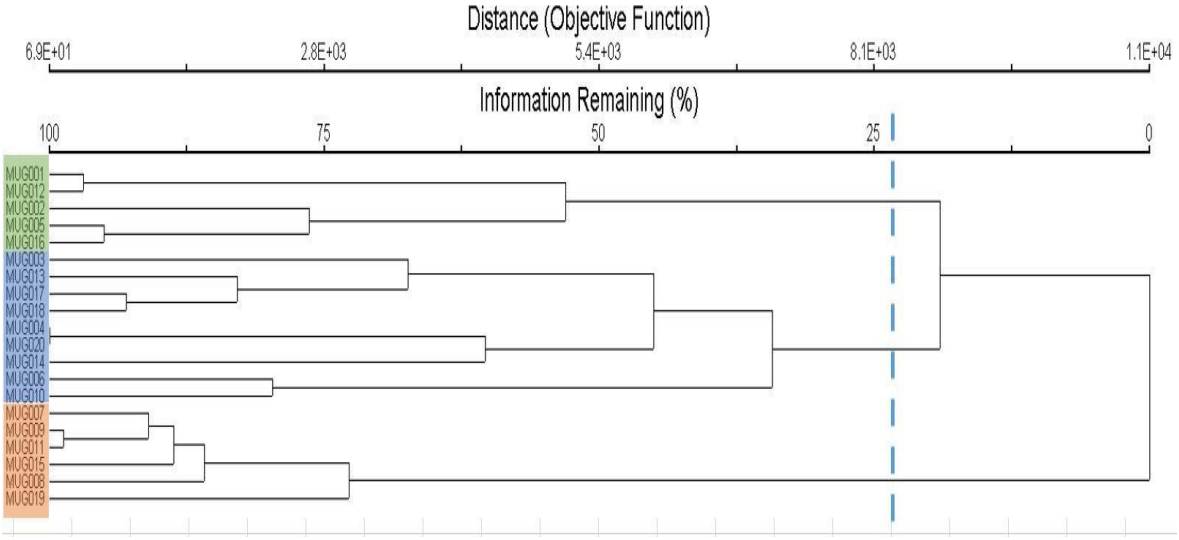

Figure 1- Dendrogram obtained by hierarchical cluster analysis of 20 samples areas

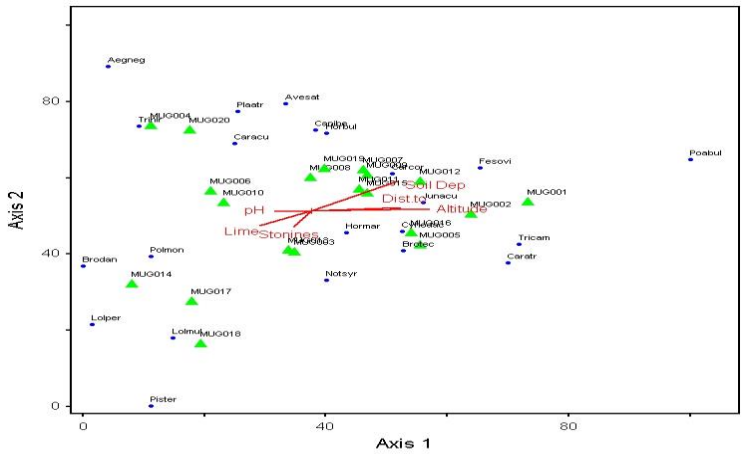

Figure 2- Ordinate distribution according to Detrended Correspondence Analysis (DCA) influenced by environmental factors in species detected in 20 sample areas

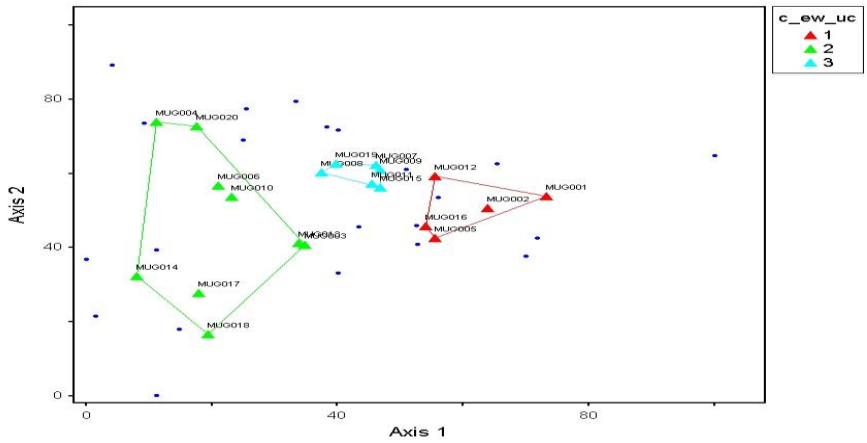

Figure 3- Group distribution of 20 sample areas by Cluster Euklidien Ward's in Detrended Correspondence Analysis (DCA)

Table 3- Correlation values of Detrended Correspondence Analysis (DCA) depending on the environmental data of matrix axis

Axis	*1*			*2*			*3*		
Soil properties in pastures	*r*	*r-sq*	*tau*	*r*	*r-sq*	*tau*	*r*	*r-sq*	*tau*
Altitude	0.624	0.389	0.108	0.100	0.010	0.162	-0.358	0.128	-0.184
pH in soil saturated with water	-0.350	0.123	-0.206	-0.049	0.002	-0.037	0.363	0.131	0.237
Lime	-0.414	0.172	-0.415	-0.274	0.075	-0.053	-0.092	0.008	0.128
Total salt	0.073	0.005	-0.016	-0.188	0.035	-0.144	0.670	0.449	0.485
EC	-0.051	0.003	-0.032	-0.228	0.052	-0.189	0.570	0.325	0.432
Plant-available potassium	0.234	0.055	0.175	0.176	0.031	0.027	-0.152	0.023	-0.111
Organic matter	-0.023	0.001	0.016	-0.045	0.002	0.005	0.168	0.028	0.132
Erodibilite	-0.073	0.005	-0.105	-0.045	0.002	-0.082	-0.390	0.152	-0.269
Hydraulic conductivity	-0.011	0.000	0.053	-0.091	0.008	-0.021	-0.021	0.000	-0.074
Field capacity	-0.011	0.000	-0.011	0.016	0.000	0.063	0.309	0.096	0.242
Permenent wilting	-0.079	0.006	-0.032	-0.041	0.002	0.063	0.336	0.113	0.242
Bulk density	0.002	0.000	0.048	-0.030	0.001	-0.069	-0.192	0.037	-0.143
Distance to village	0.541	0.293	0.242	0.134	0.018	0.038	-0.222	0.049	-0.064
Slope	-0.146	0.021	-0.130	0.081	0.007	-0.020	-0.241	0.058	-0.266
Erosion	-0.148	0.022	-0.218	0.099	0.010	0.008	-0.071	0.005	-0.121
Stoniness	-0.241	0.058	-0.216	-0.288	0.083	-0.268	0.076	0.006	0.164
Soil depth	0.532	0.283	0.392	0.388	0.151	0.166	0.106	0.011	0.126

Table 4- The results of the length and eigenvalues in Detrended Correspondence Analysis (DCA)

	Axis 1	*Axis 2*	*Axis 3*
Eigen value	0.54110	0.37308	0.14921
Gradient length	3.473	2.975	2.923

4. Conclusions

Rangeland areas have had a great impact on every period of human history from ancient times to the present day. For the suitable management of rangeland areas that have biodiversity and supply the nutritional needs of livestock, it is very important to know the species of plants in the area and investigate their relationship with environmental factors. For this purpose, the species and environmental variables were examined by vegetation study in 20 different sample areas by ordination analysis, especially in Mugla province, where is located in a known ecology with its rich in biodiversity. According to the results, it was determined that many environmental variables had an effect on botanical species composition. The results of the study revealed the species that have a growing potential in the regions. The data obtained from this study will be a resource for future studies about rangeland management and improvement in these areas.

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Abbreviations and Symbols	
DCA	Detrended Correspondence Analysis
MRPP	Multi-Response Permutation Procedures
TAGEM	General Directorate of Agricultural Research and Policies
BUGEM	General Directorate of Plant Production
TUBITAK	The Scientific and Technological Research Council of Turkey
MUG	Mugla Province
pH	Stands for the potential of Hydrogen
$CaCO_3$	Calcium Carbonate
P_2O_5	Phosphorus Pentoxide
K_2O	Potassium Oxide
r	Correlation Coefficient

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A New Pest Species *Delia platura* Meigen, (Diptera: Anthomyiidae) on Garlic (*Allium sativum* L., Alliaceae) Area in Kastamonu Province of Turkey

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ABSTRACT
Garlic (*Allium sativum* L., Alliaceae) is consumed at all stages from fresh to dry in Turkey. Major harmful factors, pests for example, bring about a significant loss in garlic production. Studies carried out in Kastamonu garlic areas to determine the harmful pests revealed that the important harmful pest was *Delia platura* Meigen, (Diptera: Anthomyiidae) in the garlic fields. Population dynamics of *D. platura* was studied using yellow sticky traps in autumn and spring seasons of planting garlic fields. Plants were checked with trap controls and searched for *D. platura* on garlic plants. Plants tested were recorded as infested and non-infested. The testing of traps finished in two weeks after the harvest. As a result, *D. platura*, the first emergence of the adult, was detected on March 21, 2017 in autumn planting and in spring planting. In the autumn planting, the highest number of adults caught in traps was on April 2015 with 4 adult/trap/week. In spring planting, the first adult in the trap was 1 adult/trap/week on 14 March, 2015. The study conducted in 2017, the first time of adult determination on trap was on March 18, 2017 in autumn planting. In spring planting, the first adult emerged on March 18, 2017.

Keywords: Seecorn maggots; Population dynamic; Garlic

1. Introduction

Garlic, *Allium sativum* (L.) (Alliaceae) consisting of sucrose, glucose, A, B and C vitamins, alliin, allicin, ajoen essential oils is an important food for people nutrition. The usage of garlic is believed to protect heart disease, cancer, and infection. The health benefits of garlic is also claimed to be lowering blood pressure and cholesterol, an protecting inflammatory reducing the risk of cancer, and a strengthening immune system (Anonymous 2018a).

There are 500 species of *Allium* genus in the world. Approximately 150 species of *Allium* genus, 57 of which fall into the group of fragrant garlic in the head or bulbs. The most important species of garlic is *Allium sativum*, which have become an important commercial product in Turkey and both fresh and dried garlic is produced in Turkey. According to the latest data, the production of fresh garlic is 25.519 tons, and dried garlic is 117.688 tons (Anonymous 2018b).

Many pests such as *Delia antiqua* Meigen (Diptera:Anthomyiidae), *Delia platura* Meigen (Diptera:Anthomyiidae), *Thrips tabaci* L. (Thysanoptera:Thripidae), *Frankliniella occidentalis* (Pergande) (Thysanoptera:Thripidae), *Rhyzoglyphus* spp., (Acari:Acaridae), *Tyrophagus* spp. (Acari:Acaridae), *Aceria tulipae* (Keifer) (Acari:Eriophyoidea) reduce the production of garlic (Anonymous 2016). Previous studies carried out in Turkey reported garlic pests as *Bactericera tremblayi* Wagner (Hemiptera: Psylloidea), *T. tabaci*, *F. occidentalis* (Thysanoptera:Thripidae), *Acrolepiopsis assectella* (Zeller) (Lepidoptera:Acrolepiidae), *Agriotes* spp. (Coleopteran:Elateridae), *Liriomyza* spp.

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(Diptera:Agromyzidae) and *Ditylenchus dipsaci* (Kühn) (Tylenchida:Anguinidae) in garlic cultivation areas (Anonymous 2008). However, in recent years, garlic producers have complained about side effects of the pest on garlic production. This pest has been become common and caused significant loss of garlic yield. Some producers have even maintained that they did not get any yields.

This study conducted in 2015 and 2017 years in Taşköprü-Kastamonu province, aimed at determining the pest in garlic production areas.

2. Material and Methods

2.1. Pest determination studies

Garlic plants were collected from garlic production areas during the production season. They were taken to laboratory for investigation. First larvae were determined and then they were fed to develop into adults and they were sent to experts for recognition. The adults were identified as *Delia platura* Meigen (Diptera:Anthomyiidae) by Hans Georg Rudzinski.

2.2. Population monitoring studies

Population monitoring methods were carried out by using yellow sticky traps (20x25 cm diameter) in autumn and spring garlic cultivation fields. During 2015, autumn planting was on September 15-18 and the spring planting was on March 10-15, 2016. Autumn planting for the year of 2017 was on September 18-25 and the spring planting was on March 5-10, 2016. Soon after the seedling emergence, yellow sticky traps were placed on a stake and placed 25 cm above the soil. When counting in traps plants were checked and searched for any larvae on garlic plants. Traps were replaced with every week. The plants were collected for 20/da and taken to laboratory. Then these plants were examined by stereo microscope and recorded as infested and non-infested. The counts of traps were completed within two weeks after harvesting (Erdoğan et al 2014). No insecticide was used in the study areas. Climate data of the study areas were obtained from the General Directorate of Meteorology (Anonymous 2017).

3. Results and Discussion

3.1. Pest determination studies

D. platura was recorded for the first time garlic fields in Turkey.

3.2. Population dynamic of Delia platura

In the autumn planting, the first adult emergence date was March 15, 2015. The highest number of adults was on March 29, 2015 with 4 adult/trap/week. The lowest number of adults was on April 19, 2015. The highest infestation rate was 7% in the same field. In the spring planting, the first adult emergence date was April 5, 2015. On the same date, the rate of infestation was 4%. The highest number of adults caught in the trap was 2 adult/trap/week. The highest infestation rate was 4% rate on the same field (Table 1). Studies in 2017, in the autumn planting, the date of first adult emergence was March 15, 2017. The highest number of adults was determined on May 5, 2017 (99 adult/trap/week). Peak activity was in May with approximately 99 adults per trap week. In the same field, the highest of rate infestation was 41%. The plants were harvested on July 7, 2017 (Table 2). In the spring planting, as shown in Table 2, the date of the first emergence of adult was March 15, 2017. The highest number was 95 adult/trap/week on April 5, 2017. The highest infestation rate (36%) was determined on April 26, 2017 in the same field (Table 2).

D. platura was first determined garlic fields in Turkey in this study. *D. platura* is the most widespread Anthomyiidae occurring all over the continents except Antarctica (Griffiths 1993). It was first reported in Germany and it is presently well established throughout the United States, including Alaska and Hawaii, and southern Canada (Gesell 2000). It is a major pest in North and South America as well as in Europe (Kornegay & Cardona 1991) and is commonly found in Japan, India, Australia and New Zealand (Trotus et al 1996). *D. platura* is reported to be hosted by *Allium* species (Griffiths 1986; Howard et al 1994). The Seedcorn maggot is on the other hand a polyphagous pest, affecting more than 40 different host plants (Ristich 1950). It is an important pest germinating soybeans and corn (Funderburk et al 1983; Bessin 2004) and may infect a wide range of horticultural crops including beans, peas, cucumber, melon, onion, pepper, potato, and other vegetables (Kessing & Mau 1991). In our country, the hosts of *D. platura* are vegetables such as bean, pumpkin, cucumber, melon and watermelon (Anonymous 2008).

Table 1- *Delia platura* Meigen adult number (adult/trap/week) and infestation rates captured in traps in garlic field of Taşköprü/Kastamonu province) (2015)

The date of counts	*The autumn planting*		*The spring planting*	
	The number of adults/trap	*The rate of Infestation (%)*	*The number of adults/trap*	*The rate of Infestation (%)*
01.03.2015*	-	-	-	-
08.03.2015	0	0	0	0
15.03.2015	2	0	2	4
22.03.2015	3	6	1	2
29.03.2015	4	7	0	0
05.04.2015	3	0	0	0
12.04.2015	2	0	0	0
19.04.2015	1	6	0	0
26.04.2015	0	1	0	0
03.04.2015	0	0	1	0
10.05.2015	0	0	0	0
17.05.2015	0	0	0	0
24.05.2015	0	0	0	0
31.05.2015	0	0	1	0
07.05.2015	0	0	0	0

*, the date of trap placement

Table 2- *Delia platura* Meigen adult number (adult/trap/week) and infestation rates captured in traps in garlic field of Taşköprü/Kastamonu province) (2017)

The date of counts	*The autumn planting*		*The spring planting*	
	The number of adults/trap	*The rate of infestation (%)*	*The number of adults/trap*	*The rate of infestation (%)*
01.03.2017	*	*	*	*
08.03.2017	0	0	0	0
15.03.2017	4	0	4	0
22.03.2017	18	3	42	9
29.03.2017	26	4	80	17
05.04.2017	33	7	95	22
12.04.2017	50	27	72	29
19.04.2017	39	32	59	24
26.04.2017	23	24	37	36
03.05.2917	90	37	23	29
10.05.2017	99	41	25	25
17.05.2017	74	36	15	22
24.05.2017	60	24	12	28
31.05.2017	22	19	11	24
08.06.2017	10	11	16	14
15.06.2017	11	7	14	9
22.06.2017	5	3	12	7
30.06.2017**	3	-	6	4
07.07.2017	2	-	6	3
15.07.2017***	3	-	4	3
20.07.2017	-	-	8	-
26.07.2017			5	

*, the date of trap placement; **, the date of harvest in autumn planting; ***, the date of harvest in spring planting

In the present study, *D. platura* population dynamic was conducted on garlic field 2015 and 2017 years in Taşköprü-Kastamonu province. The yellow sticky traps were observed to be effective at catching *D. platura*, which was present

from March to July. Our findings are consistent with those in the related literature. Ellis & Scatcherd (2007) reported, the yellow sticky traps capturing the adults *D. platura*. In addition, Vernon et al (1987) noted that yellow was the most effective color attracting vegetable-infesting insects including *D. platura*. In addition, it was *maintained* that the yellow sticky traps were effective at catching *D. platura* (Robert et al 1987; Broatch & Vernon 1997).

The population dynamic studies (2015) on *D. platura*, in this study the first emergence of the adult was detected in March in both the autumn planting and spring planting (Average temperature and humidity was 12 ºC and 95% respectively), but short after then the population of *D. platura* suddenly stopped (Figure 1, 2). We checked the trap on until May and finished counting at the end of May since there was no sign of adult *D. platura*. It might have been resulted from the heavy rainfall during the months of April and May (February: 0, March: 5, 9; April: 30, 8; May: 41, 3 kg m^{-2}, Anonymous 2015) (Figure 3). Similarly Kansu (2005), reported that some insect species spending a certain biological period in the soil by the combination of rainfall and low temperature were adversely affected by population density. To the best our knowledge, there is no study on *D. platura* related to this issue though there are a few studies on different pests. Erdogan et al (2014) revealed that total precipitation had a negative effect on population density of pest. Similarly, it was noted that the density of potato moth population was inversely related to temperature and inversely related to rainfall (Von et al 1987). In addition, in the Central Anatolia Region, a decrease of rainfall on the days of the flight of the potato moth was recorded (Has et al 1996). It was determined that the heavy rainfall during the month of May in the Aegean region caused a decrease in the population of potato tuber moths (Zümreoğlu 1996). In addition, might be a noteworthy decrease in the potato moth populations during the rainy seasons as well as in the sprinkling in the fields (Raman et al 1987).

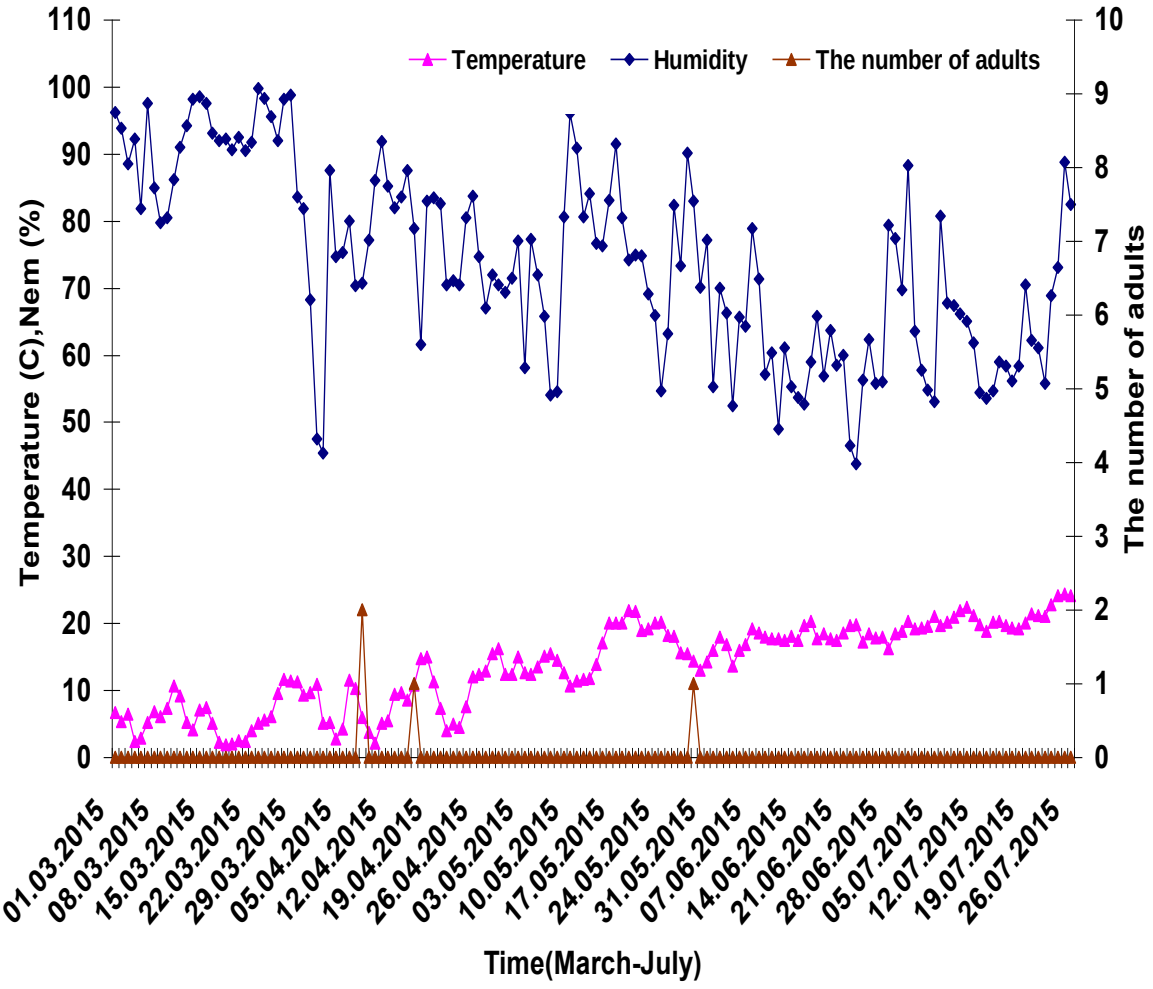

Figure 1- Temperature, humidity and the number of adults obtained from in the spring planting garlic

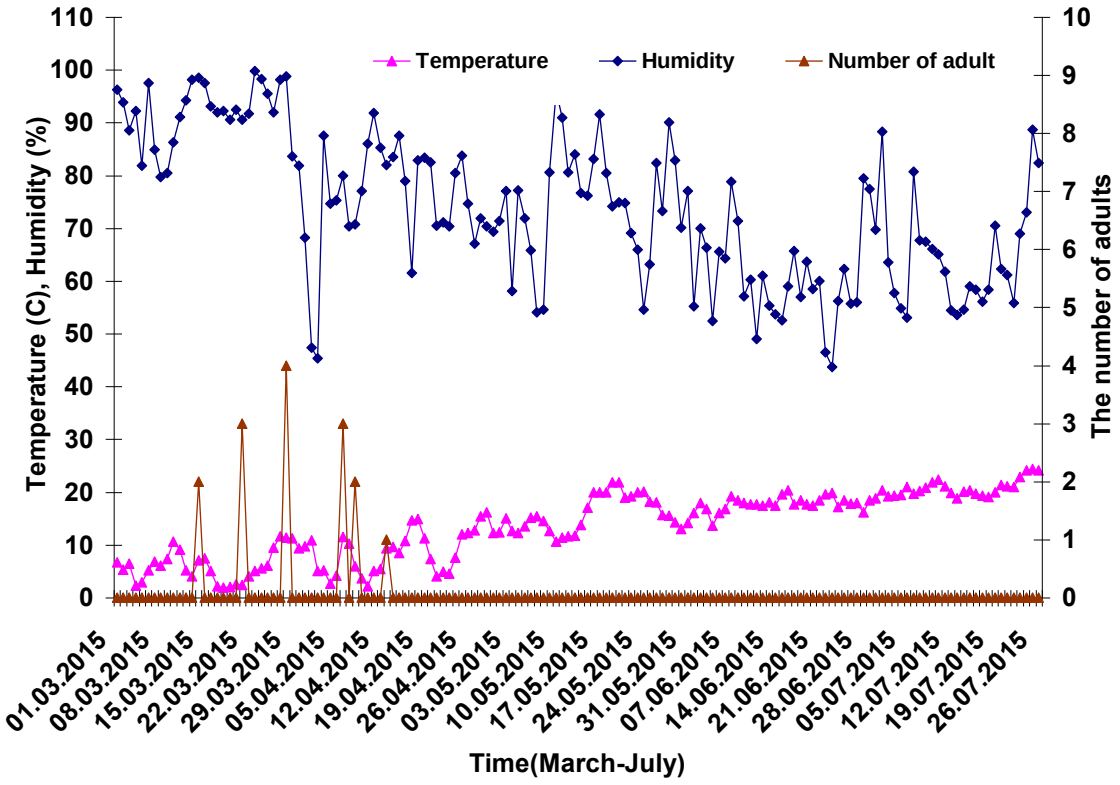

Figure 2-Temperature, humidity and the number of adults obtained from in the autumn planting garlic

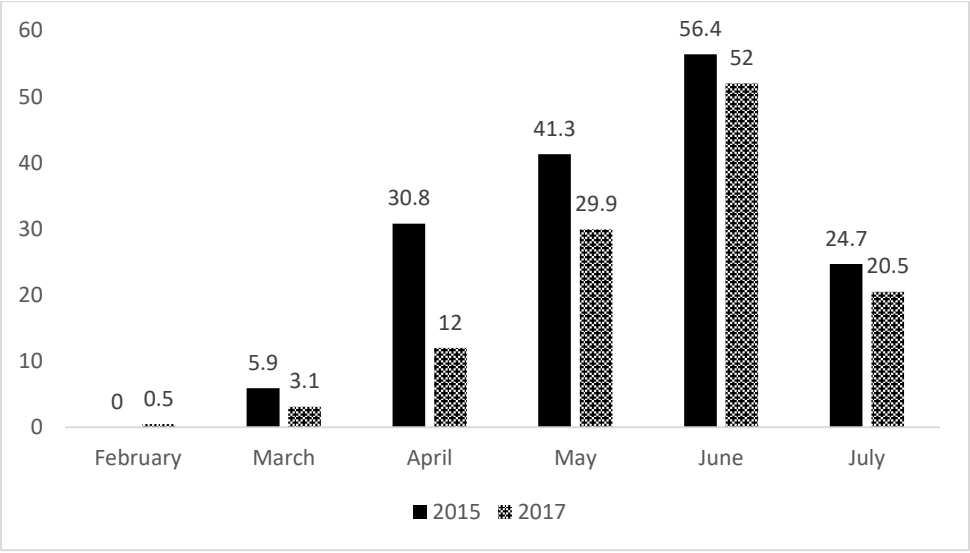

Figure 3- During the study of population dynamic determined total monthly precipitation (kg m^{-2})

In the studies carried out in 2017, it was found that the first adult emerged on March both in the autumn planting and the spring planting (Average temperature and humidity 10 ºC and 65% respectively), and the adult population increased until the beginning of March, April and May and then it decreased. A total of 614 adults were trapped in the autumn planting with peak activity in May. In the spring planting, a substantial number of adults were observed in three peaks, yet only 546 adults of *D. platura* were trapped. The highest number of adults were determined as 99 adults /week/trap in the autumn planting on May 10, 2017 (Figure 4). The average temperature and humidity were recorded as 15 ºC and 85 70%, respectively.

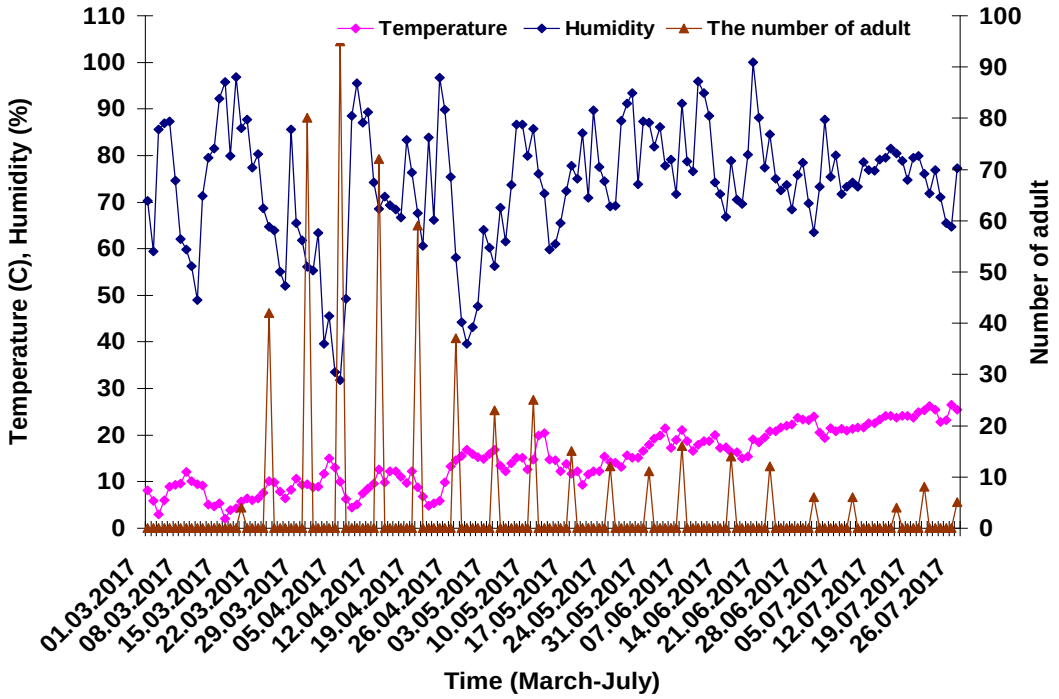

Figure 4-Temperature, humidity and the number of adults obtained from in the spring planting garlic

Furthermore, in the spring planting, the highest number of adults were 95 adults/week/trap on April 5, 2017. The average temperature and humidity were 10 ºC and 32%, respectively (Figure 5).

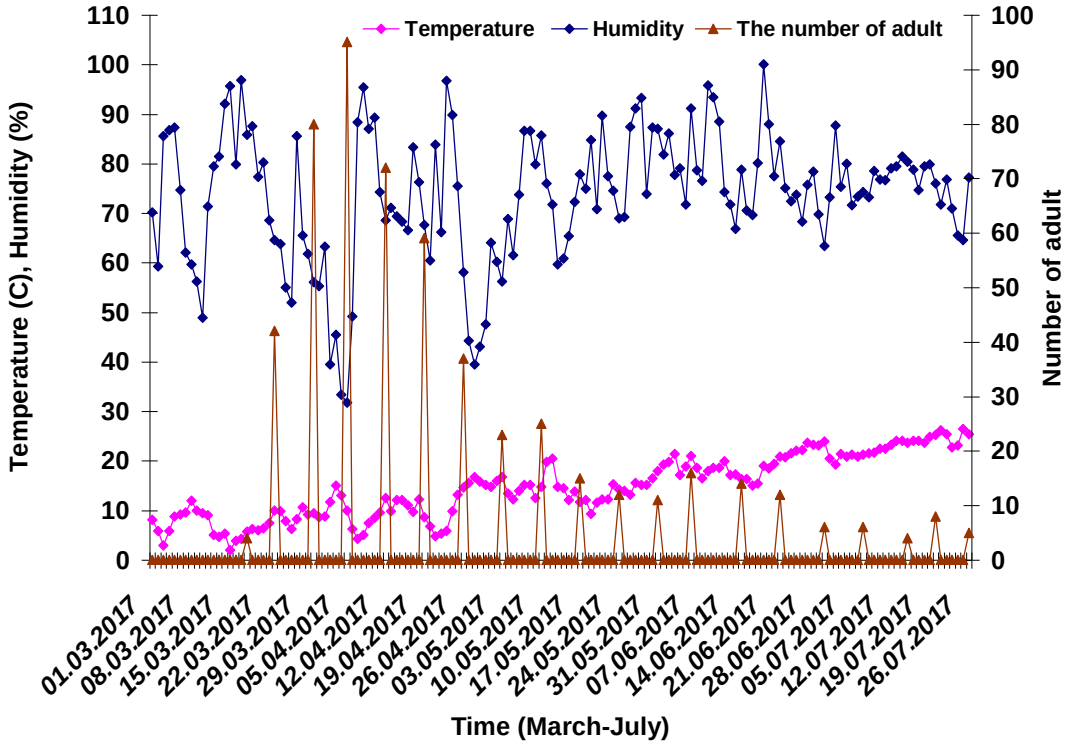

Figure 5-Temperature, humidity and the number of adults obtained from in the autumn planting garlic

These findings are consistent with those of following studies. For example, Broatch & Vernon (1997) carried out population monitoring of *D. platura* by using yellow sticky trap, and they reported that the adults of *D. platura* emergence was on the beginning of March. The authors also reported that the population density was very high in April and May, then the emergence continued until the end of the harvest even in the low population, and the average temperature was 22 ºC in the periods when the population of *D. platura* was very high. Similarly, in our study, the average temperature during the trial was 23 ºC. Moreover, adults are very numerous in the spring (two to three generations), but their population starts decreasing substantially in mid-summer (Eckenrode et al 1973; Hagel et al 1981; Sanborn 1981).

The most important harmful species in the study of garlic fields in our study was the *D. platura*, which were spread over the entire area, and the rate of harmful infestation increased to 41% in some areas. Related literature have reported similar findings. For example, Bessin (2004) reported *D. platura* damaging newly planted seeds by feeding on seed contents, often-leaving empty seed shells and preventing germination. Germinate seeds that due to damage are spindly with few leaves and die before maturation. *D. platura* induced damage is facilitated by early planting dates, heavy cover crops, and cool-wet weather (Bessin 2004). Occasionally, *D. platura* tunnel within seedling stems and germinating seeds (Funderburk et al 1983). Usually no more than 2% of the seedlings get infested by this insect yet 30% to 60% plant loss may occur in the field. Reduction in the plant stand can be seen within a week after plant emergence (Gesell 2000).

4. Conclusions

The adults of the insect was recognized as *D. platura,* which was first observed in Turkey in this study carried out in garlic area, and the population monitoring of *D. platura* was determined. In the population monitoring study were determined that that the first adult emerged on March both in the autumn planting and the spring planting (Average temperature and humidity 10 ºC and 65% respectively), and the adult population increased until the beginning of March, April and May and then it decreased. In addition, it was revealed that the amount of precipitation negatively affected the adult population of *D. platura*.

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2D Analytical Model for Evaluation of the Forces in the Three-point Hitch Mechanism

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ABSTRACT
Large investments, research efforts and time have been allocated till nowadays for innovation of agricultural tractors and machinery, with the primary aim to increase their productivity, reliability, durability, as well as the environmentally friendly, safe and easy usage. Following this general trend, present work is focused on theoretical analysis of the forces acting on the links of the three-point hitch mechanism. The simulation algorithm and computer code have been developed for calculation of draft forces by two methods (following ASAE D497.4 standard and Goryachkin approach), depending on the three point linkage geometry and mouldboard technical characteristics, and calculation of forces acting on lower and upper links of three-point hitch mechanism. Calculated forces values, based on ASAE draft, exposes smaller differences compared to experimental values, while for the Goryachkin method improvement is possible by entering experimental values for soil resistance coefficient *k* and coefficient of dynamic resistance ε, as we did in this study performing measurements on the aggregate comprehending tractor IMT 539 (IMT Belgrade, Serbia) and two furrow moldboard plough PTO 2.25 (OLT, Osijek, Croatia).

Keywords: Three hitch point; Tractor; Draft; Link forces

1. Introduction

Nowadays, large funds are allocated for innovation of agricultural tractors with the primary aim to increase their productivity, reliability, durability, as well as the safe and easy usage (Mileusnić et al 2010). Arizton Advistory & Intelligence (2018) indicates that the number of low-power tractors participate a third of total sales in 2017., having the approximate growth of 5% in some developed countries and in emerging ones in forecast period 2018-2023. Generally, about 84% of existing farms possesses less than 2 hectares, comprehending 12% of agricultural land, and even more in the less developed countries (Sarah et al 2016). Lower price, simplicity and functionality motivate owners of small farms to buy tractors with lower installed power, what was a motive to base present study on a low-power tractor.

Agricultural tractors are driving units with a wide range of possible implementations in agriculture, forestry etc. In order to provide possibility of tractors connecting with various implements applied, most of tractors are equipped with a three-point hitch mechanism (TPH). Many studies on TPH mechanisms were focused on the forces transition between tillage implements and tractors, which has the crucial influence on the operational efficiency and energy management (Dalmiş et al 2017), as we did here.

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After introducing the hydraulic system for position control, three-point linkage was improved and still remains in use in the low-power, but also in the high-power tractors. Therefore, standards recognizing four categories of these mechanisms were introduced to provide adjusting of their design to tractors having various power and traction forces.

This paper presents an analytical approach for identifying forces of three point hitch mechanism components based on the known geometry, mechanism working position, soil resistance and plough characteristics. Special computer code has been formulated, based on field measurements on the experimental parcel with known plough geometry, for assessment of forces acting on the links of the three point hitch mechanism.

During operation, the three-point linkage transfers forces acting on the implement to the tractor. Ploughing is a highly power demanding soil tillage operation (Fröba 1995), comprehending cutting and turning up and over the surface soil layer enriched with minerals and decomposed organic matter, usually in the depth range of 15÷30 cm. Going deeper increases the soil resistance and consumed energy. Beside the ploughing depth, soil reactions toward plough depend on the plough design, mechanical and other soil physical parameters and the tractor speed.

2. Material and Methods

2.1. The analytical model

Forces acting on the plough can be represented with three-dimensional force vector acting on the pull center. This is not a fixed point because of the soil parameters variability, settings of the plough and connectivity with three point linkage. The lateral *Rz* force is counteracted by a landside force. The vertical force *Ry* results from the weight of the plough body, weight of the soil volume lifted by the moldboard and vertical soil force. Part of *Ry* may be also supported by gauge wheel. The longitudinal force, so-called *draft resistance Rx,* is the rearward soil force component, that must be overcomed by tractor power. Dominant soil mechanical parameters influencing *Rx* are the soil deformation resistance and friction. The soil mechanical parameters changes with variations of soil texture and moisture content over depth (Haines 1925), affecting the forces acting on the links of three-point mechanism.

Variability of the soil properties imposes hard difficulties in the formulation of the general equations for the draft resistance evaluation. Draft resistance can be analytically estimated following standard ASAE (2003) Standard D497.4, 2003, and Goryachkin`s formula defined as a sum of static resistance and a dynamic resistance (Горячкин 1968)

$$R_x = G \cdot f + k \cdot a \cdot b \cdot n + \varepsilon \cdot a \cdot b \cdot n \cdot v^2 \quad (1)$$

Where; *G* (N) is the plough weight; *f* (-), represents the friction coefficient over the working element surface; *k* (N m^{-2}) is the specific soil resistance; *a* (m), designates the ploughing depth; *b* (m) is the width of ploughing tool; *n* is the number of tools; ε (Ns^2 m^{-4}), represents the coefficient of dynamic resistance, and *v* (m s^{-1}) is tractor velocity.

The plough friction coefficient usually has a value 0.3÷0.5 (Bernacki et al 1972), but in compact soil has a greater value.

The second member of equation corresponds to the energy used to cut and deform the slice of soil. Soil resistance coefficient *k* is a function of soil type, depth, humidity, the presence of crops or residues etc. Consequently, an experimental approach was needed.

The third term corresponds to energy required for movement the slice of soil over the mouldboard and for its throwing aside. The coefficient of dynamic resistance ε depends on tractor speed and shape of the mouldboard body. Its value ranges between 3000 and 10000 Ns^2 m^{-4} (Musil & Červinka 2007).

In the Expression (1), influence of plough geometry on draft resistance is also given by its width *b*. For more accurate determination of plough geometry influence, an experimental approach is needed (Plouffe et al 1995).

According to ASAE Standard D497.4 draft resistance can be described by the formula

$$D = F_i \cdot [A + B \cdot s + C \cdot s^2] \cdot W \cdot T \quad (2)$$

Where; F_i is parameter of soil texture (i= 1, 2, 3 for fine, medium and coarse texture respectively), *A*, *B*, *C* are the machine parameters (for moldboard plough A= 652, B= 0, C= 5.1), *W* (m) represents machine width or number of tools, *T* (cm) is the tillage depth, *s* (km h^{-1}) designates tractor velocity. This standard provides a good estimate of average implement draft. However, changes in ground profile, soil texture and tractor ride dynamics may lead to draft amplitude of up to ±50% (McLaughlin et al 2008).

The three point linkage is a 3D mechanism, consisting of the upper link, two symmetric lower links with lift rods, rockshaft arm and corresponding pins. Top and lower link set up implement into the right working position. Built in control system allows control of the implement position and/or draft force.

Fully mounted implement carries over forces to the three-hitch point. The intensity of the force depends on the position of hitching point and length of the links. Variations of the force in upper link with its length were shown by Čupera et al (2011). Tractor manufacturer usually has a test report of link length and pivot point position, measured from the rear wheel axis center. Side view of three point linkage with example of its dimensions is presented in Figure 1a. The goal of present study was to estimate forces acting on the three hitch point. Background data includes known three point linkage geometry ($l_1 \div l_6$) and position ($\alpha_1 \div \alpha_5$), estimated soil resistance (R_x, R_y, c_1, c_3), plough weight (G, c_2) and tractor rear-wheel radius (R). Position of three point linkage is determined by ploughing depth. It is not necessary to measure distance from all hitch points to the ground, but only the distance from lower link to the ground l_7, because the mast height l_6 is already known, Figure 1b. Equations (3)÷(7) were developed to determine three point positions with input data presented in Figure 1a.

$$\alpha_1 = asinQ_5 \tag{3}$$

$$\alpha_2 = acos\left[\left(l_4^2 + Q_1 + l_2^2 - 2 \cdot {Q_1}^{1/2} \cdot l_2 \cdot cos(\pi + atan\, Q_2 + asin\, Q_3) - l_3^2\right) / \left(2 \cdot l_4 \left(Q_1 + l_2^2 - 2 \cdot {Q_1}^{1/2} \cdot l_2 \cdot \cos(\pi + atanQ_2 + asinQ_3)\right)^{1/2}\right)\right] + acos\left[\left(l_2 - Q_1^{1/2} \cdot cos(\pi + atan\, Q_2 + asin\, Q_3)\right) / \left(Q_1 + l_2^2 - 2 \cdot {Q_1}^{1/2} \cdot l_2 \cdot \cos(\pi + atan\, Q_2 + asin\, Q_3\right)^{1/2}\right] \tag{4}$$

$$\alpha_3 = acos\left[\left(l_6^2 + l_5^2 - Q_4 - l_1^2 + 2 \cdot {Q_4}^{1/2} \cdot l_1 \cdot cos(atan\, Q_5 + asin\, Q_3\right) / (2 \cdot l_5 \cdot \quad l_6)\right] \tag{5}$$

$$\alpha_4 = acos\left[\left(l_1 - Q_4 \cdot cos(atanQ_5 + asinQ_3)\right) / \left(Q_4 + l_1^2 - 2 \cdot {Q_4}^{1/2} \cdot l_1 \cdot cos(atanQ_5 + asinQ_3)\right)^{1/2}\right] + acos\left[\left(l_6^2 - l_5^2 + Q_4 + l_1^2 - 2 \cdot {Q_4}^{1/2} \cdot l_1 \cdot cos(\, atanQ_5 + asinQ_3)\right) / \left(2 \cdot l_6 \cdot \left(Q_4 + l_1^2 - 2 \cdot {Q_4}^{1/2} \cdot l_1 \cdot cos(atanQ_5 + asinQ_3)\right)^{1/2}\right)\right] \tag{6}$$

$$\alpha_5 = \alpha_1 + \alpha_4 - \frac{\pi}{2} \tag{7}$$

Where; $Q_1 = (x_2 - x_1)^2 + (y_2 - y_1)^2$; $Q_2 = \frac{y_2 - y_1}{x_2 - x_1}$; $Q_3 = \frac{R - l_7 + y_1}{l_1}$; $Q_4 = (x_3 - x_1)^2 + (y_3 - y_1)^2$; $Q_5 = \frac{y_3 - y_1}{x_3 - x_1}$;

Vertical component of soil force for moldboard can be related with draft force (Martinov & Marković 2002):

$$R_y \approx 0.14\, R_x \tag{8}$$

Depending on the plough design, the vertical component of soil reaction force may be supported by the gauge force, landside heel or may be completely transferred to the three point linkage. The center point of soil resistance T_4 on a mouldboard plough is located halfway along the slice width and one-third of the ploughing depth by Bernacki & Haman (1967), while Wilkinson & Braunbeck (1977) placed center of soil resistance on one-fourth of the slice width from landside and one-fourth of the ploughing depth.

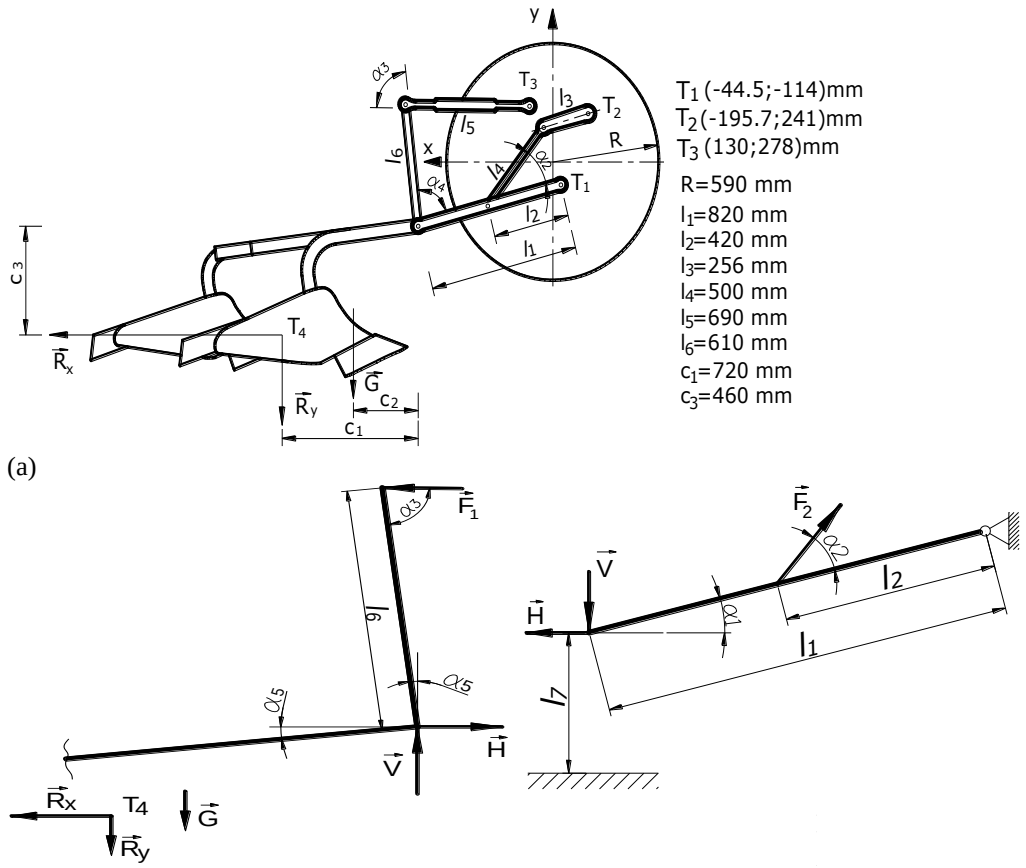

Figure 1- Three point linkage mechanism: a, three point linkage geometry; b, position of mast and lower link, and forces acting on these elements, during ploughing with forces

Implement motion is limited by three-point linkage, except it has freedom to rise up. By knowing the moving pattern (Equations (3)-(7)), it is possible to calculate forces acting on the three hitch point for each working position. The calculations only take into account the simplified 2D model of loading. Loading scenario for the mast and lower link is presented in Figure 1b. Uniform motion of tractor is assumed in this analysis, so there is no inertial force. Newton´s second law gives three independent equations of equilibrium needed to determine the forces acting on the mast.

$$F_1 = (R_x \cdot c_3 - 0{,}1 \cdot R_x \cdot c_1 - G \cdot c_2)/(l_6 \cdot sin(\alpha_3)) \quad (9)$$

$$H = (R_x + F_1 \cdot sin(\alpha_3 + \alpha_5))/2 \quad (10)$$

$$V = (G + 0{,}1 \cdot R_x - F_1 \cdot cos(\alpha_3 + \alpha_5))/2 \quad (11)$$

Where; $\overrightarrow{F_1}$ is the force acting on upper link hitch point, while *H* and *V* are horizontal and vertical component of force acting on both lower link hitch point.

2.2. Experimental procedure

Tractor IMT 539 (IMT-Belgrade, Serbia) was used in the experiment, Figure 2a. Technical characteristics of this tractor were: Diesel engine power 29.5 kW, the mass 1789 kg, front wheels 6.00-16'' and rear wheels 11.2-28''. Implement used for ploughing was two furrow semi digger moldboard plough PTO 2.25 (OLT-Osijek, Croatia), with operating width 30 cm per each moldboard and total mass 215 kg, Figure 2b. The lower link was 820 mm long with eyebolts end. It was a lower link of the first (I) category.

A field test and measuring were conducted on the flat horizontal parcel, characterized by black soil type-chernozem consisting of 35% sand, 35% silt and 30% clay, soil moisture content was 19%, volume weight 1.47 g cm^{-3}. There were remains in the ground after harvest of wheat grains.

Force was measured using custom-made force transducer based on the strain measurements using strain gauges and calibration of the developed transducer. Used strain gauges, of commercial type 3/120KY31, manufactured by Hottinger Baldwin Messtechnik, Germany, were mounted as it is explained in Cerović et al (2018), Figure 2c. Measuring resistance of these gauges are 120 Ω±1%, measuring grid length 3 mm, code number for the temperature response for ferritic steel $\alpha = 10.8 \cdot 10^{-6}$ K^{-1}, maximum bridge excitation voltage 8 V, transverse sensitivity 0.1%, gage factor 2.00±1%. Lower link of the mechanism was equipped with strain gauges as shown in Figures 2d, 2e. Prior to measurement, calibration of the lower link was performed, (Figure 2f) and dependence between the applied force and strain measurements was estimated using coefficient 0.0785 kN μm^{-1}, reaching the R-square value of $R^2 = 0.9996$, Figure 2g. Calibration was performed in the same position as the lower link is mounted on the tractor and with a depth corresponding to middle of the range of depth selected during the experiment. Force was measured using force transducer HBM U5 200 kN connected with DAQ system Quantum MX840A, (Figure 2h). Strain gauges applied on the lower link were connected in to Wheatstone bridges in order to measure horizontal force and bending caused by link geometry and consequently by eccentrically acting of forces on the link joints. Power supply for DAQ system was realized using petrol generator HONDA EU10i.

After analysing the calibration data and findings of Cerović et al (2018), it was noticed that due not only the pure axial load of the lower link but also the bending moment around the vertical axis, it is better to use (mount) only longitudinal strain gauge. Due to loading direction which does not coincide ideally with the axis, transversely placed strain gauge impose an error in establishing a connection between loading and relative deformations of the strain gauge. In order to have more accurate measurements, additional sensors should be added to measure forces in all mechanism components.

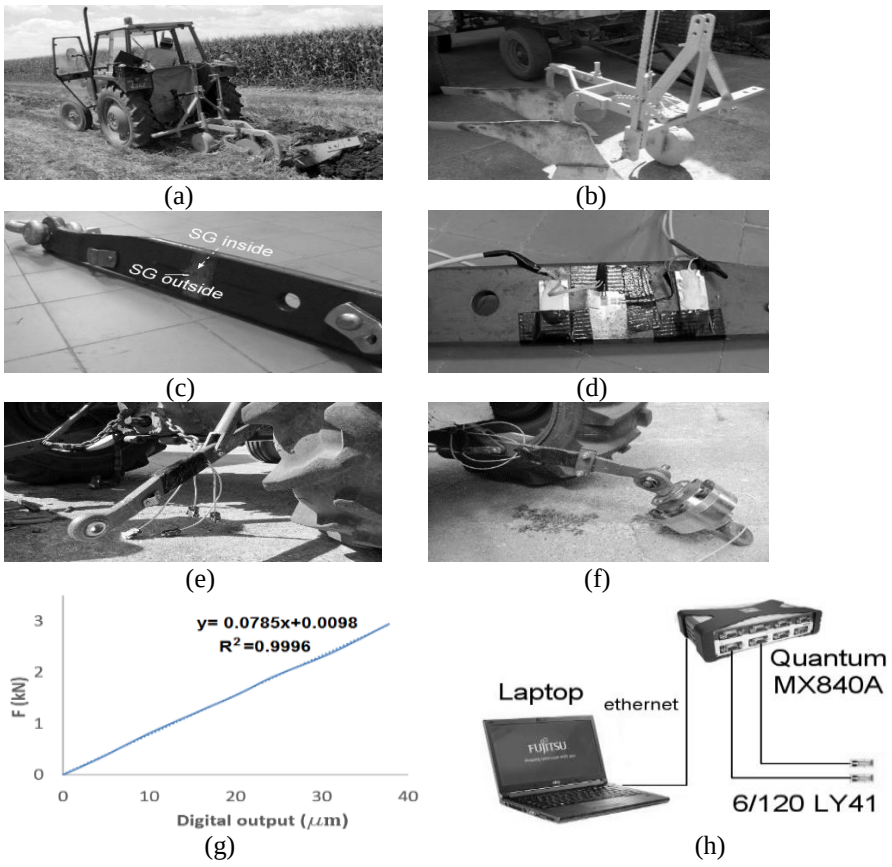

Figure 2- Experimental set-up: a, tractor in the test plot; b, two furrow moldboard plough; c, strain gauges position; d, strain gauges; e, mounting configuration directly on the lower link; f, field calibration; g, calibration line of lower link; h, measuring chain

Different ploughing depths and velocities were chosen in the field experiments, according tractor power and plough specification, Table 1.

Table 1- Ploughing depth and velocity chosen for experiment

Measurement	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Depth (cm)	20	20	20	17	15	13.5	11	11	15
Velocity (km h^{-1})	2	4	6	2	2	2	2	6	6

The plough was set in accordance with agro-technical requirements during all measurements. The tractor passed 50 m in each measurement. Measurements were repeated three times.

3. Results and Discussion

Measured values are shown in Figure 3. Recorded values of the measured forces showed strong fluctuations, which are mainly caused by the variations of soil resistance. Skalweit (1952) pointed out that ploughing depth tolerance should be ±10% of the mean value when the ploughing depth is about 18÷25 cm. Changes in ground profile and soil structure lead to variability of the measured forces. Experimentally obtained values, Figure 3, indicate a change of horizontal force in lower link with depth and velocity. Descriptive statistics of all measurements are presented in the Table 2. Such dependence is also noticeable in both formulas for soil resistance calculation, Equation (1) and Equation (2). As the soil type affects soil resistance, it is an essential factor of equations, although the ASAE D497.4 method is somewhat simpler than the Goryachkin method. Necessary parameters of soil type and machine are given in ASAE standard. Goryachkin method is based on experimental data. Yet, some regularity was noticed for specific soil resistance coefficient k: 20÷40 kN m^{-2} for light soils (sandy, silt); 40÷60 kN m^{-2} for medium soils (sandy clay); 60÷80 kN m^{-2} for heavy soils (clay) and 80÷100 kN m^{-2} for very heavy soils (Martinov & Marković 2002).

Test field chernozem is medium soil type. The third member of the equation (1) does not exceed 5% of total draft force (Borissov 2007) and therefore can be neglected. For the test, chosen value for coefficient of dynamic resistance ε was 3 $kNs^2 m^{-4}$. Next assumption for Goryachkin method was the value of 0.3 for the friction coefficient. After entering all data in Goryachkin Equation (1), it became obvious that the second term of the formula is dominant for small working velocity.

Based on Eqs. (1)÷(11) and presented data, the computer algorithm was developed for approximate calculation of average longitudinal and vertical forces in the lower link and force in the upper link, Figure 4. An important role in the transfer of forces acting on a plough onto the links has the links geometry and position. Lengths of all links and pivot point position (sketched in the Figure 1a) were entered into the Fortran program (Adams et al 1997). After the position of links were calculated according to Eqs. (3)÷(7), the program prompts how to calculate the draft: only by Equation (2) or by both Equations (1) and (2). Applied Eqs. (8)÷(11) gave a simplified solution for forces.

The program has a loop for estimation of average horizontal and vertical forces acting on the lower link and force acting on the top link for different soil depths and tractor velocities. Horizontal and vertical force dependence on the tillage depth and working velocity is presented graphically in Figure 5, while Figure 6 displayed top link force change with soil depths and tractor velocities. In the Table 2 is presented the difference between measured and the calculated values for the *H* horizontal component of force acting on the lower link hitch point. As mentioned, the ASAE standard indicates that difference up to 50% is possible between measured and calculated values for draft, and therefore for *H* as its major part.

Calculation based on ASAE method for draft determination is easier to apply because of small number of input data. Goryachkin method can also be used for approximate calculation, but for more precise value of soil resistance the experimental values of input data are needed. Developed computer program gives approximate values for observed forces, which in real condition change on the same field because of soil texture, compaction, moisture, etc. Draft can vary from 30 to 200% (Morling R W 1979). Forces in the lower left and right link are different as well. Thus, the written program can be used to estimate the average values in links of three point hitch mechanism.

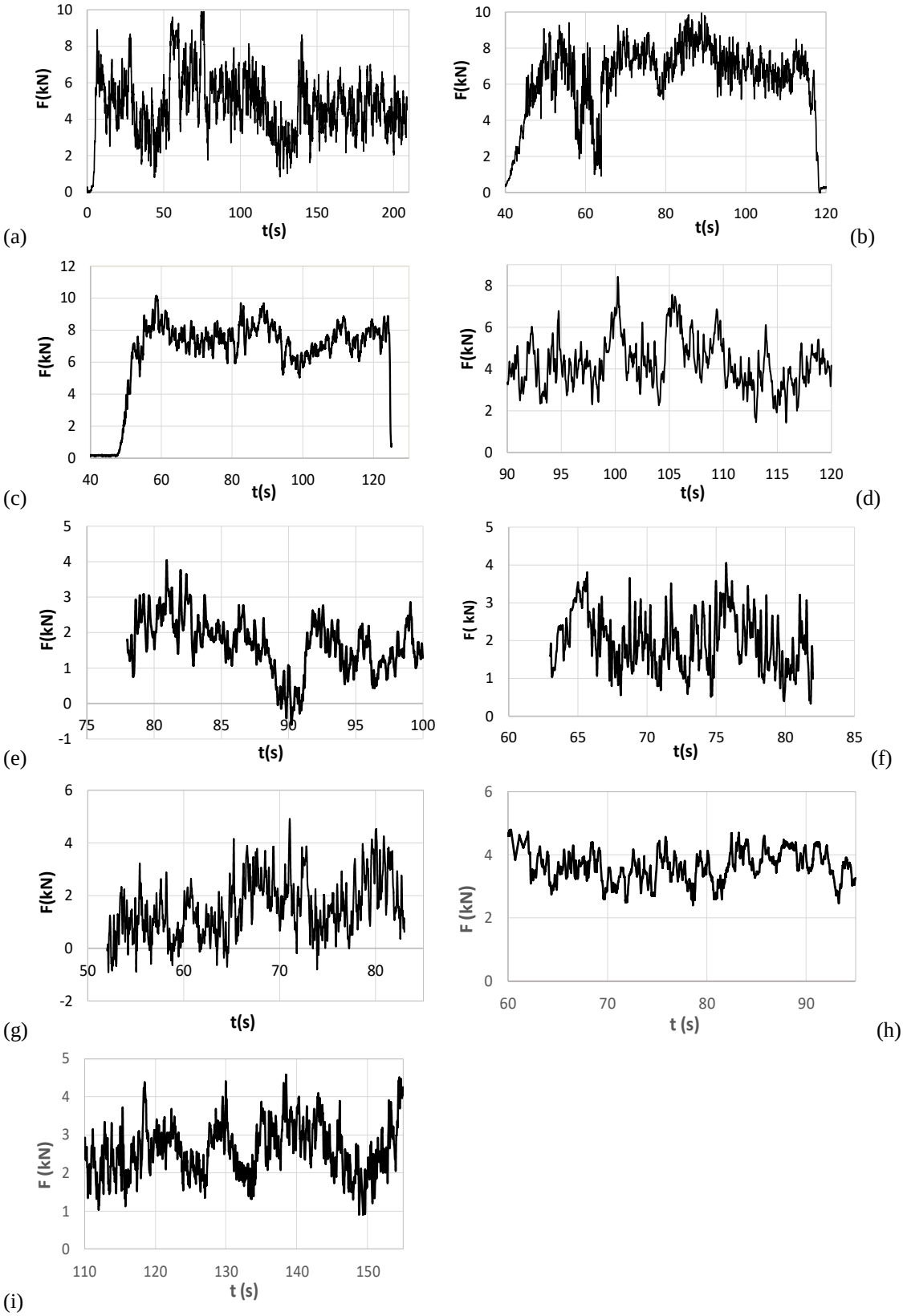

Figure 3- Sample of acquisition system data recorded for different depths and velocities. Sample measurement No.: (a) 1; (b) 2; (c) 3; (d) 4; (e) 5; (f) 6; (g) 7; (h) 8 and (i) 9, according to experimental plan listed in Table 1

Table 2- Statistical description of measurement in Table 1

Measurement	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Mean	4.8214	6.8400	7.5448	3.7706	2.5817	2.1914	1.7851	2.7027	3.6484
Standard error	0.0045	0.0068	0.0042	0.0063	0.0052	0.0044	0.0042	0.0048	0.0037
Median	4.6847	7.0145	7.5664	3.7729	2.3598	2.0921	1.6675	2.6573	3.6712
Mode	5.0570	6.4582	6.5603	4.2563	2.1485	1.7798	1.3039	1.1135	4.3490
Standard deviation	1.5632	1.4132	0.8679	1.9661	1.3629	1.2014	1.1045	0.9894	0.76806
Sample variance	2.4436	1.9973	0.7533	3.8659	1.8576	1.4434	1.2199	0.9789	0.58991
Kurtosis	0.3803	2.6026	0.0281	-0.723	0.6122	1.7088	-0.2205	-0.1901	-0.1686
Skewness	0.5081	-1.273	-0.025	0.1485	0.8008	0.6429	0.353	0.1411	-0.1615
Range	9.0881	9.0205	5.1049	10.378	9.7101	9.4947	6.737	5.2350	3.33323
Minimum	0.8081	0.9292	5.0466	-1.098	-0.948	-1.228	-1.5002	0.2399	1.86605
Maximum	9.8962	9.9498	10.151	8.8800	8.7619	8.2658	5.2369	5.4749	5.19929

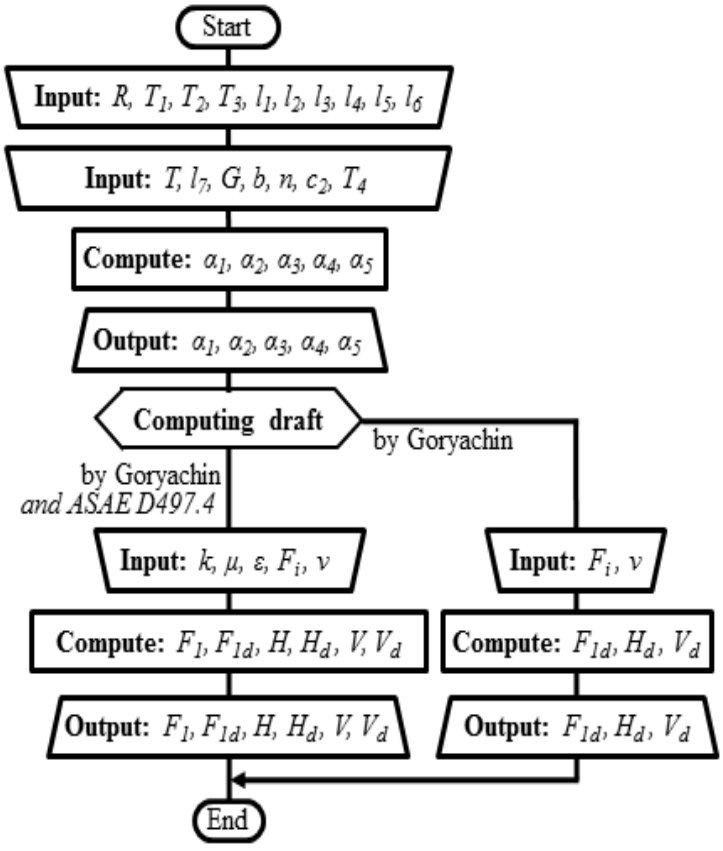

Figure 4- Block diagram representing the algorithm of the computer program

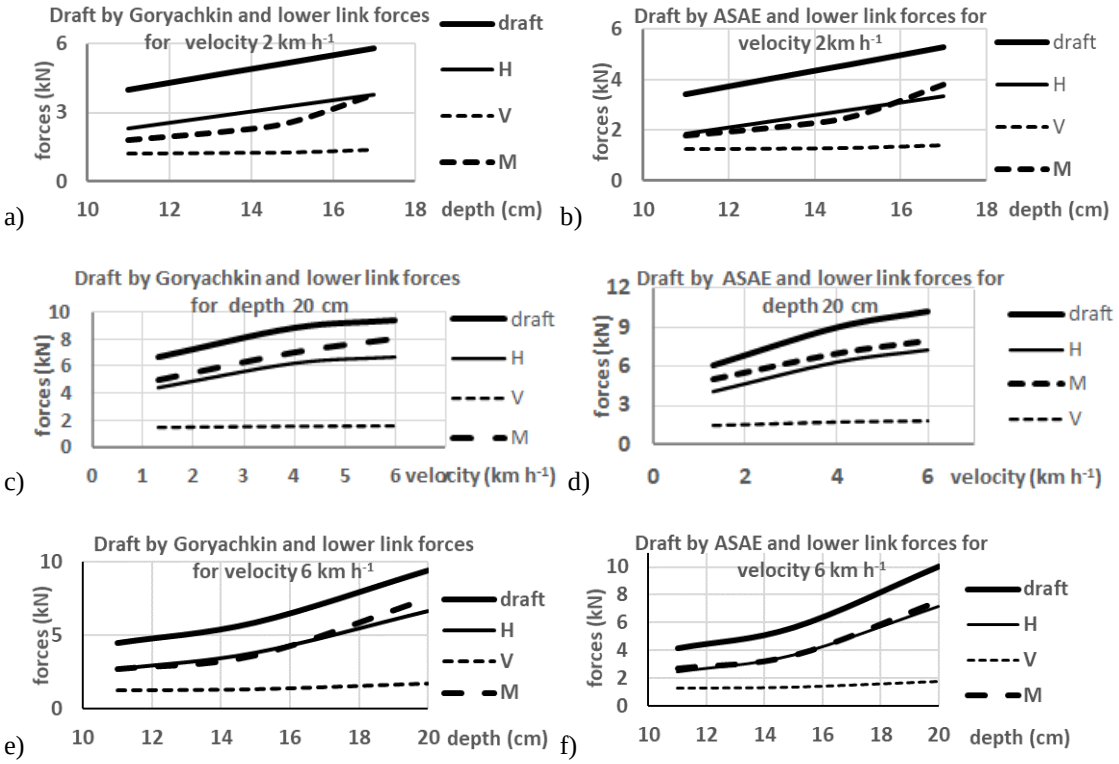

Figure 5- Graphical presentation of average values of calculated forces as dependent on the tillage depth and the tractor speed; a, change of Goryachkin draft, horizontal and vertical forces in lower link and measured forces with depth for tractor velocity 2 km h^{-1}; b, change of ASAE draft, horizontal and vertical forces in lower link and measured forces with depth for tractor velocity 2 km h^{-1}; c, change of Goryachkin draft, horizontal and vertical forces in lower link and measured forces with velocity for depth 20 cm; d, change of ASAE draft, horizontal and vertical forces in lower link and measured forces with velocity for depth 20 cm; e, change of Goryachkin draft, horizontal and vertical forces in lower link and measured forces with depth for tractor velocity 6 km h^{-1}; f, change of ASAE draft, horizontal and vertical forces in lower link and measured forces with depth for tractor velocity 6 km h^{-1}

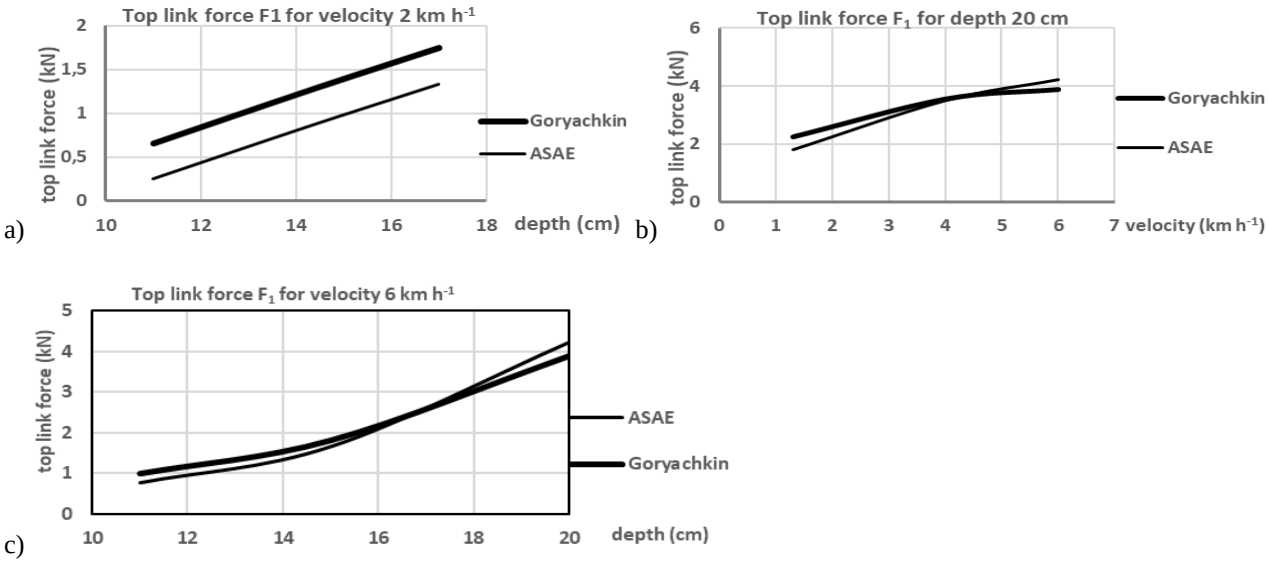

Figure 6- Graphical presentation of average values of calculated forces as dependent on the tillage depth and the tractor speed ; (a), change of top link force with depth for tractor velocity 2 km h^{-1}; (b), change of top link force with velocity for depth 20 cm; (c), change of top link force with depth for tractor velocity 6 km h^{-1}

4. Conclusions

The presented work discusses the theoretical results of analysis of the forces acting on the links of the three point hitch mechanism. Computer algorithm has been developed for the simulation of these forces. Formulated model comprehends calculation of draft force by two methods (ASAE D497.4 method and Goryachkin method) and, depending on the three point linkage geometry and mouldboard technical characteristics, calculation of forces acting on lower and upper links.

The formulated mechanical model of the THP forces, expressed by equations (3-7) and (9-11) was experimentally verified. The values of the horizontal forces in the lower link obtained by two analytical methods were compared with appropriate experimental values. The application of aproximate analytical method of ASAE D497.4 for determination of the draft force resulted in mean signed difference of 0.354 kN as it is presented in Table 3. The Goryachkin's approach of draft force evaluation generated mean signed difference of 0.449 kN. These difference can be reduced by introducing additional experimental data related to the soil properties and the mouldboard geometry in the Goryachkin's algorithm, or to apply some other more advanced method for determination of draft forces. Hence, this paper verifies the applicabillity of the analytical methods for crude estimation of the forces at the lower and upper links.

Table 3- Difference between measured and the calculated values for the *H* horizontal component of force acting on lower link hitch point

Measurement	1	2	3	4	5	6	7	8	9	*MSD* (kN)*
ASAE AD* (kN)	0.84	0.25	0.37	0.56	0.16	0.19	0.01	0.27	0.04	0.3544
Goryachkin AD* (kN)	0.29	0.69	0.85	0.02	0.7	0.73	0.52	0.01	0.23	0.4489

*AD, absolute difference; *MSD, mean signed difference

Resultes presented in this paper (Figures 5-6) also indicate a general trend of increasing the horizontal forces *H* and draft forces with plowing depth and tractor velocity. The analogue effects were evidenced with respect to the changes of top link forces F_1, with plowing depth and tractor velocity, Figure 6. Variations of the vertical lower link forces *V* were weak.

Abbreviations and Symbols	
$\alpha_1, \alpha_2, \alpha_3, \alpha_4, \alpha_5$	Link angles
a, T	Ploughing depth, m
A, B, C	Machine parameters
b, W	Width of ploughing tool, m
c_1, c_3	Coordinates of draft application, m
c_2	Coordinate of plough gravity center, m
ε	Coefficient of dynamic resistance, $Ns^2\ m^{-4}$
f	Friction coefficient
F_i	Parameter of soil texture
F_1, F_2	Top link force and lift link force, N
G	Plough weight, N
H, V	Horizontal and vertical lower link force component, N
k	Specific soil resistance, $N\ m^{-2}$
$l_1, l_2, l_3, l_4, l_5, l_6$	Three point linkage geometry, m
l_7	Lower link hitch point distance from the ground, m
n	Number of tools
R	Tractor rear-wheel radius
s, v	Tractor velocity, $m\ s^{-1}$

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The Effect of Nitrogen Deficiency on the Growth and Lipid Content of *Isochrysis affinis galbana* in Two Photobioreactor Systems (PBR): Tubular and Flat Panel

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ABSTRACT
Energy is becoming one of the most expensive production inputs nowadays. Energy reserves are starting to run out and their polluting nature has become undeniable. Therefore, there is an urgent necessity for renewable energies. One of these energy sources is algae, which are seen as promising for biofuel production. Algae can be cultured in non-agricultural land, high photosynthetic activity, harvested throughout the year high biomass production. High lipid from algae is possible by reducing some elements of growth conditions from the nutrient medium. In this study, *Isochrysis affinis galbana* species were cultured in two reactors; flat panel photobioreactors with different light paths (1, 3, 5, 7 and 10 cm) and tubular photobioreactors, with 50% nitrogen reduction and 20% inoculation densities. Biomass, lipid and protein ratios were determined. The highest lipid content of 33.13% was obtained from *I. aff. galbana* with 12.11% protein in flat panel photobioreactors with 50% nitrogen reduction and 10 cm light path, and a 0.991 g L^{-1} biomass rate was obtained. The highest optical density was found in the 10 cm light path flat panel photobioreactor with a 50% nitrogen reduction.

Keywords: *Isochrysis affinis galbana;* Photobioreactor; Lipid; N deficiency

1. Introduction

This study was conducted in order to determine the effects of N restriction on growth, lipid, protein, and chlorophyll contents in *Isochrysis affinis galbana* species of Prymnesiophyceae class and to produce renewable, non-toxic biofuels from microalgae.

Increasing interest in microalgae biotechnology in recent years is due to their high amounts of valuable bioactive metabolites (Becker 1994). In recent years, fat and fatty acid products obtained from single-celled algae have attracted considerable attention. Initially, the 'Solar Energy Research Institute' focused on the use of algal lipids as biofuels (Neenan et al 1986). Microalgae are potential sources of biodiesel with a lipid content of 20% to 50, even 80% (Chisti 2007). Efforts are underway to exploit the use of renewable, non-toxic, biodiesel fuel from microalgae as an energy source. For this purpose, in addition to the identification of microalgae species with high lipid content and growth rate, studies on determination of serious stress conditions such as N limitation, P deficiency, Si deprivation, high salinity etc. have begun (Lynn et al 2000; Zhila et al 2005; Mandal & Mallick 2009; Zhila et al 2011). Those stress conditions stimulate the increase of lipid content in the cell are being evaluated in many countries.

The purpose of the present study was to investigate the effect of N on the growth and lipid content of *I. aff. galbana* in two photobioreactor systems (PBR): tubular and flat panel. In addition, we investigated the maximum lipid and

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biomass in order to determine which PBRs are more suitable for culture of *I. aff. galbana* for biofuels production purpose.

2. Material and Methods

2.1. Algae and culture conditions

The microalgae *I. aff. galbana* UTEX LB 2307, was supplied by the University of Texas at Austin Collection, which is a single-celled marine species with two whips, with no haptonema and a single yellow-brown chloroplast. The cell size is 4-8 μm (Hoff 1987). The inoculum for the tubular and flat panel PBRs were grown under laboratory conditions on F/2 medium (Guillard et al 1973). Cells were cultured under a constant light intensity of 80 μmol photonm^{-2} s^{-1} at 20 °C were used. The irradiation was measured using the Radiation Sensor LI-COR (LI-250, Inc. USA). The microalgae stock culture was cultured in an 8 L glass jar and air was continuously supplied. The volume of the tubular photobioreactor system is 110 L. The tubular photobioreactor system was a horizontally installed reactor made of transparent acrylic tubes with an inner diameter of 2.6 cm. In order to keep the craw flow rate constant, a flow rate of the circulation pump was set to 0.3 m s^{-1}. A collection tank of about 150 L was built for the culture collection chamber. CO_2 gas inlet flowmeter was provided. pH and temperature were measured continuously by probes. Outdoor flat panel PBRs were 10 mm thick transparent glass material, 50.0 cm wide and 50.0 cm high with 1, 3, 5, 7 and 10 cm light paths (Hu et al 1996a) in batch systems. The volumes of the PBR (without bubbling gas) was 2 L in 1 cm, 6 L in 3 cm, 10 L in 5 cm, 15 L in 7 cm and 21 L in 10 cm. The culture mixture was provided with 2% CO_2 enriched air as described by Hu et al (1996b). pH was arranged with a pH controller as 7 and light intensity was measured 3 times a day.

In the experiment, *I. aff. galbana* was cultured in different flat panel and tubular PBRs. Experimental work is carried out on 50% N deficiency according to F/2 medium and with inoculation densities of 20%. All the applications were made in three replicates. The experiments were completed on different days and indicated in the tables.

2.2. Analytical methods

Chlorophyll *a*, total carotenoids, dry weight (biomass) and optical density (OD) analysis were performed daily. The dry weight was determined according to Hu & Richmond (1994). The *I. aff. galbana* cell concentration was determined daily by measuring the optical density at 680 nm (Lin et al 2007), by a UV-visible spectrophotometer (Shimadzu, UV mini, 1240 model, Japan). Chlorophyll *a* and total carotenoids contents were determined on a spectrophotometer at 665, 645, 630 and 480 nm as described by Parsons & Strickland (1963). All measurements were made in three replicates.

Microalgae were harvested for lipid and protein analysis in the stationary growth phase. *I. aff. galbana* cells were separated by centrifugation at 7500 rpm for 10 min, using the centrifuge model of Hereaus Supragufe 22. However, the biomass was dried at 55 °C for 2 hours, triturated with a grinder and then stored at -20 °C for analysis. Lipid extraction from microalgae cells was performed by the method described by Bligh & Dyer (1959). The total protein was calculated by the determination of N content (Nx6.25) according to Kjeldahl method (AOAC 1995).

2.3. Statistical analysis

The data were subjected to a one-way analysis of variance and Duncan's multiple range test was used as a post-hoc test. Statistical Package for the Social Sciences (SPSS) (Version 12.0, SPSS, Chicago, IL) (Zar 1999) was adapted to a personal computer. The differences were considered at a significance level of $\alpha = 0.05$.

3. Results and Discussion

The measured temperatures were between 21.7 ºC and 24.7 ºC and light intensities were recorded between 224 μmol photonm^{-2} s^{-1} and 284 μmol photonm^{-2} s^{-1} in culture at different light path lengths in flat PBR systems. In tubular photobioreactor systems, the temperature was determined between 21.6 ºC and 22.3 ºC and the light intensity between 281 μmol photonm^{-2} s^{-1} and 286 μmol photonm^{-2} s^{-1}.

The biomass, protein, and lipid contents of *I. aff. galbana* were presented in Table 1 and Table 2. In the control group and N deficiency group, the highest biomass was determined as 1.068 and 0.991 g L^{-1}, respectively, in flat panel PBR with 10 cm light path. The highest amount of lipid was detected in the flat panel PBR with 10 cm light path where the highest biomass was obtained. The highest lipid in the control group was 15.19%, while the N deficiency group was

33.13%. The lowest amount of protein was obtained with 12.11% in the flat panel PBR with 10 cm light path where the highest lipid was obtained. The protein and total lipid ratios were similar in all control groups (P>0.05). In N deficient cultures, the lowest biomass and lowest lipid amount was obtained in the flat panel PBR with 1 cm and 3 cm light path.

Table 1- Main parameters of biomass, lipid and protein content of *I. aff. galbana* at control groups in flat panel PBRs and tubular PBR

Reactors	*Biomass (g L^{-1})*	*Protein (%)*	*Total lipid (%)*
Tubular PBR (16 days)	0.902±0.02^{c}	28.65±10^{a}	15.09±0.70^{a}
FP-PBRs 1 cm light path (15 days)	0.801±0.02^{d}	28.67±0.60^{a}	15.31±0.30^{a}
FP-PBRs 3 cm light path (16 days)	0.813±0.03^{d}	28.80±0.50^{a}	14.98±0.50^{a}
FP-PBRs 5 cm light path (16 days)	0.893±0.02^{c}	28.83±0.20^{a}	15.07±0.60^{a}
FP-PBRs 7 cm light path (18 days)	0.958±0.01^{b}	28.65±0.60^{a}	15.12±0.60^{a}
FP-PBRs 10 cm light path (19 days)	1.068±0.02^{a}	28.80±0.50^{a}	15.19±0.70^{a}

Means values, n= 3, different letters between the lines indicate significant difference at 5% by Duncan multiple range test

Table 2- Main parameters of biomass, lipid and protein content of *I. aff. galbana* at N deficiency groups in FP-PBRs and tubular PBR

Reactors	*Biomass (g L^{-1})*	*Protein (%)*	*Total lipid (%)*
Tubular PBR (12 days)	0.875±0.03^{bc}	13.50±0.70^{b}	32.10±0.40^{b}
FP-PBRs 1 cm light path (11 days)	0.772±0.01^{d}	15.05±0.50^{a}	30.01±0.10^{c}
FP-PBRs 3 cm light path (13 days)	0.785±0.01^{d}	15.01±0.40^{a}	30.16±0.30^{c}
FP-PBRs 5 cm light path (13 days)	0.832±0.02^{c}	13.07±0.20^{b}	32.09±0.80^{b}
FP-PBRs 7 cm light path (14 days)	0.913±0.01^{b}	13.02±0.40^{b}	32.18±0.50^{ab}
FP-PBRs 10 cm light path (15 days)	0.991±0.02^{a}	12.11±0.30^{c}	33.13±0.80^{a}

Means values, n= 3; different letters between the lines indicate significant difference at 5% by Duncan multiple range test.

Optical density, chl *a* and total carotenoid amounts in the control and N deficiency groups were summarized in Table 3 and Table 4.

Table 3- Main parameters of optical density, chl *a* and total carotenoid amounts of *I. aff. galbana* at control groups in flat panel PBRs and tubular PBR

Reactors	*OD*	*Chl a (μg L^{-1})*	*Total carotenoid (μg L^{-1})*
Tubular PBR (16 days)	0.487±0.001^{c}	489±2^{a}	0.626±0.001^{a}
FP-PBRs 1 cm light path (15 days)	0.457±0.010^{d}	358±5^{c}	0.408±0.002^{d}
FP-PBRs 3 cm light path (16 days)	0.497±0.020^{c}	361±2^{c}	0.419±0.001^{d}
FP-PBRs 5 cm light path (16 days)	0.568±0.001^{b}	422±6^{b}	0.459±0.002^{b}
FP-PBRs 7 cm light path (18 days)	0.625±0.002^{a}	484±9^{a}	0.438±0.001^{c}
FP-PBRs 10 cm light path (19 days)	0.588±0.010^{b}	501±10^{a}	0.467±0.001^{b}

Means values, n= 3; different letters between the lines indicate significant difference at 5% by Duncan multiple range test.

Table 4- Main parameters of optical density, chl *a* and total carotenoid amounts of *I. aff. galbana* at N deficiency groups in flat panel PBRs and tubular PBR

Reactors	*OD*	*Chl a (μg L^{-1})*	*Total carotenoid (μg L^{-1})*
Tubular PBR (12 days)	0.398±0.002^{c}	180±3^{a}	0.810±0.001^{a}
FP-PBRs 1 cm light path (11 days)	0.301±0.001^{d}	130±1^{b}	0.479±0.002^{c}
FP-PBRs 3 cm light path (13 days)	0.437±0.003^{b}	130±1^{b}	0.476±0.001^{c}
FP-PBRs 5 cm light path (13 days)	0.441±0.001^{b}	172±1^{a}	0.543±0.002^{b}
FP-PBRs 7 cm light path (14 days)	0.453±0.001^{a}	130±2^{b}	0.534±0.001^{b}
FP-PBRs 10 cm light path (15 days)	0.458±0.001^{a}	125±1^{b}	0.546±0.001^{b}

Means values, n= 3; different letters between the lines indicate significant difference at 5% by Duncan multiple range test

Initial optical density values were 0.263 for all groups. At the end of the experiment, the highest optical density value was be 0.625 in the flat panel PBR with 7 cm light path in the control group. The highest optical density was 0.458±0.001 in the flat panel PBR with 10 cm light path in the N deficiency group. The minimum optical density was determined in the flat panel PBR with a 1 cm light path as 0.457±0.01 and 0.301±0.001 the control group and N deficiency, respectively.

The initial chl *a* and total carotenoid were determined as 245±2 µg L^{-1} and 0.242±0.001 µg L^{-1} in the control group and in the N deficiency group, respectively. At the end of the experiment, the lowest chl *a* was detected in the N deficient groups in flat panel PBRs with 1, 3, 7 and 10 cm light path. But the highest total carotene was measured in the tubular PBR with 0.810 µg L^{-1} in the N deficient group. The lowest carotene in the group with N deficiency was determined in the flat panel PBR with 1 cm light path.

The primary objective of producing phototrophic organisms is to provide a continuous culture with optimal cell density. Algae requires that cultured cells constantly react to these conditions, as the various environmental factors during the cultivation of an algae show great changes both daily and seasonally. The biochemical composition of algal biomass is affected by environmental factors. The most important of these are growth conditions such as light, temperature, nutrient medium, salinity and pH (Brown et al 1989; Roessler 1990; Sukenik 1991; Cohen et al 1988; Lourenço et al 2002). Nutrient elements and their concentrations used in culture media with physical conditions may cause changes in microalgae growth and their biochemical structure. Growth affects concentrates as well as the nutrient content used in media (Brown et al 1989). Nitrogen (N) limitation has been shown to cause changes in the biochemical structure of many algal groups. Especially the increase in the amount of lipids (Illman et al 2000). For this purpose, in this study, N concentration in F/2 medium was reduced to fifty percent. It is known that different N levels can affect the biochemical composition and growth of microalgae (Fidalgo et al 1995; Xu et al 2001).

Microalgal biomass is very important for the study of lipid, and lipid is the main objective of increasing both simultaneously biomass. In this study, the reduction in the amount of N in the medium caused an increase in the amount of lipids in the cell and a decrease in the amount of protein. The highest biomass in the study was determined to be 0.991 g^{-1} and the highest lipid was 33.13% with a 50% N deficiency in the flat panel PBR with 10 cm light path. However, the lowest amount of protein was found in this group. In another study, it was determined that low N concentration decreased the growth rate in *N. oculata* and did not affect growth rate in *C. vulgaris* (Converti et al 2009). In a research conducted by Xu et al (2001), *Ellipsoidion* sp. was cultured in different N sources and in a N free medium, the growth in the N free medium was low. Adenan et al (2016) reported that lipid ratio is increased in *Chlorella* and *Chaetoceros* species cultured with N deficiency applied to culture, but growth with protein and carbohydrate amounts decreases. The amount of biomass obtained due to the lipid content of the algae is also important. Biomass is generally reduced in cultures where N deficiency is applied. Thomas et al (1984) reported that *P. tricornutum* cultured in medium containing N and in the medium where N deficiencies were applied, and that the biomass amount was low in the nutrient medium where N deficiency was applied. Similar studies have suggested that the N restriction is responsible for the decrease in cell density and the decrease in biomass quantities in species (Kilham et al 1997; Pruvost et al 2009). In this study, the lowest biomass amount in *I. aff. galbana* was determined as 0.772 g L^{-1} in the flat panel PBR with 1 cm light pathway, in the 50% N (-) group with 30.01% lipid. N and P are the most essential elements for cell growth. These two elements are involved in the synthesis of intracellular structure. Therefore, cell growth instead of lipid synthesis will be dominant when these nutrients are present. Nitrogen deficiency limits algal growth and protein synthesis, resulting in increased lipid content (Converti et al 2009). In similar studies it was reported that the N limitation caused increases lipid; *D. tertiolecta* contained maximum total lipid in low N containing medium conditions (Fábregas et al 1989); *P. tricornutum* accumulates a high amount of total lipid in N deficiency (Thomas et al 1984); when the amount of N in *I. galbana* was increased from 0.04 mmol L^{-1} to 0.7 mmol L^{-1}, the total lipid content decreased from 22% to 16.9% (Utting 1985). Tornabene et al (1983) reported that freshwater algae *N. oloeabundas* was cultured in growth medium with N deficiency at different rates, and the total fat percentage in N deficient groups varied between 35-54% by dry weight. Ben-Amotz et al (1984) reported that different algal species were cultured in a nutrient-deprived medium and showed significant increases in total lipid content in all species. Sukenik & Wahnon (1991) reported that when *I. aff. galbana* was cultured in a N deprived medium, both carbohydrate and total lipid ratios increase. Zhila et al (2005) reported that *B. braunii* was cultured in 75% N reduced medium and reported a 21% increase in total lipid. Weldy & Huesemann (2007) reported that *D. salina* was cultured in a photobioreactor system with N deficiency and observed the change in total lipid ratio, which increased from16% at the beginning of the stagnation phase to 44%. In the study conducted by Rodolfi et al (2009), studied the dry matter and total lipid ratio of 4 species (2 marine and 2 freshwater species) by culturing them in a 20 L flat alveolar panel reactor with N deficiency. In the marine species, *Nannochloropsis* sp. a total lipid content of 60% was determined in N deprived medium. Gouveia et al (2009) found

that the maximum total lipid ratio in *N. oleabundans* species was 56% when N restriction was applied for 6 days. Damiani Cecilia et al (2010) reported that when they cultured *H. pluvialis* under different stress conditions (high light and high light-nitrogen deficiency), the total lipid ratio increased from 15% to 32.99% in N deficiency and high light intensity. Bulut Mutlu et al (2011), in their study, cultured *C. vulgaris* for five in different nutrient media under laboratory conditions, they found that the highest total lipid content was 35.6% in the group with 100% N excision. In a study conducted by Uslu et al (2011), *S. platensis* increased total lipid (17.05%) when cultured in a 100% N starvation medium. Uslu et al (2013), also investigated *C. vulgaris* in tubular photobioreactors in a 50% reduced N medium; lipid ratios were 12.34% and 38.16% for the control and 50% N limitation groups, respectively. In another research Uslu et al (2014) cultivated *P. tricornutum* with different light path lengths of 1, 3, 5, 7 and 10 cm and with a deficiency of 50% N in order to determine lipid, protein and biomass contents. The highest lipid, protein and biomass of *P. tricornutum* was 34.6%, 8.50% and 1.064 g L^{-1}, respectively, in the flat panel PBR system with 7 cm light path. Ak et al (2015) studied *P. tricornutum* tubular reactor systems and in medium containing 50% N, and found 35.04% lipid, 0.980±0.02 g L^{-1} biomass and 8.87% protein ratios. whereas 16.93% lipid, 1.036±0.025 g L^{-1} biomass and 31.05% protein were detected in the control group. Kamalanathan et al (2016) observed physiological changes on *Chlamydomonas* cultured in N and phosphorus (P) deficiency. They have reported that the physiology of *Chlamydomonas reinhardtii*, with N starvation and also with N plus P starvation combined, shows a deeper influence on the P starvation. At the same time, the photosynthetic performance of *C. reinhardtii* showed major changes under N starvation but was comparatively unchanged by P starvation. The lipid concentration per cell was at least 2.4 times higher in the N deficient groups than in the control group, however, the amount of protein is lower in groups with N deficiency. In general, N deficiency has a more dramatic effect on *C. reinhardtii*'s physiology and lipids and protein levels than P deficiency.

In this study, similarly, the N limitation reduces the growth rate, which increases the cellular lipid rate. However, the amounts of biomass were not very low in groups with N deficiency. What is important is that the biomass ratio can be obtained at reasonable levels so that the increased lipid content can be economically assessed.

The algae biomass is affected by many parameters including the nutrient medium used for the culture of algae, the surface area and material of the system used, and the path taken by the light in the water column. Light is an important parameter especially in algal cultures. The angle at which the photobioreactor receives the light is important, in fact the surface area of the material plays a significant role in the efficiency to get enough light for the algal culture. According to Zijffers et al (2008), it is important for the reactor to take sunlight in the most efficient way. They generally recommend the use of flat and rectangular systems or linear and cylindrical shaped systems for good light energy distribution. Linear and cylindrical systems can be effective in focalizing light (Richmond 1986; Zou & Richmond 1999; Durmaz & Erbil 2017). In the study of Durmaz (2000), he investigated the growth of *Chlorella* sp. in natural light/dark periods outside the laboratory in reactors with different light path lengths (10 cm, 15 cm and 20 cm). *Chlorella* sp. reached the highest concentration of cells density of $49.5x10^6$ mL^{-1} in the photobioreactors with 20 cm light path. While, the highest cells density was $49x10^6$ cells mL^{-1} in the panels with 15 cm and $36.5x10^6$ cells mL^{-1} in the panels of 10 cm. In his study, it was stated that the length of light path should be adjusted according to the type of culture. It was determined that panels with 15 cm and 20 cm light paths have a high specific growth rate. It is emphasized that the sunlight intensity and duration is important in microalgae culture which is made in glass panels with different light path lengths and it is stated that the appropriate light path length for the species is important for efficiency. In this study, it was also determined that the growth increased as light path length increased.

In a study investigating the effects of N restriction on metabolites in microalgae cultures, an increase in the proportion of organic carbon compounds such as lipid was observed, while a decrease in cell number and chl *a* was detected. However, yellowing of colors was observed in cultures due to increased carotene content (Shifrin & Chisholm 1981; Sukenik et al 1989). Marín et al (1998) investigated the effects of *D. salina* on different N rates on carotene and chl *a* amount. As the N deficiency increased, total carotene and chl *a* decreased. In this study, chlorophyll content decreased in 50% N (-) cultures and increases in total carotene content occurred in *I. aff. galbana*. The highest OD values in *I. aff. galbana* was obtained in the control group and in the panel system with 7 cm light path. The lowest OD value was obtained in the group of 50% N (-) in the tubular photobioreactor system in *I. aff. galbana*. This provides us with the result that the optical density is lower in the N deficiency groups, as in other parameters.

4. Conclusions

As a result, microalgae biomass can be used as an alternative source for the production of renewable energy. The studies on microalgae lipid production are mostly carried out under laboratory conditions. It is important to ensure the commercial production of microalgae. However, it is also significant to reduce the cost of culture to produce lipids.

Finally, the results obtained in this study have shown that the lipid content of *I. aff. galbana* may be a good source of biodiesel. It would be more appropriate to use algae which can be cultured in nonagricultural lands for year-round instead of production of oil crops in agricultural lands as energy source.

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Investigations on the Use of Kairomone and Pheromone Attractants for Control of *Thrips* Species (Thysanoptera: Thripidae) by Mass-trapping in Nectarine Orchards

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ABSTRACT
Thrips species (Thysanoptera: Thripidae) cause superficial, brown colored scars and discoloration called silvering on the surface of nectarine fruit which reduce market quality. In cases of high thrips populations, cracking and splitting of the fruit in accompany to superficial damage causes the fruit to be discarded. The study which was based on non-chemical control of thrips species was conducted in a commercial nectarine orchard in Tarsus county of Mersin province in 2013 and 2014. In the study, pest management effect of mass trapping by yellow colored sticky traps baited with semiochemical-kairomone and pheromone-were detected. Each semiochemical was tested in a particular plot. One baited trap per tree was hung at both kairomone and pheromone plots and one bait-free trap per tree at the control plot. The traps were all hung at the pink bud period and were recovered after harvest. The amount of thrips adult and larvae in the flower buds were checked 4-7 day intervals and the traps were checked weekly to count the individuals caught on traps. The results showed that the lowest number of thrips was detected in nectarine flowers in the kairomone plot and kairomone-baited traps captured the highest number of thrips adults. To the contrary, flowers taken from the control plot had the highest number of thrips and control traps captured the lowest number of thrips adults. The success of mass trapping by adding semiochemicals to sticky traps was evaluated by comparing fruit damage in baited and unbaited trap plots by observing 100 fruits on each of five replicate trees for 500 fruit total at each plot before harvest. The fruit damage was 9.0% and 9.8% in the kairomone plot and was 11.2% and 18.2% in the pheromone plot while it was 23.4% and 20.0% in the control plot in years 2013 and 2014 respectively. Mass trapping by baited traps for thrips control in nectarine orchards seems to be encouraging for integrated pest management especially when considering the easily occurring pesticide resistance of thrips species.

Keywords: Nectarine; Thrips control; Semiochemical; Lurem-tr; Thripline-ams

1. Introduction

The East Mediterranean Region of Turkey has a large potential of nectarine production for both domestic and external markets with 42226 tons of yearly production (Anonymous 2018). Numerous pest species like Thrips (Thysanoptera: Thripidae), Peach twig borer-*Anarsia lineatella* Zeller and Oriental peach moth-*Cydia molesta* (Busck.) (Lepidoptera: Gelechiidae), Aphids (Hemiptera: Aphididae), White peach scale-*Pseudaulacaspis pentagona* (Targ.-Tozz.) (Hemiptera: Diaspididae) put a strain on nectarine growing. Thrips species are considered a serious pest of nectarine. Thrips adults and larvae feed on nectarine flower organs, ovarium and fruitlet which cause brown scars on the fruit surface and also cause deformation of fruit (Gonzales et al 1994; Pearsall 2000; Pearsall & Myers 2000; Tommasini et al 2004; Sengonca et al 2006; Hazır & Ulusoy 2008; Atakan 2008). Thrips feeding on matured fruit causes discoloration damage called silvering (Gonzales et al 1994; Atakan 2008). Hazır et al (2011) notified that 3 flower thrips species among 12-*Frankliniella occidentalis* (Pergande), *Thrips tabaci* Lindemann and *Thrips major* Uzel (Thysanoptera: Thripidae)

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were present in high numbers in nectarine flowers that cause scarred fruit in Mersin (Tarsus county) province of Turkey. According to the study of Tommasini et al (2004) the silvering damage was caused by *T. major, T. tabaci* and *F. occidentalis* on nectarine fruit in Nothern Italy. Similarly, according to Hazır & Ulusoy (2008), *F. occidentalis* and *T.major* caused silvering damage in Mersin, Turkey. Both types of damage are not desired by growers and consumers because of the decrease in value in domestic fruit marketing and during exportation. *F. occidentalis* is listed on the quarantine list of many countries including Turkey and regulated as a quarantine pest. Thrips management depends mostly on insecticide applications but the effect of insecticides is low due to some characteristics of thrips species such as the eggs are laid in the plant tissue, adults and nymphs are feeding in the interior parts of the flower and the pupa stage undergo in the soil.

As of 2011 there were no chemicals licensed against thrips on nectarines in Turkey. Some of the growers were using some pesticides *licensed* for *thrips* in other crops *like grape and strawberry* while some other growers were using any kind of pesticides that were licensed for nectarine pests other than thrips. Economic losses were being reported by technical experts because of unsuitable chemicals and ineffective control strategies (unpublished reports). Besides, thrips resistance to insecticides was a known fact (Herron & James 2007; Darnell-Crumpton et al 2018). This study was planned under these conditions to offer a control method as an alternative to chemical control. Effect of using sticky traps baited with pheromone and kairomone was tested for the purpose of breaking up the thrips populations in nectarine orchards in this study.

Adding pheromone and kairomone attractants to sticky traps are licensed for monitoring the thrips populations on protected crops in greenhouses in many countries. The attractants are slowly released from the dispenser and makes adult thrips more active. Adults appear from their shelters and are more attracted to sticky traps based on visual stimulant (color traps) and odor stimulant (pheromone or kairomone) (Anonymous 2019b and c). We planned to benefit this feature of attractants in thrips management in nectarine orchards by having thrips adults landing on traps instead of nectarine flowers and in this way decreasing fruit damage caused by thrips feeding. Kirk (2009) notified that aggregation pheromone of *F. occidentalis* increases capture of both male and female thrips on traps and were useful for early detection of thrips. Broughton (2009) tested monitoring efficacy of traps baited with Thripline-ams (*F. occidentalis* pheromone) and Lurem-Tr (kairomone) in a study in New Zealand nectarine orchards. The researcher notified that more thrips were captured on the traps combined with lures when compared with no-lure traps. Many other researchers tested and compared the capture capacity of baited and unbaited traps for the purpose of monitoring thrips adults earlier (Teulon et al 2008; Broughton & Harrison 2012; Sampson & Kirk 2013; Muvea et al 2014; Abdullah et al 2015) A few number of researchers tested the semiochemical baited traps for controlling thrips populations. Harbi et al (2013) detected that thrips populations in pepper greenhouse and Broughton et al (2015) notified that thrips in rose greenhouses can be successfully controlled by using sticky traps baited with semiochemicals. Within our study, the efficacy of mass-trapping by adding attractant (pheromone and kairomone) to yellow colored sticky traps was investigated in a nectarine orchard as a pest management strategy alternative to insecticide applications.

2. Material and Methods

2.1. Study site

Studies were conducted in a nectarine orchard located in Yenice/Tarsus county of Mersin province in East Mediterranean Region of Turkey in 2013 and 2014 during the nectarine growing season between pink bud and harvest period. The trial orchard was established with 'Early Sprite' nectarine cultivar having 4x5 m row spacing. The trees were 6 years old. A drip irrigation system was installed in the orchard. No insecticide had been applied in the study site and there weren't any weed species on the orchard base because of continued weed control by mowing.

2.2. Traps and semiochemicals

The sticky traps were yellow in color, self-adhesive, 10x25 cm in size, commercially supplied from Russell IPM (Anonymous 2019a). Traps were baited with two kinds of semiochemical attractants-pheromone and kairomone. Aggregation pheromone of *F. occidentalis* is composed of neryl (**S**)-2-methylbutanoate. Its' commercial name is Thripline-ams, producer company is Syngenta (Anonymous 2019b). Kairomone is an allelochemical attracting various thrips species like, *F. occidentalis, T. tabaci, T. major* and some other species. It is composed of plant volatiles derived from host plants, commercial name is Lurem-Tr, producer company is Koppert (Anonymous 2019c). The trial consisted of three characters [1.Mass-trapping by using yellow sticky traps baited with pheromone-Thripline-ams (PMT); 2.Mass-

trapping by using yellow sticky traps baited with kairomone-Lurem Tr (KMT); 3.Mass-trapping by using attractant free yellow sticky traps-Control (CMT)]. All characters were replicated five times in a randomised block design. Each plot (block) consisted of 30 trees. Plots were seperated, having 50 meters between each other.

2.3. Method

The orchard was divided into 3 plots-kairomone (KMT), pheromone (PMT) and control (CMT). Five trees located in the middle of every trial plot were determined as replicates and 3 types of counts were conducted on these five trees: 1- Amount of thrips adult and larvae existed in the flower samples, 2- Amount of thrips adults caught on the traps, 3- Damage rates on the fruits.

2.3.1. Amount of thrips adults and larvae in nectarine flowers

One hundred randomly selected flower buds (20 buds from each of five replicate trees) were picked every 6-7 days during pink bud-petal fall period at each plot and brought into the laboratory in paper bags. A piece of cotton treated with ethyl acetate was placed in each paper bag to immobilize any thrips present in the flowers. Flower samples were knocked into a white plastic container to get the thrips inside. The hidden thrips were detected by tearing the flowers into pieces. The amount of thrips adult and larvae were recorded, then adults were saved in vials containing 8 parts 60% ethyl alcohol+1 part acetic acid+1 part glycerin for later identification (Hazır & Ulusoy 2012).

2.3.2. Amount of Thrips adults captured on traps

2.3.2.1. Mass trapping trial using yellow coloured sticky traps baited with Thripline-ams pheromone capsule

This trial was conducted in a plot consisted of 30 trees (PMT). A buffer zone of 15-20 meters was constituted by placing traps to the trees adjacent to the trial plot to prevent external infestations. One Thripline-ams pheromone capsule was attached to each yellow colored sticky trap. One baited trap per nectarine tree was placed at pink bud period on 29.01.2013 and 30.01.2014 at 1.5-2.0 m height towards the southwest-predominant wind direction. Pheromone lures were replaced with fresh ones monthly and the sticky traps on five sample trees (sample (replicate) trees were selected for counts) were replaced with clean ones weekly. The rest of the traps were replaced with clean ones when needed. Five traps/plot on the sample trees were brought to the laboratory weekly. The amount of thrips adults captured on traps was counted weekly in the laboratory under binocular microscope until harvest. Average amount of thrips per trap was calculated and recorded.

2.3.2.2. Mass trapping trial using yellow coloured sticky traps baited with Lurem-TR kairomone attractant

The method used in pheromone mass-trapping (PMT) plot was also used for kairomone mass-trapping (KMT) plot by hanging 1 Kairomone (Lurem-Tr) baited trap on each of the 30 trees on 30.01.2014. All counts were done in the same way as in the PMT plot.

2.3.2.3. Mass trapping trial using unbaited yellow coloured sticky traps

In the control plot (CMT), one unbaited trap was hanged on each tree. Thrips adults captured on traps on sample trees were counted weekly.

2.3.3. Evaluation of damage rate

Damage rate was determined by monitoring 500 matured fruits (100 fruits/tree x 5 trees). Fruits located in the 4 particular directions and in the center of each sample tree were randomly selected and monitored/inspected for thrips injury (russetting/silvering) a week before harvest on 25.04.2013 and 29.04.2014. Damage rate was classified by 0-3 scale (Atakan 2008). According to the scale, scar areas smaller than 0.5 cm² were called light damage (scale 1), scar areas between 0.5-2.0 cm² were called medium damage (scale 2) and larger than 2.0 cm² were called severe damage (scale 3).

Statistical analysis was performed by using SPSS statistics package software. Variance analysis was performed after logarithmic and arcsin transformation was done to the data of the trial and the difference between treatments was evaluated by using the Tukey multiple comparison test. T-test was used for paired comparisons.

3. Results and Discussion

3.1. Amount of thrips adults and larvae in nectarine flowers

The flowering period started on the last days of January in both years. Amount of thrips in the flower samples in 2013 and 2014 is shown in Table 1. As seen in the table, the highest number of thrips adult/larvae in the flower samples at petal fall in both years were obtained from the CMT plot where unbaited traps were used (Table 1). The lowest number of thrips were detected in the flower buds taken from KMT plot at petal fall in both years. The odor of semiochemicals activate thrips adults and direct them towards an object like colored sticky traps that attract attention. According to previous studies in the world, adding semiochemicals (pheromone or kairomone) to sticky traps attracts thrips adults to traps (Broughton 2009; Kirk 2009; Anonymous 2019b; c). While capture of thrips adults on traps increases, the amount of thrips landing on and feed in flower buds reduces. This phenomena results in less fruit damage. Results of two trial years showed that mass trapping by using baited traps reduced thrips existence in flowers when compared with control. Amount of thrips in flowers were the lowest in the KMT plot in both years which lead to the lowest fruit damage at harvest. The highest damage was noted in the CMT plot where the flower samples consisted of the highest amount of thrips.

Table 1- Amount of thrips adults and larvae in nectarine flowers in 2013 and 2014

Sampling time	*2013*			*2014*		
	Kairomone (KMT)	*Pheromone (PMT)*	*Control (CMT)*	*Kairomone (KMT)*	*Pheromone (PMT)*	*Control (CMT)*
- Beginning of flowering	0	0	0	8 a	0	0
- Full bloom	6 a*	3 a	10 a	7 a	11 a	16 a
- Petal fall	3 a+6 l**	4 a+8 l	8 a+16 l	11 a+3 l	26 a+6 l	29a+9 l

*a, adult; **l, larvae

Thrips adults collected from flowers were identified into species by the expert Prof. Dr. Ekrem ATAKAN (Çukurova University, Agriculture Faculty, Plant Protection Department). Four thrips species-*T. major, F. occidentalis, T. tabaci* and *Haplothrips subtilissimus*-were identified in the nectarine flowers. The incidence percantages of *T. major, F. occidentalis* and *Haplothrips subtilissimus* in flower samples were 75.9%; 20.7%; 3.4% respectively in 2013 and 52.6% for *T. major*; 36.8% for *F. occidentalis;* 10.5% for *T. tabaci* in 2014 during petal fall period.

3.2. Amount of thrips adults captured on traps

3.2.1. Results of the first year (2013)

Thrips population peaked three times in 2013. The first peak flight was seen at petal fall on 12 March 2013 simultaneously with increases in the average air temperature (15.8 °C). The second and the greatest peak was on 02 May 2013 at harvest period when the air temperature peaked to 25.2 °C and the last peak flight was seen on 23 May (Table 2). The amount of thrips adults captured on traps at the petal fall period on 12 March 2013 was 390.4±55.0 adults/trap on the KMT traps while it was 41.8±9.3 and 22.0±2.6 in the PMT and the CMT traps respectively. Amount of thrips on traps increased and peaked on 02 May 2013 in all plots. The highest capture was on the kairomone plot (KMT) with 1196.8±245.7 adults/trap while it was 270.0±27.0 and 240.0±56.9 adults on the PMT and CMT plots respectively. According to statistical analysis, PMT and CMT were placed in the same group (a) on 12 March, 2 May and 23 May while KMT and CMT were in seperate groups on all dates (Table 2). The amount of thrips captured on the KMT trap was 5 times more than on CMT traps on 2nd May (Table 2). According to the total number of thrips captured on traps between flower bud and fruit harvest, it is seen that 9 times more thrips adults were captured on KMT traps (7375.4) in comparison to CMT traps (813.1). Number of adults captured on PMT trap (1230.1) was 1.5 times more than CMT traps.

Table 2- Amount of thrips captured on traps in trial plots in 2013 and 2014 (means of 5 traps)

Date	*Amount of thrips adults on traps (2013)*				*Date*	*Amount of thrips adults on traps (2014)*			
	Kairomone (KMT)	*Control (CMT)*	*df*	*P value*		*Kairomone (KMT)*	*Control (CMT)*	*df*	*P value*
*12 March	390.4±55.0a***	22.0±2.6b	8	0.0001	*04 March	455.8±97.2a	64.6±5.8b	8	0.004
25 April	884.6±161.2a	55.2±8.9b	8	0.001	22 April	3564.0±362.6a	315.2±92.3b	8	0.0001
**02 May	1196.8±245.7a	240.0±56.9b	8	0.005	**07 May	1895.2±235.7a	316.2±36.4b	8	0.0001
23 May	855.5±42.7a	102.0±13.9b	8	0.0001	29 May	492.8±67.4a	38.0±4.4b	8	0.0001
Date	*Pheromone (PMT)*	*Control (CMT)*	*df*	*P value*	*Date*	*Pheromone (PMT)*	*Control (CMT)*	*df*	*P value*
12 March	41.8±9.3a	22.0±2.6a	8	0.074	04 March	148.2±7.5a	64.6±5.8b	8	0.0001
25 April	196.8±24.9a	55.2±8.9b	8	0.001	22 April	672.8±82.7a	315.2±92.3b	8	0.0001
02 May	270.0±27.0a	240.0±56.9a	8	0.647	07 May	672.8±62.2a	316.2±36.4b	8	0.001
23 May	180.0±64.5a	102.0±13.9a	8	0.090	29 May	105.0±22.7a	38.0±4.4a	8	0.045

*, Petal fall; **, harvest; ***, data are means of 5 replicate per treatment; Means followed by the same letters do not differ significantly (P≤0.05); Independent samples t test has been performed for comparing the kairomone/control and pheromone/control plots separately. Logarithmic transformation has been done before performing analysis

3.2.2. Results of the second year (2014)

Thrips population peaked four times in 2014. The first peak was seen during petal fall period in all plots when the air temperature was 13 °C. Two more population peaks occured synchronous with increases in temperature. The last peak was on 22 April 2014 (2 weeks before harvest) when the temperature increased to 18 °C (Table 2). The highest amount of thrips at the end of blooming period (petal fall) on 04.03.2014 was captured on kairomone traps. Average thrips density on the kairomone traps was 455.8±97.2 while it was 148.2±7.5 on the pheromone and 64.6±5.8 on the control traps on 04.03.2014. According to statistical analysis, KMT and CMT placed in seperate groups-CMT (group a) and KMT (group b) (Table 2). When compared with control traps, kairomone traps captured 7 times and pheromone traps captured 2.3 times more thrips adults. The highest number of thrips adults captured on the kairomone traps was 3564.0±362.6 adults on 22.04.2014 while it was 672.8±82.7 and 315.2±92.3 on pheromone and control traps respectively. According to statistical analysis, the differences between treatments (KMT-CMT and PMT-CMT) were significant at 5% level (P≤0.05). According to Tukey multible comparison test, KMT and CMT; PMT and CMT were placed in seperate groups (Table 2). When compared with control traps, it was seen that kairomone traps captured 11.3 and pheromone traps captured 2.1 times more thrips than control traps on 22 April (Table 2). The total thrips density captured on kairomone traps from pink bud stage till harvest was 8.3 times more than control traps. Total amount of thrips on pheromone traps was 2.3 times more than control traps.

Findings of some previous studies confirm our results. Harbi et al (2013) recorded that number of thrips adults trapped on traps baited with kairomone were higher than unbaited traps in a study conducted in a pepper greenhouse in Tunisia. Broughton & Harrison (2012) conducted a study in nectarine orchards in West Australia and tested capture efficacy of sticky traps baited with pheromone (Thripline-ams) and kairomone (Lurem Tr). Researchers notified that traps baited with lures attracted and captured more thrips adults than no lure traps and adding lures enhances the capture efficacy of traps. Similarly, Broughton et al (2015) recorded that in a greenhouse of roses, 1.2-4.0 times more F. occidentalis were caught on traps baited with aggregation pheromone and kairomone compared to unbaited traps. Muvea et al (2014) conducted a study in a bean field in Kenya and determined that attaching Lurem Tr kairomone lure to sticky traps caused significant increases in capture rate of all thrips species. They recorded negative correlation between trap captures and direct plant sampling.

Likewise in the study conducted by the authors of this article in Turkey, capture of thrips on kairomone traps was the highest while thrips existence in the flower samplings was the lowest in the KMT plot. Due to low thrips density in the flower buds based on high thrips capture on traps, the damage rate on the nectarine fruit in this plot was the lowest.

3.3. Damage rate on fruit

3.3.1. Results of the first year (2013)

In order to score the rate of thrips damage, observations on fruits were done on sample trees. The percentage

and intensity of damage is shown in Table 3. Table 3 indicates that fruit damage were mostly light (scale 1) in kairomone (KMT) and pheromone (PMT) plots while most damage was medium grade (scale 2) in the control (CMT) plot in 2013. The highest damage was noted in control plot where 23.4% of the fruit were damaged. The damage was 11.2% and 9.0% in PMT and KMT plots respectively. Independent samples t-test was carried out to the data of damaged fruit. The difference between treatments appeared significant at 5% Alpha level (P≤0.05). According to the t-test shown in Table 3, treatments were placed in seperate groups when compared to control (kairomone group b/control group a and pheromone group b/control group a.

Table 3- Rates (%) and intensity of the damage in means of 0-3 scale in 2013 and 2014

Damage rate and intensity (%)	*2013*					*2014*				
	Treatments			*df*	*P value*	Treatments			*df*	*P value*
	*CMT	*PMT	*KMT			*CMT	*PMT	*KMT		
**Scale 1	***8.8	***6.2	***5.0	-	-	10.6	8.0	4.6	-	-
**Scale 2	11.8	2.8	3.4	-	-	8.4	6.4	3.8	-	-
**Scale 3	2.8	2.2	0.6	-	-	1.2	0.6	0.4	-	-
Total	23.4a		9.0b	8	0.0001	20.2a		8.8b	8	0.0001
	23.4a	11.2b		8	0.020	20.2a	15.0b		8	0.0001

*CMT, Control mass trapping; PMT, Pheromone mass trapping; KMT, Kairomone mass trapping; **Scale 1, light damage; Scale 2, medium damage; Scale 3, severe damage; ***, data, are percentage of damaged fruits; percentages followed by the same letters do not differ significantly (P≤0.05); Independent samples t test has been performed for comparing kairomone/control and pheromone/control plots separately. Arcsin transformation has been done before performing analysis

The aim of this study is to draw adults away from flowers and pull them towards baited traps by using olfactory and visual sitimulants. Capturing maximum amount of thrips on the traps especially during full bloom, petal fall and pre-harvest period would result in decreased amount of damaged fruit. To understand the relationship between the fruit damage and trap capture, Table 2 and Table 3 are evaluated together. According to Table 3, the amount of damaged fruit in 2013 was the lowest (9.0%) in the KMT plot (Table 3) where total trap capture throughout blooming period was the highest (477.2 thrips/trap) (Table 2) and similarly damage was the highest (23.4%) in the CMT plot (Table 3) where trap capture attracted to yellow traps baited with kairomone (Lurem Tr) instead of nectarine flowers in the KMT plot which resulted in lower fruit damage.

Obtaining lower number of damaged fruit in kairomone and pheromone plots when compared with control plot is a promising and encouraging result for the applicability of mass trapping of thrips adults by using baited colored traps for thrips management in nectarine orchards.

3.3.2. Results of the second year (2014)

Number of damaged and undamaged fruit in the trial area in 2014 is shown in Table 3. It is seen in the table that thrips damage was 'light'(Scale 1) in all plots. The highest damage was in the control plot where the damaged fruit rate was 20.2%. It was followed by pheromone and kairomone plots where 15.0% and 8.8% of the fruit was damaged respectively (Table 3). According to statistical tests, the difference between treatments was significant at Alpha 5% level. PMT and CMT were placed in different groups (PMT group b-CMT group a). Similarly KMT and CMT were placed in different groups (Table 3). To understand the interaction between trap capture and damage rate, table 2 and 3 should be evaluated together. The highest trap capture was seen in the kairomone plot on all dates of checking. This was followed by pheromone and the lowest capture was seen in the control plot (Table 2). According to the correlation between trap capture and fruit damage, it was seen that number of damaged fruit was the lowest in kairomone plot where trap capture was the highest and damage was the highest in the plot where thrips capture was the lowest (control plot). This showed that thrips adults were attracted to kairomone baited traps instead of nectarine flowers. In other words, kairomone bait attracted thrips adults by means of the specific odor and adults prefered traps instead of flowers to land on-which in turn caused less fruit damage.

Findings of a previous study confirms our results. Sampson & Kirk (2013) found out that, in semi-protected strawberry crops, mass trapping of F. occidentalis by using blue sticky roller traps reduced adult thrips numbers per flower by 61% and fruit bronzing by 55%. As to the researchers, the addition of the F. occidentalis aggregation pheromone, neryl (**S**)-2-methylbutanoate to the traps doubled trap efficacy, reduced number of thrips adults per flower by 73% and fruit bronzing by 68%.

The results of the two years study in nectarine orchard in Tarsus/Turkey revealed that fruit damage was lower in the KMT and PMT plots when compared to the control plot by attracting adults towards sticky traps using visual and olfactory cues and by this way taking the adults away from the flowers. The kairomone baited traps caught 8 to 16 times more thrips than control traps throughout the trial, starting from blooming period until harvest. The higher capture of kairomone baited traps is due to its' feature not being specific to any particular thrips species but attracting several species like *F. occidentalis*, *T. tabaci*, *Thrips imaginalis* (Broughton & Harrison 2012), *T. major* and etc. In the study, four thrips species appeared together in the nectarine flowers in 2013 and 2014. Therefore, an effective thrips management could be achieved in the plot where Lurem-Tr kairomone attractant was used.

Thripline-ams pheromone attractant is an aggregation pheromone of *F. occidentalis*, and could attract only this species. It is obvious that the higher fruit damage in pheromone plot is due to attracting only one specific thrips species-*F. occidentalis.* In the study area four thrips species responsible of the fruit damage were detected in flowers. For this reason Thripline-ams could be limitedly effective on thrips management in nectarine orchard.

4. Conclusions

Thrips management depends mostly on insecticide applications but the effect of insecticides is low due to some characteristics of thrips species such as the eggs are laid in the plant tissue, adults and nymphs are feeding in the interior parts of the flower and the pupa stage undergo in the soil. Thrips resistance to insecticides is another problem. For a proper thrips management, an alternative method instead of insecticide usage should be implemented.

In this study, the effect of mass-trapping of thrips adults with sticky traps baited with attractants was tested for thrips control in nectarine orchards. Two years' output of the study revealed that kairomone baited traps are much more successful than control traps in mass trapping of thrips species. Based on these results, the usage of kairomone baited traps for thrips control in nectarine orchards seems to be encouraging for integrated pest management. Thrips pheromone-Thripline-ams is also a promising attractant that can be used for thrips control in the orchards where predominant thrips species is *F. occidentalis*. This study revealed that mass trapping of thrips species by using semiochemicals would be an alternative control strategy when easily developing pesticide resistance of thrips species is considered. Besides, usage of baited traps could be a supplementary agent that can help to increase the success of insecticides in commercial orchards.

It is a known fact that colored traps are attractive for natural enemies and has a negative effect on their population (Atakan et al 2016). Kairomone added traps were found out more attractive to natural enemies than pheromone added traps and bait free traps during this study. But, it is another known fact that insecticides also knock down most of natural enemy populations. For this reason it would be wrong to pass judgement on this issue without a comparison study being conducted on the effect of sticky traps and the effect of insecticides on natural enemies. For all that, using semiochemical baited traps are recommended for thrips control in nectarine orchards and should be in hand as a tool for management strategy.

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Abbreviations and Symbols	
PMT	Pheromone mass trapping
KMT	Kairomone mass trapping
CMT	Control mass trapping

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Evaluation of Integrated Control Methods of Purple Nutsedge (*Cyperus rotundus* L.) In Transplanted Onion

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ABSTRACT
Onion (*Allium cepa* L.) is one of the most important vegetable crops in the world. Weeds are the most global problem in onion production. Purple nutsedge (*Cyperus rotundus* L.) is one of the most damaging weeds, which propagates rapidly through extensive underground system and tubers. The aims of this study were to evaluate the effect of solarization duration and tuber weight on characters of *C. rotundus* and assessed different treatments on the weed control and onion yield. For this purpose, two separate experiments were conducted in South of Kerman Agricultural Research and Education Center, Jiroft, Iran. The solarization experiment conducted to investigate the effects of solarization duration [0 (control), 5, 10, 15, and 20 days] and tuber weight (small, medium and large) on tuber viability and the number of produced tubers of *C. rotundus*. The weed management experiment conducted as a randomized complete block design with eight weed management methods with three replications. The results of the solarization experiment showed that in all solarization duration tuber viability eliminated except for control. The maximum percentage of tuber viability found in the interaction of non-solarization with large and medium tuber weights. Also, the results of the weed management experiment revealed that after hand plucking treatment, deep disking twice with 20 days interval followed by application of Glyphosate twice after each disk treatment (T8) was the best weed management method. The highest onion yield was obtained in hand plucking and T8 treatments by 96.53 and 70.67 ton ha^{-1}, respectively.

Keywords: Solarization; Purple nutsedge; Onion; Tuber weight; Glyphosate

1. Introduction

Onion (*Allium cepa* L.) is one of the most important vegetable crops in the world. It belongs to Magnoliophyta division, Liliopsida class, Liliales order, and Liliaceae family. Weeds are the most global problem to onion production and reduced it to 70-75% (Mani & Gautam 1976; Chattopadhyay et al 2016). There are many different weeds associated with this plant such as purple and yellow nutsedges, cockspur grass, feverfew, gooseberry and common purslane (Sahu et al 2017; Sahu et al 2018).

Purple nutsedge (*Cyperus rotundus* L.) is one of the most important weeds and a perennial sedge which propagates rapidly through extensive underground system and tubers (Nelson & Renner 2002). Also, it has a high ability to compete with other plants as well as to survive in different conditions. This weed grows in some regions of Iran that

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possess fertile soils and adequate temperature and humidity, in which, due to the extent of damage, farmer leave their fields (Najafi et al 2010).

Tuber viability elimination or prevent tuber production are the ways to successful management of *C. rotundus* (Webster et al 2017). Temperature treatments (e.g., solarization, steam and electromagnetic radiation) have been proposed as alternatives to methyl bromide for pest management (Kokalis-Burelle et al 2016). The tubers of *C. rotundus* died when they exposed to 50 °C for 96 h, whereas exposure for 48 h did not affect tuber viability. Also, the tubers died at 60 °C for one hour (Smith & Fick 1937). In another study, the tubers were died at 90 °C for 30 min, whereas at 50 and 60 °C it was reduced by 10 to 20%, respectively (Rubin & Benjamin 1984). Ransom et al (2003) reported that the plant densities at five locations ranged from 28 to 67 shoots ft^{-2}, and onion yields were 23 to 64 percent less with yellow nutsedge compared to weed free conditions.

Kumar et al (2012) studied about *C. rotundus* management in a soybean-wheat cropping system. The authors reported that a significant reduction in the plant density and increase in soybean and wheat yields were observed due to solarization followed by the application of glyphosate. Soil solarization, herbicide and tillage independently show variable effects on *C. rotundus* (Grichar & Sestak 2000; Edenfield et al 2005; Bangarwa et al 2008; Das & Yaduraju 2008; Gill et al 2009; Gill & McSorley 2010). Soil solarization covered by polyethylene mulch is a method to increase soil temperature and its effect has proven against many weeds such as *Orobanche crenata*, *O. ramose* and *C. rotundus* (Abouziena & Haggag 2016). Solarization during summer controls *C. rotundus* due to deadly temperatures near the soil surface (Chase et al 1999; Webster 2003). Onion yield was significantly enhanced by solarization (Abouziena & Haggag 2016).

Frequent tillage and application of polyethylene film with or without turnip resulted in a lower density of large tubers of *C. rotundus* in bell pepper cultivation (Bangarwa et al 2008). In order to decrease the multiplication of *C. rotundus* tubers, shallow tillage at frequent intervals is needed (Bangarwa et al 2008). *C. rotundus* was effectively managed by glyphosate and paraquat (Iqbal et al 2012). The density of *C. rotundus* was reduced due to the glyphosate application in soybean (Reddy & Bryson 2009). The viability of tubers was reduced by 80% and 65% in soybean and cotton, respectively, due to glyphosate application (Edenfield et al 2005).

Although many studies have been carried out on the use of different treatments to control of *C. rotundus*, to the best of our knowledge there is no comprehensive research on the use of combination treatments. Therefore, the objectives of this study were to evaluate the effects of solarization duration and tuber weight on tuber viability and the number of produced tubers of *C. rotundus* and effect of different treatments on the weed control and onion yield.

2. Material and Methods

2.1. Plant material, experimental location and soil properties

Two separate experiments were conducted in south of Kerman Agricultural Research and Education Center, Jiroft, Iran, during the summer 2017. Onion transplanting was carried out using Rio Bravo variety from Nun Hems Company. Also, The *C. rotundus* tubers were collected from 0-30 cm depth of the field soil. The result of the physical and chemical properties of the location soil was present in Table 1. The experimental soil of the location was sandy loam in texture and a pH of 7.4. Some meteorological data for the experimental area were present in Table 2.

Table 1- Physical and chemical characteristics of the experimental soil

Physical properties					
Clay (%)	Silt (%)	Fine sand (%)		Soil texture	
12	18.5	69.5		Sandy loam	
Chemical properties					
pH	ECe (ds m^{-1})	Organic matter (%)	N (%)	P (mg kg^{-1})	K (mg kg^{-1})
7.4	2.23	0.48	0.48	7.5	2.1

Table 2- Some meteorological data for the experimental area

Month	*Temperature (°C)*			*Totalmonthly precipitation (mm)*
	Average	*Maximum*	*Minimum*	
March	20.9	30.8	11.1	64.1
April	27.1	39.8	14.4	2.9
May	33.8	46.4	21.3	6.1
June	37.5	48.2	26.8	0.0
July	37.4	46.2	28.6	0.8
August	35.6	44.4	26.8	2.0
September	32.4	43.2	21.7	0.0
October	28.6	39.4	17.8	0.0
November	21.6	33.6	9.6	6.0
December	21.0	37.9	4.2	0.0
January	13.7	18.1	9.3	10.0
February	19.5	23.8	15.1	22.0

2.2. Solarization experiment

The solarization experiment was conducted to investigate the effects of solarization duration and tuber weight on tuber viability and the number of produced tubers of *C. rotundus*. A factorial experiment based on randomized complete block design was carried out with two factors including three levels of tuber weight including 0.2 g (small), 0.5 g (medium) and 1 g (large) and five levels of solarization duration [0 (control), 5, 10, 15, and 20 days] with three replications. The *C. rotundus* tubers were placed under solarization on the plowing surface for different treatments. The maximum temperature of the location during the August and September months was more than 40 °C (Table 2). In order to assay the tuber viability and the number of produced tubers, the fifteen tubers from each treatment were randomly selected and cultured in 15×30 cm pots in the 2.5 cm depth. The pots were irrigated by drip irrigation.

Tuber viability was calculated by the following formula:

$$Tuber\quad viability\quad = \frac{The\quad number\quad of\quad sprouted\quad tubers}{Total\quad tubers\quad sown} \times 100$$

At the end of the experiment, the soil from pots was washed under tap water onto a metal net (3×3 mm) and the number of produced tubers were counted.

2.3. Weed management experiment

The weed management experiment was conducted as a randomized complete block design with eight treatments and three replications in a field with high contamination of *C. rotundus*. The treatments were included:

T1: Deep disking twice with 20 days interval followed by application of Glyphosate 410 g a.i. L^{-1} at 3360 g a.i. ha^{-1}, T2: Deep plowing followed by disk two weeks after plowing, T3: Hand plucking, T4: Soil solarization with transparent polyethylene plastic in the second half of August, T5: T4 treatment followed by glyphosate two weeks after removing the plastic before onion transplanting, T6: Un-weeded control, T7: Three times plowing at three weeks intervals, T8: Deep disking twice with 20 days interval followed by application of glyphosate twice after each disk.

Each plot consisted of four furrows with 6 m long and 50 cm apart, each furrow contained four rows. In order to study the weed density, after the treatments, a fixed quadrate was used before weed emergence. Also, during the experiment all other weeds were controlled. In the end of growing season, the following characters were measured, the weed density, shoot and tuber dry weights (g), weed control (%), number of tubers, and onion yield. The tuber populations from each plot were counted in an area of 25 cm^2 by 20 cm deep volumes of the soil. The soil was sifted by a 5 mm sieve to record the total number of tubers. In order to measure dry weights of shoot and tuber, they were placed in the oven for 72 h at 75 °C. As suggested by Üstüner & Güncan (2002), in the present study the high weed density was used (The average plant more than 10 in per square meter). *C. rotundus* control index (CCI) (Das 2008) which estimate treatment efficiency based on the reduction in *C. rotundus* dry weight, was calculated using the following formula.

CCI (%)= [($CDW_c - CDW_t$) ×100 / CDW_c]

Where; CDW_c and CDW_t are the weed dry weight (g m^{-2}) of *C. rotundus* in control and treated plots, respectively.

2.4. Statistical analysis

Two experiments were performed. The experiments were carried out based on the factorial experiment in a randomized complete block (RCBD) and RCBD, respectively. Analysis of variance was done using SAS software (SAS Institute, Inc., Cary, NC). Then, post-hoc Fisher LSD (least significant difference) test at a 5% probability level was carried out to compare the difference among the treatments.

3. Results and Discussion

3.1. The solarization experiment

The results of the analysis of variance indicated that the interaction effects of solarization duration and tuber weight was significant for both measured characters (data not shown). There was a significant difference between control treatment (non-solarization) and different solarization durations. In all solarization durations, tuber viability was eliminated. The maximum percentage of tuber viability was found in the interaction of non-solarization with large and medium tuber weight (Table 3).

Table 3- Interaction of solarization duration and tuber weight on tuber viability and the number of produced tuber of *C. rotundus*

Character	*Control*			*5 days*			*10 days*			*15 days*			*20 days*		
	Tuber weight (g)														
	0.2	*0.5*	*1*	*0.2*	*0.5*	*1*	*0.2*	*0.5*	*1*	*0.2*	*0.5*	*1*	*0.2*	*0.5*	*1*
Tuber viability	85b	100a	100a	0c	0c	0c	0c	0c	0c	0c	0c	0c	0c	0c	0c
No. of produced tuber	5b	5b	7a	0c	0c	0c	0c	0c	0c	0c	0c	0c	0c	0c	0c

The same letter within each column indicates no significant difference among treatments (P<0.05)

The solarization of tubers at a high temperature for a short period of solarization duration leads to effective control and reduction in the tuber viability of *C. rotundus*. Therefore, any farming activities that can place more tubers on the soil surface and exposed them to solarization in warm and dry seasons, such as plowing and repeated discs even at a short period, can dramatically and effectively reduce the damage of this weed. As the area temperatures in August and September months reaches above 40 °C, therefore these temperatures are sufficient to eliminate the weed tubers. Same as our results, previous studies showed that the high temperature prevented the tuber viability of *C. rotundus* (Webster 2003). The high temperature affects the hydrogen and disulfide bonds in proteins, lipids and the membrane structure (Ahmad et al 1996). The lateral mechanisms of thermal death or reduction in the number of tubers are due to the inactivation of respiratory enzymes, damage to the synthesis of proteins, as well as damage to nucleic acids (Katan 2015). The previous study revealed that *C. dactylon* was eliminated when exposed to a high temperature (40 °C for 30 min), whereas the tuber viability of *C. rotundus* at high temperature (above 60 °C) was decreased, indicating the survival potential of this weed (Rubin & Benjamin 1984). All the interaction treatments between solarization durations with tuber weights (except the interaction between the control with the three tuber weights), prevented the tuber production. Successful management of purple and *C. esculentus* is related to the elimination of tuber viability and tuber production (Roozkhosh et al 2017). Solarization, steam and electromagnetic radiation treatments that produced high temperature can be used instead of methyl bromide for different pests control (Webster 2003; Stapleton et al 2000; Kumar et al 2012; Díaz-Hernández et al 2017).

3.2. The weed management experiment

The results of ANOVA indicated that the effect of management methods on the measured characters including weed density, shoot and tuber dry weights, and control percentage and onion yield was significant at 1% level (Table 4). The low values of the coefficient of variation (CV) showed that the traits were measured with high accuracy. The results of means comparison showed that hand plucking (T3) was the best treatment for *C. rotundus* management. Also, the highest onion yield was obtained in this treatment (96.53 ton ha^{-1}; Table 5). Although T3 was the best treatment, it had some

problems such as high cost and time consuming (personal observation). Hand plucking method is not possible on large plots as a method. Among the other treatment, T8 was the best treatment for weed control. It seems that application of this treatment in successful weed control was due to two main reason; first, the double disc during the warm and dry months causes more tubers to be exposed to hot sunshine, and secondly, Glyphosate has eliminated the tubers. The tubers of *C. rotundus* are weakened by soil solarization and will be susceptible to Glyphosate herbicide (Peerzada 2017; Johnson et al 2007). Many researchers have suggested that among the management strategies, the solarization and many summer plowing can be more effective to reduce vegetative growth and tuber production of *C. rotundus* (Wang et al 2009). After T3, it had the lowest plant density (8n m^{-2}) and shoot dry weight (7.9 g), and the characters were reduced by 86 and 88% compared to control treatment, respectively. After un-weeded control treatment (T6), T4 had the lowest effect on weed control. No weed plant was observed during the application of T4 treatment, but after removing the plastic and planting the onions, this treatment showed the highest weed density and dry weight than other treatments, except for T6 treatment. It seems that solarization can stimulate tubers germination. Egley (1983) reported that 3 to 4 weeks of solarization not only did not decrease *C. rotundus* emergence but also in some cases it was increased. It also seems that solarization cannot affect the tubers which located at downward depths. Light and sandy texture of the soil allowed the tubers to penetrate and distribute at the downward depths. Therefore, a low percentage of the tubers were affected in this soil by solarization. The tubers that located at depths of more than 10 cm, not only escaped from solarization due to the absence of lethal temperature but also they were stimulated to germinate (Rubin & Benjamin 1984). When the depth of tubers increased from 10 to 15 cm, *C. rotundus* control was reduced by 32%. After hand plucking treatment (T3; 0 tubers) the T8 treatment had the minimum number of produced tubers (13 tubers 0.05 m^{-3} soil), whereas the control treatment (T6) had the highest number of tubers (98 tubers 0.05 m^{-3} soil). The previous study showed that transparent polyethylene mulch reduced the *C. rotundus* density by 79% compared with control (Webster et al 2008). Solarization effectively controlled the *C. esculentus* and significantly reduced the number of tuber in the soil (Johnson et al 2007). The production of the new *C. esculentus* tubers was stopped after exposure at 50, 55 and 60 °C for 1, 4 and 16 h, respectively (Webster 2003). After hand plucking (0 g), the T8 treatment had the lowest tuber dry weight (6.85 g), and control treatment had the highest value for this trait (52.55 g). The results showed that the highest control percentage was obtained from hand plucking treatment (100%) followed by T8, T7 and T1 treatments (88.6, 82.03 and 77.03%, respectively). The lowest control percentage was achieved from un-weeded control (T6) and soil solarization using transparent polyethylene treatment (T4) by 0 and 43.3%, respectively.

Table 4- ANOVA for the effect of different weed managements on the weed control and onion yield

Source of variation	*df*	*Mean square of traits (MS)*					
		Weed density	*Weed shoot dry weight*	*Weed control %*	*Number of produced tuber*	*Weed tuber dry weight*	*Onion yield*
Replication	2	6.5^{ns}	2.5^{*}	14.51^{*}	18.3^{*}	9.29^{*}	$9.6^{n's}$
Treatment	7	937^{**}	1449^{**}	799^{**}	2780^{**}	779.26^{**}	2579^{**}
Error	14	2.3	4.21	3.70	6.05	3.97	17.69
CV%		5.6	8.44	2.95	5.3	8.1	10.47

ns, no significant; ** and *, are significant at 1 and 5% level, respectively

Table 5- Means comparison of the *C. rotundus* measured characters

Treatment	*Density (plant m^{-2})*	*Shoot dry weight (g m^{-2})*	*Control (%)*	*Tuber dry weight (g 0.05 m^{-3})*	*Number. of produced tuber (0.05 m^3 soil)*
T1	25.6^{d}	16^{d}	77.03^{d}	22.97^{d}	45^{d}
T2	31.3^{c}	23.8^{c}	65.76^{e}	27.71^{c}	53^{c}
T3	0^{f}	0^{f}	100^{a}	0^{f}	0^{f}
T4	37.6^{b}	39.6^{b}	43.33^{f}	29.15^{c}	54^{c}
T5	31.3^{c}	24.7^{c}	64.43^{e}	33.65^{b}	67^{b}
T6	57.6^{a}	69.9^{a}	0^{g}	52.55^{a}	98^{a}
T7	25^{d}	12.5^{d}	82.03^{c}	23.34^{d}	42^{d}
T8	8^{e}	7.9^{e}	88.6^{b}	6.85^{e}	13^{e}
LSD (P≤0.05)	2.65	3.59	3.37	3.49	4.30

Within each column, the same letter indicates no significant differences among treatments (P<0.01)

Application of different management methods of *C. rotundus* had many fluctuations in onion yield. The highest onion yield was obtained in T3 treatment by 96.53 ton ha^{-1}, followed by T8, T7 and T1 treatments by 70.67, 40.67 and 34.13 ton ha^{-1}, respectively (Figure 1). Also, after un-weeded control treatment, the lowest onion yield was obtained from T5 treatment (14.47 ton ha^{-1}). T4 treatment had more weed density as well as onion yield than T5 treatment, and this reduced of onion yield in T5 than T4 was due to the effects of glyphosate phytotoxicity on the onion plant in T5.

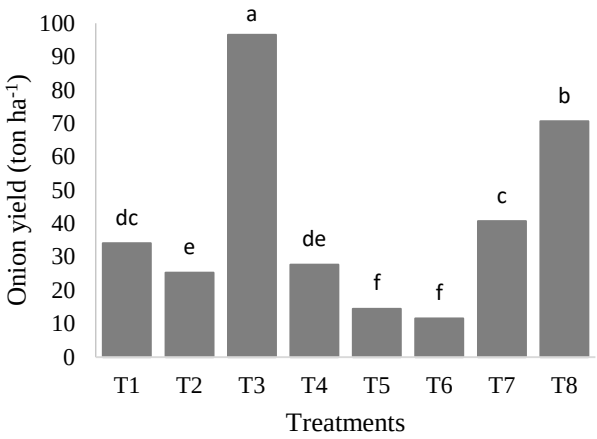

Figure 1- Mean comparison of onion yield under different *C. rotundus* managements

4. Conclusions

The tubers are one of the main ways of *C. rotundus* reproduction. The results of the solarization experiment showed that the solarization of tubers at a high temperature for a short period of time leads to effective control and reduction in the tuber viability of the weed. Therefore, any farming activities that can place more tubers on the soil surface and exposed them to solarization in warm and dry seasons, such as plowing and repeated discs, can dramatically and effectively reduce the damage of this weed. Also, the result of the weed management experiment revealed that the lowest weed density and the highest onion yield were achieved in T3 and T8 treatments. Whereas hand plucking (T3) was very laborious and costly, therefore deep disking twice with 20 days interval followed by application of glyphosate twice after each disk (T8) was found to be the most effective treatment in controlling weeds.

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Effects of Marigold (*Tagetes erecta*) and Synthetic Carotenoid on Growth Performance and Skin Coloration of Blue Streak Hap (*Labidochromis caeruleus)* and Pindani (*Pseudotropheus socolofi*) Fry (Cichlidae)

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ABSTRACT
This study was designed to determine the ideal dosages of marigold (*Tagetes erecta*) and synthetic carotenoid in blue streak hap (*Labidochromis caeruleus*) and pindani (*Pseudotropheus socolofi*) fry (Cichlidae), and to compare effects on the growth and skin coloration. In the first experiment, the blue streak hap and pindani fry fed with different levels of water-soluble marigold flower meal (0, 2, 4, 6, 8, 10 and 12%) were tested for 30 days to find the optimum skin coloration and growth parameters. Then, in the experiment, the most effective doses of water-soluble marigold flower meal (2, 4, 8%) which obtained from the first experiment were compared with the different synthetic carotenoid dosages (50, 100 and 150 mg kg^{-1}) for 30 days. At the end of the first experiment, weight gain and the skin coloration degrees for both fish species were increased significantly by supplementation of 4% water-soluble marigold flower meal supplemented diet ($P<0.05$). In the second experiment, 50 and 100 mg kg^{-1} of synthetic carotenoid and 4% water-soluble marigold flower meal supplementation showed better performances concerning growth and skin coloration ($P<0.05$). This study showed that the 4% water-soluble marigold flower meal could be used as an alternative and useful pigmentation source for blue streak hap and pindani.

Keywords: Cichlidae; Marigold flower meal; *Tagetes erecta*; Coloration; Growth

1. Introduction

Ornamental fish are considered among the most popular pets worldwide, and also ornamental fish culture is known as one of the essential sectors of aquaculture. The aquarium fish production sector can be expressed as an important source for the gross national income of underdeveloped and developing countries (Yanar et al 2008). Thus, there is a steadily increasing in culture and trade of ornamental fish globally. The blue streak hap (*Labidochromis caeruleus* Fryer 1956) and pindani (*Pseudotropheus socolofi* Johnson 1974) are freshwater perciform fishes, belong Cichlidae family, and this family are described by a broad diversification of colors and color designs. The origins of both fishes are Lake Malawi in Africa. The blue streak hap and pindani are the most commercially valuable ornamental fish species and cultured all over the world (Ergün et al 2010). The market prices and acceptance of cultured aquarium fish are generally dependent on the vibrant colors. One of the main problems in cultured ornamental fish in the market is related to the inadequate coloration because the paleness of the color has a negative impact on the demand and market price. It's known that to achieve consumer acceptance and optimal price; cultured fish must be pigmented. For this reason, recent studies have been concentrated on coloration in the ornamental fish. The color of aquarium fish come from carotenoid pigments which are predominantly astaxanthin. Like other animals, also fish cannot biosynthesize carotenoids in their tissues. So, the pigmentation of fish generally depends on carotenoid content within the ingested food (Goodwin 1984; Moorhead & Zeng

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2010; Ansarifard et al 2018; Pezeshk et al 2019). Fish species and size, feed content, colorant concentration in the ration, feeding rate, hereditary and environmental factors are also useful in the coloration of aquarium fish (Yeşilayer et al 2008). Several studies have been conducted to the use of carotenoids as pigments and also their effects on fish growth, maturation, reproduction and enhancing the immune system (Gupta et al 2007; Sinha & Asimi 2007; Singh et al 2016; Singh & Kumar 2016; Maiti et al 2017; Pezeshk et al 2019). In addition to coloring effects, the carotenoids are playing an essential role in animals such as pro-vitamin-A, antioxidant and immune-regulator. Also, they can be effective against the bacterial and fungal diseases (Shahidi et al 1998). Synthetic carotenoids are not only expensive but also it has limited usage in the feed formulations due to deteriorating effects on the environment (Gupta et al 2007). However, natural carotenoids are mainly herbal origin and generally derived from microalgae-based sources. The yellow corn, corn gluten meal, alfalfa flour and extract, red pepper flour and extract, marigold flour and extract, meadow grass and aquatic macrophytes are known as the conventional carotenoid-rich plant products (Kırkpınar & Erkek 1999; Ezhil et al 2008; Velasco-Santamaría & Corredor-Santamaría 2011). It is thought that natural carotenoid sources should be used to prevent potential harmful effects of synthetic carotenoids. Therefore, this study aimed to determine the effective dose of water-soluble marigold flower meal (*Tagetes erecta*) in blue streak hap (*Labidochromis caeruleus*) and pindani (*Pseudotropheus socolofi*) fry and to identify the comparative effects of synthetic carotenoid dosages on the growth performance and skin coloration.

2. Material and Methods

2.1. Experimental design

The study was carried out at Fisheries Research and Application Unit (FRAU, Faculty of Agriculture, Ankara University), Ankara, Turkey. In the two trials, 21 plastic tanks (32x25x13 cm, 10 L, Çankaya plastic, Istanbul, Turkey) were used. In this study, a total of 420 fish (210 blue streak hap, *Labidochromis caeruleus* and 210 pindani, *Pseudotropheus socolofi*) were obtained from the commercial producer (Ulus, Ankara) and used in the experiments. The stock rate for each tank was set to 10 fish/10 L (5 blue streak hap, 5 pindani). In the experiments, the water temperature was kept constant at 27±1 °C, 10% of the tank volume was siphoned, and the water was renewed daily. The water quality parameters such as pH, dissolved oxygen (YSI ProPlus 20 multi-parameter), oxidation-reduction potential (American Marine USA Pinpoint ORP Monitor) were monitored daily, nitrite and ammonium were analyzed weekly with the standard method (Eaton et al 2005). Throughout the experiments, the variations of water quality parameters such as pH: 8.2±0.5; DO: 7.8±0.4 mg L^{-1}; ORP: 200±14 mV; NO_2^-: 0.03±0.02 mg L^{-1}; NH_4^+: 0.06±0.03 mg L^{-1} were recorded.

2.2. Feed additives, carotenoid analysis and feeding

A commercial basal diet (500 µm trout, 55% protein, Skretting Feed Production Inc. Mugla, Turkey) was used for the feeding of fish. Colorants, water-soluble marigold flower meal (M: from *Tagetes erecta*, Aksuvital Natural Products Ltd., Turkey) and synthetic carotenoid (SC) (Carophyll® pink, DSM Nutritional Products Ltd. Basel, Switzerland) were used as feed additives. The carotene content of the water-soluble marigold flower meal was calculated as 1040 mg kg^{-1} total carotenoid and synthetic carotene source of Carophyll®pink was 8% astaxanthin. To prevent the protein imbalance, fish meal (65% protein) were added to the experimental feeds (Göçer et al 2006; Büyükçapar et al 2007; Yeşilayer et al 2011). Different doses of M (2, 4, 6, 8, 10, 12% water-soluble marigold flower meal) and SC (50, 100, 150 mg kg^{-1} as astaxanthin per kg of feed) were dissolved in 10 mL of distilled water and added to the grounded basal feed (100 g) by the mixer. For the control feed, only distilled water was added. The prepared feed was dried in the incubator (20 hours at 35 °C), ground and used after a 500 µm mesh sieve (stored in the refrigerator at 4±1 °C). For the analysis of total carotenoids in water-soluble marigold flower flour and basal diet, 1 g of the sample was dissolved in 10 mL of acetone solution (80%; 80/20: Acetone/Distilled water). The concentration was diluted to 1 mg mL^{-1}, and the absorbances were read at 450, 645 and 663 nm on the Biobase BK-D560 Uv-Vis spectrophotometer (CI Scientific Pty Ltd., Australia) (Kocaçalışkan & Kadıoğlu 1990). The amount of these pigments and the supplementation dosages in experimental feeds were calculated as below (Table 1).

In the experiments, fish were fed ad libitum three times per day in the morning (08:00 am), at noon (12:30) and in the evening (06:00 pm). After one hour of feeding, faeces and feed wastes at the bottom of the tanks were syphoned.

Table 1- The supplementation levels of the different experimental feeds

Groups	*Ratio (unit kg^{-1})*	*Total carotenoids (mg kg^{-1})*	*Astaxanthin (mg kg^{-1})*	*Total pigment in feed (mg kg^{-1})*
Control	0 g	180	NA	180.0
M2	20 g	20.8	NA	200.8 (180+20.8)
M4	40 g	41.6	NA	221.6 (180+41.6)
M6	60 g	62.4	NA	242.4 (180+62.4)
M8	80 g	83.2	NA	263.2 (180+83.2)
M10	100 g	104.0	NA	284.0 (180+104)
M12	120 g	124.8	NA	304.8 (180+124.8)
SC50	625 mg	NA	50	230.0 (180+50)
SC100	1250 mg	NA	100	280.0 (180+100)
SC150	1875 mg	NA	150	330.0 (180+150)

Control, basal diet without supplementation (180 mg kg^{-1} total carotenoid); M, water-soluble marigold flower meal (1040 mg kg^{-1} total carotenoid); M2, 2% M (20.8 mg total carotenoid); M4, 4% M (41.6 mg total carotenoid); M6, 6% M (62.4 mg total carotenoid); M8, 8% M (83.2 mg total carotenoid); M10, 10% M (104 mg total carotenoid); M12, 12% M (124.8 mg total carotenoid); SC, Synthetic carotene source (Carophyll®pink, DSM, Basel, Switzerland 80 g astaxanthin kg^{-1}); SC50, 50 mg astaxanthin; SC100, 100 mg astaxanthin; SC150, 150 mg astaxanthin; NA, Not available. Chlorophyll a: (12.7 x A663 nm)-(2.69 x A645 nm), Chlorophyll b: (22.9 x A645 nm)-(4.68 x A663 nm), Total carotenoids: (4.07 x A450 nm)-((0.0435 x Chlorophyll a)+(0.367 x Chlorophyll b))

2.3. Experiment I: The determination of the effective dosage for water-soluble marigold flower meal

The aim of this experiment I was to determine the ideal dosage of the water-soluble marigold flower meal (M) added to the fish feed. In totally, 105 blue streak hap (initial weight: 0.246-0.262 g) and 105 pindani (initial weight: 0.274-0.294 g) were used. A total of 10 fish (5 blue streak hap and 5 pindani) were stocked in each plastic tank (10 L). All trial were triplicated in 7 groups (0, 2, 4, 6, 8, 10, 12% M) and tested for 30 days.

2.4. Experiment II: Comparison of effective water-soluble marigold flower meal dosage and synthetic carotenoid

The aim of the second trial was to compare the synthetic carotenoid (SC) with effective dosages of water-soluble marigold flower meal that have been taken from the results of the first trial. Experiment II was designed as 7 groups in triplicate. Except for the control group, fish were fed with diet contained a different ratio of M (2, 4, 8%) and SC (50, 100 and 150 mg kg^{-1}) during 30 days.

2.5. Growth performance analysis

All the fish (5 blue streak hap and 5 pindani in each replicate) were anaesthetized with a moderate dose of clove oil (Eugenol 5 mg L^{-1}) to prevent possible damage during the measurements of live weight (W) and total length (TL) at the beginning and at the end of the trials. For both experiments survival rates (SR), growth parameters such as weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were calculated by following formulas (Güroy et al 2012);

SR (survival rate) = number of fish at the day 0 - Number of fish at the end of the period
WG (weight gain) = Wi-Wf (Wi, initial body weight; Wf, final body weight, g)
SGR (specific growth rate) = [(*ln* Wf−*ln* Wi)/days] x 100
FCR (feed conversion ratio) = Total feed given (g)/Total weight gain (g)

2.6. Color analysis

Color measurements were performed by Chroma Meter (the color system mode: International Commission on Illumination). The lightness (*L*), redness (*a*) and yellowness (*b*) values of the groups were determined. The maximum number of the light in the system is 100, corresponding to the white color; the lowest is 0 corresponding to black. The device (Time Group Inc. Beijing, China) was calibrated using the black and white inductor caps before measurements (CIE 1976; Güroy et al 2012). For the color analyses, three blue streak hap and three pindani from each replicate were randomly sampled and deeply anaesthetized with 10 mg L^{-1} Eugenol (clove oil). For the color measurement, the median portion of the lateral region of fish skin was chosen. Then, the color measurements were recorded for the statistical analysis.

2.7. Statistical analysis

At the end of both the 30-days experiment periods data were analyzed by the SPSS 14 for Windows package program (SPSS Inc., Chicago, IL, USA). The differences of variance between the groups were performed by one-way ANOVA and DUNCAN post-hoc test (with a significance level of 0.05, and the results were presented as mean±s.d).

3. Results and Discussion

3.1. Experiment I: The determination of the effective dosage for water-soluble marigold flower meal

3.1.1. The growth and coloration parameters of blue streak hap

At the beginning of experiment I; the initial weights of blue streak hap were varied between 0.246-0.262 g (P>0.05). After 30 days, the best live final weight (Wf) with 0.580±0.018 g and also the final total length (TLf), the live weight gain (WG), and the specific growth rate (SGR) were taken from the M4 group (4% water-soluble marigold flower meal) (P<0.05). It was determined that there was not a significant difference between the groups in terms of feed conversion ratio (FCR) (P>0.05). The highest FCR was obtained from the control group with 3.837±0.101%, whereas the best (lowest) rate was achieved by the M4 group, with 2.580±0.657% (P>0.05). The water-soluble marigold flower meal supplemented diets did not appear to have any effect on survival rates (SR). In experiment I for blue streak hap, there were no significant differences between the control group and the increasing doses of water-soluble marigold flower meal regarding the comparison of lightness (*L*) values. Also, it was seen that the darkest *L* value was obtained from M4 (4% water-soluble marigold flower meal) group (P>0.05). According to the comparison of the redness indicator, *a*, M4 group were found closest to red (P<0.05). The yellowness indicator *b* presents the highest value for also the M4 group. However, no statistically significant differences were observed between the trial groups for yellowness indicator *b* (P>0.05) (Table 2).

Table 2- Experiment I: Growth and coloration parameters of blue streak hap (*L. caeruleus*) fed with water-soluble marigold flower meal supplemented diets for 30 days

Experiment I: blue streak hap							
	Control[1]	*M2*	*M4*	*M6*	*M8*	*M10*	*M12*
Wi	0.262±0.019^{a}	0.252±0.021^{a}	0.250±0.019^{a}	0.252±0.022^{a}	0.252±0.015^{a}	0.252±0.021^{a}	0.246±0.022^{a}
Wf	0.484±0.031^{a}	0.516±0.031^{a}	0.580±0.018^{b}	0.487±0.024^{a}	0.477±0.029^{a}	0.506±0.024^{a}	0.509±0.041^{a}
TLi	2.480±0.084^{a}	2.540±0.134^{a}	2.600±0.100^{a}	2.520±0.084^{a}	2.560±0.114^{a}	2.600±0.158^{a}	2.540±0.114^{a}
TLf	3.160±0.152 ^{a}	3.480±0.837^{bc}	3.600±0.187^{c}	3.380±0.164^{abc}	3.400±0.255^{bc}	3.440±0.152^{bc}	3.340±0.134^{ab}
WG	0.222±0.001^{a}	0.266±0.018^{c}	0.328±0.010^{d}	0.232±0.017^{ab}	0.223±0.015^{a}	0.254±0.001^{bc}	0.262±0.010^{bc}
SGR	2.057±0.090^{a}	2.404±0.128^{b}	2.789±0.100^{c}	2.170±0.176^{ab}	2.106±0.164^{ab}	2.326±0.019^{ab}	2.411±0.101^{b}
FCR	3.837±0.101^{a}	3.232±1.116^{a}	2.580±0.657^{a}	3.630±0.770^{a}	3.784±0.822^{a}	3.347±0.953^{a}	3.233±0.791^{a}
SR	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}
L	71.920±2.950^{a}	70.960±1.630^{a}	67.070±5.480^{a}	70.620±2.380^{a}	69.550±0.100^{a}	71.140±1.04^{a}	70.540±3.450^{a}
a	-6.450±0.570^{a}	-5.600±0.390^{ab}	-3.730±0.850^{c}	-5.090±0.120^{b}	-4.420±0.460^{bc}	-4.590±0.88^{bc}	-4.930±0.740^{b}
b	26.700±0.240^{a}	27.689±0.500^{a}	28.190±0.860^{a}	27.510±1.680^{a}	27.930±2.260^{a}	26.880±0.670^{a}	26.390±0.360^{a}

[1], values (means±s.d.) in same line with different superscripts are significantly different (P<0.05). For the growth and for the color parameters five and three fish were measured from each replicates respectively. Control, basal diet without supplementation (180 mg kg^{-1} total carotenoid); M, water-soluble marigold flower meal (1040 mg kg^{-1} total carotenoid); M2, 2% M (20.8 mg total carotenoid); M4, 4% M (41.6 mg total carotenoid); M6, 6% M (62.4 mg total carotenoid); M8, 8% M (83.2 mg total carotenoid); M10, 10% M (104 mg total carotenoid); M12, 12% M (124.8 mg total carotenoid); Wi (g), initial weight; Wf (g), final weight; TLi (cm), initial total length; TLf (cm), final total length; WG, wet weight gain; SGR, specific growth rate; FCR, feed conversion ratio; SR, survival rate; *L*, lightness (white=100, black=0); *a* = redness (positive value = red, negative value= blue); *b* = yellowness (positive value= yellow, negative value= blue)

3.1.2. The growth and coloration parameters of pindani

In experiment I; the best live weight gains of the pindani, which initial weight varied between 0.280-0.300 g were obtained from M4 group (4% water-soluble marigold flower meal) at the end of 30 days. The best TLf, Wf, WG, and SGR results were obtained from the M4 group with statistically significant differences compared to the other groups (P<0.05). It was calculated that there were no significant differences between the groups in terms of FCR and SR (P>0.05). In experiment I, for pindani, there were statistically significant differences regarding the lightness (*L*) values (P<0.05). The highest amount of *L* was read from the control group, whereas the lowest *L* values belong to the groups M4 and M10 (P<0.05). According to the redness (*a*) results, we obtained the best color from M4 group, which closer to green (P<0.05).

Considering the yellowness indicator (*b*) values used as, although there were no differences between the groups, the closest value to blue was also obtained from M4 group (Table 3).

Table 3- Experiment I: Growth and coloration parameters of pindani (*P. socolofi*) fed with water-soluble marigold flower meal supplemented diets for 30 days

Experiment I: pindani							
	Control[1]	*M2*	*M4*	*M6*	*M8*	*M10*	*M12*
Wi	0.280±0.021^{a}	0.290±0.016^{a}	0.300±0.019^{a}	0.280±0.024^{a}	0.280±0.010^{a}	0.290±0.013^{a}	0.290±0.023^{a}
Wf	0.512±0.016^{a}	0.554±0.015^{b}	0.582±0.022^{c}	0.532±0.023^{ab}	0.520±0.019^{a}	0.530±0.014^{ab}	0.518±0.021^{a}
TLi	2.540±0.167^{a}	2.600±0.141^{a}	2.600±0.158^{a}	2.640±0.152^{a}	2.600±0.100^{a}	2.640±0.114^{a}	2.600±0.158^{a}
TLf	3.320±0.084 ^{a}	3.520±0.130^{ab}	3.620±0.205^{b}	3.500±0.122^{ab}	3.460±0.167^{ab}	3.480±0.148^{ab}	3.440±0.089^{ab}
WG	0.233±0.003^{a}	0.264±0.001^{c}	0.279±0.06^{d}	0.248±0.003^{b}	0.240±0.000^{ab}	0.238±0.002^{a}	0.233±0.004^{a}
SGR	2.020±0.055^{ab}	2.154±0.025^{bc}	2.184±0.021^{c}	2.75±0.155^{abc}	2.064±0.000^{abc}	1.987±0.003^{a}	1.980±0.002^{a}
FCR	3.664±1.090^{a}	3.220±0.925^{a}	3.036±0.797^{a}	3.428±0.922^{a}	3.542±1.002^{a}	3.572±1.044^{a}	3.664±0.759^{a}
SR	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}
L	78.450±01.340^{b}	75.680±2.060^{ab}	72.160±1.47^{a}	75.300±0.400^{ab}	75.750±1.190^{ab}	73.700±3.190^{a}	75.300±4.240^{ab}
a	-6.000±0.2800^{b}	-6.520±0.130^{ab}	-6.960±0.270^{a}	-5.830±0.250^{b}	-6.480±0.460^{ab}	-6.300±0.850^{ab}	-6.430±0.250^{ab}
b	-1.720±0.3700^{a}	-1.700±0.330^{a}	-1.990±0.130^{a}	-1.950±0.160^{a}	-1.570±0.300^{a}	-1.550±0.100^{a}	-1.640±0.400^{a}

[1], values (means±s.d.) in same line with different superscripts are significantly different (P<0.05). For the growth and for the color parameters five and three fish were measured from each replicates respectively; Control, basal diet without supplementation (180 mg kg^{-1} total carotenoid); M, water-soluble marigold flower meal (1040 mg kg^{-1} total carotenoid); M2, 2% M (20.8 mg total carotenoid); M4, 4% M (41.6 mg total carotenoid); M6, 6% M (62.4 mg total carotenoid); M8, 8% M (83.2 mg total carotenoid); M10, 10% M (104 mg total carotenoid); M12, 12% M (124.8 mg total carotenoid); Wi (g), initial weight, Wf (g), final weight; TLi (cm), initial total length; TLf (cm), final total length; WG, wet weight gain; SGR, specific growth rate; FCR, feed conversion ratio; SR, survival rate; *L*, lightness (white=100, black=0); *a* = redness (positive value= red, negative value= blue); *b* = yellowness (positive value= yellow, negative value= blue)

3.2. Experiment II: Effects of different levels of water-soluble marigold flower meal and synthetic carotenoid

3.2.1. The growth and coloration parameters of blue streak hap

In experiment II; the blue streak hap (0.274-0.294 g), were fed with different levels of water-soluble marigold flower meal (M) and synthetic carotenoid (SC) during 30 days. We obtained the best Wf and TLf from M4 group, which was significantly different compared to the other groups (P<0.05). However, there was not a significant difference between the groups in terms of SGR, FCR, and SR (P>0.05). At the end of the 30 days, the difference concerning lightness (*L*), redness (*a*) and yellowness (*b*) values between the groups was not statically significant (P>0.05) (Table 4).

Table 4- Experiment II: Growth and coloration parameters of blue streak hap (*L. caeruleus*) fed with water-soluble marigold flower meal and synthetic carotenoid supplemented diets for 30 days

Experiment II: blue streak hap							
	Control[1]	*M2*	*M4*	*M8*	*SC50*	*SC100*	*SC150*
Wi	0.274±0.017^{a}	0.290±0.012^{a}	0.292±0.019^{a}	0.288±0.018^{a}	0.294±0.009^{a}	0.282±0.009^{a}	0.290±0.016^{a}
Wf	0.576±0.009^{a}	0.610±0.016^{bc}	0.638±0.008^{d}	0.596±0.011^{b}	0.620±0.014^{cd}	0.600±0.016^{bc}	0.610±0.021^{bc}
TLi	2.640±0.114^{a}	2.560±0.152^{a}	2.540±0.114^{a}	2.580±0.179^{a}	2.480±0.148^{a}	2.580±0.164^{a}	2.540±0.167^{a}
TLf	4.120±0.084^{ab}	4.180±0.130^{ab}	4.280±0.084^{b}	4.100±0.141^{a}	4.160±0.055^{ab}	4.180±0.148^{ab}	4.100±0.158^{a}
WG	0.302±0.002^{a}	0.319±0.006^{a}	0.348±0.011^{b}	0.307±0.009^{a}	0.327±0.005^{ab}	0.321±0.020^{ab}	0.322±0.012^{ab}
SGR	2.469±0.059^{a}	2.474±0.032^{a}	2.628±0.144^{a}	2.415±0.067^{a}	2.495±0.054^{a}	2.540±0.163^{a}	2.482±0.029^{a}
FCR	2.815±0.775^{a}	2.657±0.704^{a}	2.458±0.767^{a}	2.761±0.699^{a}	2.601±0.774^{a}	2.678±0.917^{a}	2.658±0.845^{a}
SR	100±0.00^{a}	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}	100±0.000^{a}
L	71.920±2.950^{a}	70.960±1.630^{a}	67.070±5.480^{a}	70.620±2.380^{a}	69.550±0.100^{a}	71.140±1.040^{a}	70.540±3.450^{a}
a	-6.450±0.570^{a}	-5.600±0.390^{ab}	-3.730±0.85^{c}	-5.090±0.12^{b}	-4.420±0.460^{bc}	-4.590±0.880^{bc}	-4.930±0.740^{b}
b	26.700±0.240^{a}	27.689±0.500^{a}	28.190±0.860^{a}	27.510±1.680^{a}	27.930±2.260^{a}	26.880±0.670^{a}	26.390±0.360^{a}

[1], values (means±s.d.) in same line with different superscripts are significantly different (P<0.05). For the growth and for the color parameters five and three fish were measured from each replicates respectively; Control, basal diet without supplementation (180 mg kg^{-1} total carotenoid); M, water-soluble marigold flower meal (1040 mg kg^{-1} total carotenoid); M2, 2% M (20.8 mg total carotenoid); M4, 4% M (41.6 mg total carotenoid); M8, 8% M (83.2 mg total carotenoid); SC, Synthetic carotene source (Carophyll®pink, DSM, Basel, Switzerland 80 g astaxanthin kg^{-1}); SC50, 50 mg astaxanthin; SC100, 100 mg astaxanthin; SC150, 150 mg astaxanthin; Wi (g), initial weight; Wf (g), final weight; TLi (cm), initial total length; TLf (cm), final total length; WG, wet weight gain; SGR, specific growth rate; FCR, feed conversion ratio; SR, survival rate; *L*, lightness (white=100, black=0); *a* = redness (positive value= red, negative value= blue); *b* = yellowness (positive value= yellow, negative value= blue)

3.2.2. The growth and coloration parameters of pindani

During 30 days, pindani (0.286-0.294 g), were fed with 2, 4 and 8% rates of M. In experiment II; this natural colorant M was compared to a SC (50, 100, 150 mg SC feed kg^{-1}). The best Wf and WG were obtained from M4 group (4% M) ($P<0.05$). It was determined that there were no significant differences between the groups regarding TLf, SGR, FCR, and SR ($P>0.05$). The highest value of lightness (*L*) was measured from group control. In contrast, the lowest *L* value belongs to the groups SC50 ($P<0.05$). According to the results given by the redness (*a*), the lowest value (nearest to green) was read from group SC50 (-8.920±0.150), the highest *a* value was taken from the control group, and the difference between the groups was statistically significant ($P<0.05$). Concerning the yellowness (*b*), it found its lower value (closest to blue color) in group SC50 and a statistically significant difference between the groups was determined ($P<0.05$) (Table 5).

Table 5- Experiment II: Growth and coloration parameters of pindani (*P. socolofi*) fed with water-soluble marigold flower meal and synthetic carotenoid supplemented diets for 30 days

Experiment II: pinhandi							
	Control	*M2*	*M4*	*M8*	*SC50*	*SC100*	*SC150*
Wi	0.290±0.010^{a}	0.286±0.013^{a}	0.286±0.011^{a}	0.294±0.013^{a}	0.292±0.008^{a}	0.292±0.013^{a}	0.294±0.018^{a}
Wf	0.608±0.008^{ab}	0.618±0.037^{ab}	0.632±0.016^{b}	0.612±0.019^{ab}	0.606±0.009^{ab}	0.608±0.011^{ab}	0.604±0.011^{a}
TLi	2.600±0.158^{a}	2.620±0.130^{a}	2.560±0.114^{a}	2.540±0.114^{a}	2.560±0.182^{a}	2.540±0.114^{a}	2.640±0.114^{a}
TLf	3.880±0.164^{a}	4.000±0.173^{a}	4.000±0.187^{a}	3.880±0.164^{a}	3.860±0.182^{a}	3.880±0.164^{a}	3.940±0.134^{a}
WG	0.317±0.009^{a}	0.332±0.020^{ab}	0.349±0.020^{b}	0.318±0.002^{a}	0.313±0.005^{a}	0.316±0.001^{a}	0.311±0.007^{a}
SGR	2.456±0.087^{a}	2.541±0.210^{a}	2.651±0.172^{a}	2.436±0.063^{a}	2.427±0.047^{a}	2.441±0.027^{a}	2.441±0.060^{a}
FCR	2.674±0.680^{a}	2.565±0.574^{a}	2.419±0.550^{a}	2.673±0.775^{a}	2.707±0.727^{a}	2.690±0.751^{a}	2.742±0.826^{a}
SR	100±0.000^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}
L	77.750±0.610^{d}	73.320±3.480^{bc}	70.900±2.710^{b}	76.870±2.780^{cd}	65.620±1.580^{a}	70.210±1.140^{b}	71.170±0.360^{b}
a	-6.230±0.080^{e}	-6.490±0.660^{de}	-7.920±0.190^{b}	-6.980±0.160^{cd}	-8.920±0.150^{a}	-7.550±0.420^{bc}	-7.470±0.430^{bc}
b	-1.440±0.300^{e}	-1.890±0.170^{de}	-2.140±0.140^{cd}	-2.010±0.100^{cd}	-3.260±0.460^{a}	-2.440±0.330^{bc}	-2.640±0.220^{b}

[1], values (means±s.d.) in same line with different superscripts are significantly different ($P<0.05$). For the growth and for the color parameters five and three fish were measured from each replicates respectively; Control, basal diet without supplementation (180 mg kg^{-1} total carotenoid); M, water-soluble marigold flower meal (1040 mg kg^{-1} total carotenoid); M2, 2% M (20.8 mg total carotenoid); M4, 4% M (41.6 mg total carotenoid); M8, 8% M (83.2 mg total carotenoid); SC, Synthetic carotene source (Carophyll®pink, DSM, Basel, Switzerland 80 g astaxanthin kg^{-1}); SC50, 50 mg astaxanthin; SC100, 100 mg astaxanthin; SC150, 150 mg astaxanthin; Wi (g), initial weight; Wf (g), final weight; TLi (cm), initial total length; TLf (cm), final total length; WG, wet weight gain; SGR, specific growth rate; FCR, feed conversion ratio; SR, survival rate; L, lightness (white=100, black=0); a = redness (positive value= red, negative value= blue); b = yellowness (positive value= yellow, negative value= blue).

Many aquatic organisms take natural carotenoids through their food for pigmentation of skin and muscle. The coloration of fish mainly depends on several factors such as carotenoid source and chemical structure, the lipid content of the feed, fish species and environmental conditions (Harpaz & Padowicz 2007; Yanar et al 2007; Yanar et al 2008; Del Villar-Martínez et al 2013; Swian et al 2014; Kumar et al 2017). In aquaculture applications, the supplementation of nutritional carotenoid is essential for skin and meat pigmentation. Since these pigment substances cannot be synthesized by fish. As it is known that the most important criteria determining the market value of aquarium fish is the color viability of the skin. The studies carried out on natural and artificial pigment sources as a feed additive for different culture fish species showed that the marigold and synthetic carotenoids provided coloration and improved the growth parameters (Büyükçapar et al 2007; Moorhead & Zeng 2010; Ansarifard et al 2018; Pezeshk et al 2019). Many studies in the literature have no adverse effects on the survival rate of natural carotenoids and in general terms, fish and crustaceans fed with diets containing carotenoids exhibit a higher survival rate (Ako et al 2000; Moorhead & Zeng 2010). It has been reported that Pacific white shrimp (*Litopenaeus vannamei*) fed with 350 mg kg^{-1} marigold-supplemented diet for five weeks and achieved a better survival rate compared to the control group (Arredondo-Figueroa et al 1999). There are also reported a better survival rate without statistical significance for Koi, *Cyprinius carpio* fed with marigold oleoresin additive diet (Swian et al 2014) and goldfish, *Carassius auratus* fed with *Spirulina* supplemented food (Kumar et al 2017). The current study results showed that the non-negative effect of carotenoid supplementation on survival rate for tested cichlids.

In the present study, according to the experiment I, results showed that the low dosages (M2, M4 and M6) water-soluble marigold flower meal supplemented groups were significantly improved regarding final mean weight and SGR values compared to the control group ($P<0.05$). It can be claimed that at the level of 4% water-soluble marigold flower meal supplemented group showed better growth performance than other groups for experiment II. As shown in the overall data evaluation; in the first and second experiment, the best final live weight and length gains were also found in the M4 group for both fish species. This study result was in parallel with the report that effects of carotenoids on the growth rates of Atlantic salmon, *Salmo salar* fry (Christiansen et al 1995), rainbow trout, *Oncorhynchus mykiss* (De La Mora et al

2006), shrimp, *Litopenaeus vannamei* (Ponce-Palafox et al 2006) goldfish, *Carassius auratus* (Sinha & Asimi 2007), and certain freshwater ornamental fish species (Ako et al 2000; Velasco-Santamaría & Corredor-Santamaría 2011).

It has been reported that natural carotenoids can be used in fish for promoting the skin coloration such as dwarf cichlid, *Microgeophagus ramirezi* (Harpaz & Padowicz 2007), red swordtail, *Xiphophorus helleri* (Ezhil et al 2008), clown anemonefish, *Amphiprion ocellaris* (Ramamoorthy et al 2010), yellow tail cichlid, *Pseudotropheus acei* (Güroy et al 2012), goldfish, *Carassius auratus* (Del Villar-Martínez et al 2013; Kumar et al 2017).

In the first experiment, regarding the comparison of lightness (*L*) value for blue steak hap, there were no significant differences between the control group and the increasing doses of water-soluble marigold flower meal groups ($P>0.05$) (Table 1). However, for pindani, shown in Table 2, 4% and 10% water-soluble marigold flower meal supplemented groups were better than the control group in terms of the darkest *L* values (lightness: white=100, black=0) ($P<0.05$). The redness (*a*) values from M4 group were found better than all groups for both cichlid fry ($P<0.05$). Besides, there were no significant differences between all groups in terms of yellowness (*b*) value ($P>0.05$). When the results of the experiment I, were evaluated, it can be assumed that the 4% water-soluble marigold flower meal supplemented diet had a positive effect on the skin coloration. This situation can also be explained as an indication of the carotenoid intake or transfer to the tissues to reach saturation levels (Yanar et al 2008).

In experiment II, there were no differences between the M and SC groups regarding the lightness (*L*), and yellowness (*b*) values of blue streak hap ($P>0.05$) except redness (*a*) values. However, for pindani, the best color values were taken from SC50 group in terms of lightness (*L*: lightness white=100, black=0), redness (*a*: positive value= red, negative value= blue) and yellowness values (*b*: positive value= yellow, negative value= blue), and this group followed by 4% water-soluble marigold flower meal supplemented group. It can be stated that using the 4% water-soluble marigold flower meal in the diet will improve the skin coloration of blue streak hap and pindani without any adverse effects on growth performance.

4. Conclusions

In conclusion, this study showed that the dietary supplementation of 4% water-soluble marigold flower meal as a natural carotenoid source could be used instead of the tested synthetic carotenoid, in cichlids, for the skin coloration, growth, and survival rate.

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Antibacterial Activity of Different Kefir Types Against Various Plant Pathogenic Bacteria

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ABSTRACT
Kefir is a probiotic, dairy product produced by the fermentative activity of a diverse range of lactic acid bacteria, acetic acid bacteria, and yeast. In this study, we revealed the antimicrobial spectra of five types of kefir supernatants (EG, AN, KF, KY and SD) from different regions of Turkey fermented for 24 and 48 h against seven plant pathogenic bacteria and one bacterial biocontrol agent *in vitro* and *in vivo* for the first time. *In vitro*, antibacterial activity was investigated by the disk diffusion agar method. Their antibacterial potencies varied according to the type of kefir and the fermentation time. Also, we showed that the antimicrobial activity of kefir could be attributable to antimicrobial substances in supernatants rather than the low pH. *In vivo*, studies using the most potent kefir type on cucumber and common bean with their pathogenic bacteria in the climate chamber showed no remarkable decrease in diseases but revealed an increase in some plant growth parameters. The application resulted in an increase of 22% in shoot fresh weight, 20% in shoot dry weight, 79% in root fresh weight and 113% in root dry weight in common bean, on the other hand, 25% in shoot fresh weight, 34% in root fresh weight and 30% in shoot dry weight in cucumber.

Keywords: Kefir; Probiotic; Plant pathogens; Biological control

1. Introduction

Bacteria cause diseases in plants which are generally characterized by morphological symptoms such as galls and overgrowths, wilts, leaf spots, specks and blights, soft rots, as well as scabs and cankers. In general, the most common and devastating plant pathogenic bacteria belong to genera Erwinia, Pectobacterium, Agrobacterium, Pseudomonas, Ralstonia, Burkholderia, Acidovorax, Xanthomonas, Clavibacter, Streptomyces, Xylella, Spiroplasma, and Phytoplasma (Bull et al 2010). The diseases caused by bacteria in essential crop plants cause financial problems on a global scale. For example, The U.S. government spent US$1 billion to eradicate citrus canker disease during 1995-2005, while the Indian government spent more than INR20 million to combat bacterial blight diseases in pomegranate during 2003-2008. These and other examples indicate the importance of controlling bacterial plant diseases throughout the world (Borkar & Yumlembam 2017).

There are many approaches to controlling the plant bacterial diseases but the most commonly used one is chemical control (Kannan & Bastas 2015). The chemical management of individual bacterial diseases has been largely driven by the use of copper compounds and the antibiotics (Sundin et al 2016). However, as a result of extensive use of these compounds, bacteria have started to evolve resistance either through mutation or the acquisition of a resistance gene(s) (Förster et al 2015; Sundin & Wang 2018). Also, the use of antibiotics in plant disease control is banned in European

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Union because of potential impacts on human health and the transfer of antibiotic resistance into clinical pathogens (Sundin et al 2016; Sundin & Wang 2018).

Kefir is a fermented milk product that contains both mainly lactic acid bacteria (*Lactobacillus*, *Lactococcus* spp., *Leuconostoc* spp., *Acetobacter* spp. and *Streptococcus* spp.) and yeasts (*Kluyveromyces* spp., *Torula* spp., *Candida* spp. and *Saccharomyces* spp.) (Güzel-Seydim et al 2011; Fiorda et al 2017). Various functional properties of kefirs such as cholesterol-lowering effects (Liu et al 2006) and the antitumor activities (Renner & Munzner 1991; Liu et al 2005; Guzel-Seydim et al 2006) were reported. There are also studies focusing on the effects of different kefir products. Jeong et al (2017) showed the antimicrobial activities of the exopolysaccharide (EPS) produced by a *Lactobacillus kefiranofaciens* strain which is a member of the microflora of kefir granules against *Listeria monocytogenes* and *Salmonella enteritidis* in their study.

Rodrigues et al (2005) conducted a study to examine the antimicrobial activity of the kefir and kefiran which is the polysaccharide extracted from kefir beads on various types of human pathogenic bacteria. Ulusoy et al (2007) studied the antibacterial effect of 24 h and 48 h fermented kefir produced from commercial starter culture against several food pathogens. Kim et al (2016) reported a study to elucidate the optimal fermentation time and conditions for antimicrobial activities of kefirs from different origins against food-borne pathogens. They found that the spectra and potencies of kefirs varied according to the type of kefir and the fermentation time. In addition to those, Marquina et al (2002) reported that the consumption of kefir caused to decrease in *Enterobacteriaceae* and *Clostridia* population in the mucosa of mice bowel. Furthermore, other than the main ones mentioned above various functional properties such as improving lactose tolerance, stimulation of immune system and control of irritable bowel symptoms are exhibited by kefir (Güzel-Seydim et al 2011).

Although kefir has been shown to have an antibacterial effect on human and food pathogens in several studies, there is no evaluation of such application for plant pathogenic bacteria, to the best of our knowledge. We investigated the *in vitro* antimicrobial activities of kefirs from different origins fermented for 24 and 48 h against seven plant pathogenic strains and one bacterial biocontrol agent. Additionally, we studied the effects of the kefir product on common bean and cucumber diseases and plant growth parameters in a climate chamber. This study aimed to elucidate the antimicrobial activities of kefirs against plant pathogenic bacterial strains and evaluate the potential of this dairy product to be used in plant pathology for the first time.

2. Material and Methods

2.1. Kefir and supernatant preparation

Five types of kefir grains, i.e., SD, AN, EG, KY and KF, were used in this study. Among them, kefir SD is the one which is being sold commercially and the others were collected from private households in a different region of Turkey. Starter grains (5 g) were continuously cultured in pasteurized, low-fat milk for 15 days prior to experiments. The medium was changed at 24 h intervals and the grains washed with sterile water. For antibacterial activity tests, viable kefir grains, 10% w/v, were inoculated in pasteurized, low-fat milk and cultured at 25 °C for 24 and 48 h. At the end of the fermentation process, the grains and milk were separated using a sterilized plastic filter (2-mm pore size).

For antimicrobial activity tests, kefir milk was centrifuged at 6,000 g for 10 min and the supernatant was sterilized by filtration using a 0.45-µm pore-size syringe filter (Millipore Co., USA). The pH of the filtered kefir supernatant was determined with a pH meter.

2.2. Bacterial strains

All bacterial strains were provided by the bacteriology laboratory in the Department of Plant Protection, Faculty of Agriculture, Van Yuzuncu Yil University. The pathogens; *Pseudomonas syringae* pv. s*yringae* (*Pss*), *Pseudomonas syringae* pv. *lachrymans (Psl), Pseudomonas syringae* pv. *tomato (Pst), Xanthomonas axonopodis* pv. *phaseoli (Xap), Xanthomonas euvesicatoria (Xe), Erwinia amylovora (Ea), Clavibacter michiganensis ssp. michiganensis (Cmm) and* the biocontrol agent of plant diseases, *Bacillus spp.* 66/3 (66/3*),* were used as the test strains in this study. All strain cultures were grown either in Nutrient Broth (NB) broth (Difco, Detroit, MI, USA) or on Nutrient Broth agar plates at 25 °C.

2.3. In vitro antibacterial activity

Antibacterial activity of kefir supernatants was evaluated using the disk diffusion method as described by the National Committee for Clinical Laboratory Standards (NCCLS 2002). Cell suspensions were prepared with the spectrophotometric method and the final bacterial cell concentration applied on the agar surface was approximated to 10^6 CFU. Sterile test disks (Bioanalyse Co., Ltd., Ankara, Turkey) were applied to the agar surface previously inoculated with 0.1 mL bacterial suspension. 20 µL of each kefir supernatant was directly dropped onto the surface of disks. The plates were incubated for 48 h at 25 ºC, and the inhibition zones were measured. All experiments were done in nine replicates. The presence of a clear zone around the disks was considered as inhibition.

To see whether the antimicrobial activity of kefirs could be attributable to low pH value or not, we conducted a control experiment. For this, lactic acid solution (Sigma-Aldrich, USA), acetic acid solution (Merck, Germany), and absolute ethyl alcohol (JT Baker, USA) were diluted with sterilized distilled water. The pH of the diluent was adjusted to 3.5 for both acid solutions. Ethyl alcohol was diluted to 2.0% v/v. All solutions were sterilized by filtration using a 0.45-µm pore-size syringe filter before use. Antibacterial activity of control solutions was evaluated as mentioned above.

2.4. In vivo antibacterial activity

In this part of the study, kefir AN fermented for 48 h which was one of the most potent kefir types on *Psl* and *Xap* in *in vitro* tests were investigated in climate room. Bacterial blight disease of common bean (*Phaseolus vulgaris cv.* Gina) caused by *Xap* and angular leaf spot disease of cucumber (*Cucumis sativus cv.* Gordion F1) caused by *Psl* were tested in a climate chamber.

2.5. Cultivation of plants, application of pathogens and kefir supernatant

Pesticides free cucumber and bean seeds were planted in 250 mL volume containers filled with sterile peat and left in a climate room at 24±2 °C with 60% humidity and 14-h light conditions. During the study, nutrition required by seedlings was provided as recommended by Akköprü & Özaktan (2018).

The supernatant was diluted with sterile distilled water at a rate of 1 to 3 just before application to prevent phytotoxicity due to its low pH. The supernatant was applied to the plants three times in two different ways. First, the suspension was applied twice to the seedlings by drenching method with 10 mL plant^{-1} and 20 mL plant^{-1} 48 h prior to the pathogen application. The second, the suspension was sprayed onto plants 24 h prior to the pathogen application.

For the application of pathogens, 48-h *Psl and Xap* cultures grown on Nutrient Broth medium were prepared in suspension with 10^8 cfu mL^{-1} and 0.01% Tween 80 was added as a surfactant. Pathogens were inoculated with a hand sprayer 24 h after last application of kefir supernatant. The *Psl* suspension was applied to the cucumber seedlings when the second true leaves began to open (Akköprü & Özaktan 2018). The *Xap* culture was applied to the bean seedlings during the trifoliate leaf period (Akköprü et al 2018). Immediately after pathogen application, the plants were left in high relative humidity for 48 hours.

2.6. Determination of disease severity and plant development parameters

The diseases severity ratings were based on the infected leaf area. Three weeks after *Xap* application, disease symptoms on common bean plants were assessed by scale 1-5 (1: no disease symptoms, 2: a few necrotic spots or ≤ 5%, 3: 6-25%, 4: 26-50%, 5: ≥ 50% and defoliation) (Abbasi et al 2002). 14 days after Psl application, disease symptoms on cucumber plants were assessed by scale 1-6 (1: no disease symptoms, 2: a few necrotic spots or < 10%, 3: 10-25%, 4: 26-50%, 5: 51-75%, 6: > 76% or fallen or dead leaves) (Akköprü & Özaktan 2018). The disease index (1) and % efficacy (2) were calculated using the following formulas;

$$Disease\ \ index = \frac{\sum (Rating\ \ number \times Number\ \ of\ \ leaves\ \ in\ \ the\ \ rating)}{Total\ \ number\ \ of\ \ leaves \times Highest\ \ rating} \times 100 \quad (1)$$

$$Efficacy\% = \frac{Control\ \ value - treatment\ \ value}{Control\ \ value} \times 100 \quad (2)$$

The effect of Kefir supernatant on cucumber and common bean were determined in the 15th day and 21st day, respectively. After the root parts of the seedlings cut from the root collar were washed, roots and shoots were weighed separately to determine fresh weight. Then they were dried at 65 ºC for 72 h and weighed again to obtain dry weights.

2.7. Statistical analysis

All in vitro experiments were performed in nine replicates. In vivo experiments were set up according to completely randomized with ten replicates. SPSS 17.0 package was used for statistical analysis.

3. Result and Discussion

3.1. In vitro antibacterial activity

Antimicrobial spectra of the kefir supernatants for 24 h and 48 h against the plant pathogens are presented in Table 1 and 2, respectively. Antimicrobial activity generally increased along with prolonged fermentation time in all types of kefirs; this effect was evaluated by using Student's t-test and found statistically important (Table 3). This test indicated that all kefir types showed higher antibacterial activity against *Pss* and *Xe* after 48 h fermentation compared with 24 h fermentation. On the other hand, not all kefir types showed a statistically significant increase in antibacterial activity with time against all bacterial strains. For example, the antibacterial activity of kefir types increased with prolonged fermentation time against *Cmm*, except for kefir KF. It did not exhibit a statistically significant increase in antibacterial activity with time against this pathogen. The similar situation was also observed for *Ea*, *Pst, Psl*, *Xap* and *66/3* (Table 3).

Table 1- Antibacterial spectrum of five types of kefir supernatants fermented for 24 h against eight bacterial strains. The results represent the mean zone diameters (in mm) using the disk diffusion method

Kefir Types	*EA Sig. 0.0001*	*PST Sig. 0.0001*	*PSL Sig. 0.0001*	*XAP Sig. 0.0001*	*XE Sig. 0.0001*	*66/3 Sig. 0.0001*	*CMM Sig. 0.001*	*PSS Sig. 0.001*
EG	9.78±0.15^{ab}	8.33±0.24^{b}	9.33±0.26^{c}	10.20±0.15^{b}	7.00±0.01 ^{c}	6.40±0.18^{bc}	8.33±0.24^{c}	8.00±0.29^{bc}
KY	9.89±0.11^{ab}	7.11±0.26^{c}	9.78±0.15^{bc}	9.50±0.33^{b}	6.11±0.33^{d}	5.67±0.24 ^{c}	9.33±0.41 ^{b}	7.33±0.67^{c}
AN	10.67±0.75^{a}	10.11±0.39^{a}	11.00±0.65^{ab}	9.56±0.50^{b}	7.67±0.17^{c}	6.44±0.44^{bc}	6.11±0.48 ^{d}	8.22±0.70^{bc}
KF	8.60±0.71^{b}	8.89±0.35^{b}	11.33±0.67^{a}	5.00±0.01^{b}	13±0.29^{a}	8.50±0.90^{a}	16.20±0.28 ^{a}	11.40±0.96^{a}
SD	9.78±0.40^{ab}	10.9±0.35^{a}	10.00±0.17^{bc}	9.22±0.49^{a}	9.67±0.33^{b}	7.56±0.80^{ab}	6.44±0.18^{d}	9.89±0.26^{ab}

*Mean values followed by the same letter were not significantly different based on the Duncan's Multiple Range Test at P< 0.05 significance level.

Table 2- Antibacterial spectrum of five types of kefir supernatants fermented for 48 h against bacterial strains. The results represent the mean zone diameters (in mm) using the disk diffusion method

Kefir Types	*EA Sig. 0.001*	*PST Sig. 0.001*	*PSL Sig. 0.001*	*XAP Sig. 0.001*	*XE Sig. 0.0001*	*66/3 Sig. 0.001*	*CMM Sig. 0.001*	*PSS Sig. 0.001*
EG	10.00±0.65^{c}	9.33±2.06^{c}	12.00±0.69^{c}	8.00±0.26^{cd}	16.89±0.89^{a}	6.56±0.24^{d}	15.78±0.68^{b}	8.67±0.37^{d}
KY	10.00±0.17^{c}	10.3±0.50^{c}	10.22±0.37^{d}	9.00±0.17^{c}	12.78±0.70^{b}	9.44±0.50^{c}	10.11±0.35^{c}	10.78±0.64^{c}
AN	15.44±0.40^{a}	15.67±1.66^{a}	18.22±0.87^{a}	16.11±0.79^{a}	15.33±0.53^{a}	13.89±0.98^{a}	7.89±0.26^{d}	13.56±1.04^{b}
KF	13.33±0.60^{b}	12. 67±1.00^{b}	15.89±0.62^{b}	6.67±0.37^{d}	16.17±1.05^{a}	11.78±0.43^{b}	18.78±0.36^{a}	16.22±0.88^{a}
SD	12.89±0.56^{b}	15.33±0.28^{a}	14.22±0.41^{b}	13.44±0.44^{b}	17.44±1.02^{a}	12.11±0.35^{b}	10.11±0.72^{c}	14.89±0.48^{ab}

*Mean values followed by the same letter were not significantly different based on the Duncan's Multiple Range Test at P< 0.05 significance level.

Our results are consistent with those of the study conducted by Kim et al (2016). They found that the spectra and potencies of kefirs against several food pathogens varied according to the type of kefir and the fermentation time. Also, Silva et al (2009) reported that the antimicrobial activities of kefirs generally increased with prolonged fermentation times. However, Ulusoy et al (2007) showed no difference in the antimicrobial activities of kefirs fermented for 24 or 48 h against some food-borne pathogens *in vitro.*

In general, all kefir types showed a remarkable antibacterial effect against the bacteria we used. Kefir AN was the most potent one on *Ea*, *Pst, Psl*, *Xap*, and *Xe*. *Xe* was inhibited by all kefir types strongly except kefir KY which gave a slightly smaller zone compared to others. On the other hand, kefir KF gave the highest activity against *Cmm*, *Pss*, and *Xe* (Table 2). Those results suggest that kefirs from different origins have different antimicrobial spectra against a particular

strain. Also, the results indicate that the same kefir type may have different antibacterial potencies against different species and even subspecies.

Table 3- Comparasion of fermentation time (24 and 48 hours) on antibacterial activities of kefir supernatants against bacterial strains using Student's t-test

Strain/Kefir	*EG*	*KY*	*SD*	*KF*	*AN*
EA	-0.34*±0.741	-0.56*±0.588	-4.49±0.001	-4.80±0.001	-4.27±0.001
PST	-1.38*±0.001	-10.42±0.001	-9.77±0.001	-7.80±0.001	-8.22±0.001
PSL	-3.67±0.002	-1.13*±0.283	-9.73±0.0001	-4.82±0.001	-6.70±0.001
XAP	8.06±0.0001	1.41*±0.179	-6.36±0.001	-4.47±0.001	-4.16±0.001
XE	-13.24±0.001	-6.47±0.001	-5.44±0.001	2.36±0.034	-6.27±0.001
66/3	1.48*±0.163	-10.51±0,001	-3.47±0.003	-13.18±0.001	-2.80±0.015
CMM	-9.30±0.001	-4.24±0.001	-10.68±0.001	0.05*±0.961	-12.88±0.001
PSS	-1.41±0.176	-3.73±0.002	-9.09±0.001	-3.67±0.002	-4.24±0.001

One-way variance analysis was carried out using Student's t-test ($P<0.05$) to detect a significant difference between variables; *, means no statistically significant effect of prolonged fermentation time on antibacterial activities of kefir

The microbial composition of kefir varies according to kefir origin, the substrate used in the fermentation process and the storage conditions also (Prado et al 2015). Pintado et al (1996) found different microbiological diversity in terms of yeast and Lactobacilli species content in Portuguese kefir grains compared to some other kefir grains such as Russian, Yugoslavian and Bulgarian grains. Also, it was shown that the composition of Tibetan kefir differs from that of others such as Russian, Taiwan or Turkey kefir. (Gao et al 2012; Altay et al 2013). Since the substrate used in the fermentation, storage and handling conditions were all the same for each kefir grain in our study, it can be concluded that different kefir types have variable antibacterial spectra against the bacterial strains due to their different origins.

The probiotic species, especially lactobacilli, are known to produce a wide range of antimicrobial compounds such as organic acids (lactic and acetic acids), hydrogen peroxide, ethanol, and bacteriocins (Guzel-Seydim et al 2011; Kim et al 2015). In this study, during the fermentation process, the pH gradually decreased in all kefir samples (Table 4). However, our overall data suggested that the antimicrobial activity of kefirs could not be attributable to low pH value simply. To demonstrate this, the antimicrobial activities of lactic and acetic acid solutions against the test strains were also investigated. All strains were resistant to both acid solutions at pH 3.5. The growth of test strains was inhibited by kefir supernatants, although the lowest pH value of kefirs in our study was 3.67. Additionally, since some yeast strains produce ethyl alcohol during kefir fermentation, we wanted to use ethyl alcohol solution (2% v/v) to see the antibacterial activity of it on the test strains. There are variations between 0.01% and 0.1% (v/v) among the reported ethanol contents of kefir (Guzel-Seydim et al 2000). The growth of all strains, however, was not affected by even higher ethyl alcohol solution. Therefore, we concluded that the antimicrobial activity of kefir could be attributable to the effect of antimicrobial substances in the kefir supernatants rather than the low pH.

Table 4- pH values of five different kefir supernatants fermented for 24 and 48 hours. Measurements were performed in supernatants at temperatures of 24±2 °C

Kefir types	*pH value according to fermentation times*	
	24 h	*48 h*
EG	3.80	3.70
KY	3.95	3.75
AN	3.91	3.67
KF	4.50	4.20
SD	3.82	3.70

It could be postulated that the antimicrobial effect may be caused by the antagonistic action of metabolites and inhibitory compounds synthesized and released into the supernatant by various microorganisms present in kefir, and possibly interact with each other to enhance or antagonize their antimicrobial effects.

3.2. In planta studies

The effect of using Kefir AN, one of the most potent kefir types, on *Psl* and *Xap* were also investigated in the climate

room. *Xap* causes common bacterial blight disease in bean and *Psl* causes angular leaf spot disease in cucumber.

The application of kefir supernatant to bean seedlings resulted in a statistically significant increase in all plant growth parameters. It resulted in an increase of 22% in shoot fresh weight, 20% in shoot dry weight, 79% in root fresh weight and 113% in root dry weight. However, no effect on plant growth parameters was observed under disease pressure (Figure 1).

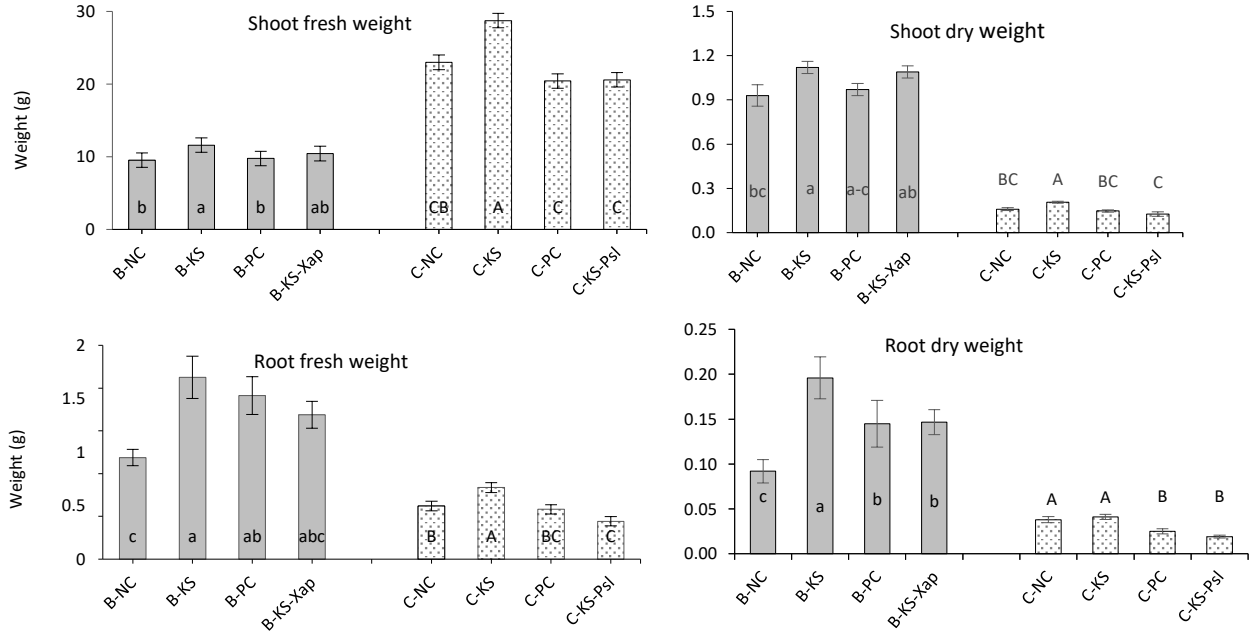

Figure 1- The effect of the supernatant on common bean and cucumber growth parameters with and without the disease pressure in a climate chamber. NC, negative control; KS, kefir supernatant only; C, cucumber, B, bean; PC, positive control. Mean values followed by the same letter are not significant (P<0.05) (N≥15)

On the other hand, in cucumber experiments, kefir supernatant application significantly increased at the rate of 34% in root fresh weight, 25% in shoot fresh weight and 30% in dry weight but did not cause an increase in root dry weight. However, no effect on plant growth parameters was observed under disease pressure (Figure 1).

Also, the application did not show any inhibitory effect on common bacterial leaf blight disease symptoms in bean and angular leaf spot disease symptoms in cucumber (Figure 2).

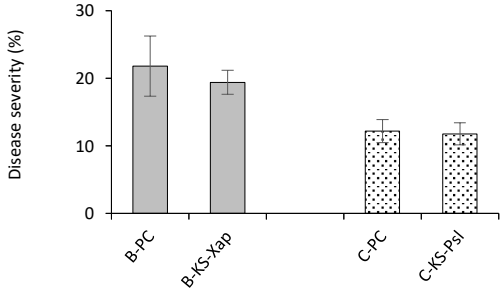

Figure 2- The effect of the supernatant on Angular leaf spot of cucumber caused by *Psl* and Common bacterial leaf blight disease of bean caused by *Xap* in climate room. The severity of diseases on bean and cucumber were assessed by scale 1-5 and 1-6, respectively. B, common bean; C, cucumber; KS, kefir supernatant; PC, positive control. Mean values followed by the same letter are not significant (P<0.05) (N≥15)

The positive effect of kefir supernatant on plant development parameters may be because of the presence of many organic and bioactive compounds in the supernatant. These compounds might have been used by plant cells and plant-associated bacteria in many ways, such as carbon and other plant nutrients sources. Also, many undefined bioactive

compounds of kefir supernatant might have affected metabolic pathways of plant cells or plant-associated bacteria which resulted in an increase of those parameters we measured.

On the other hand, we did not observe any inhibitory effect of kefir supernatant on common bacterial leaf blight disease of bean caused by *Xap* and angular leaf spot disease of cucumber caused by *Psl*. At first, we used supernatant without any dilution on plants, however; it produced slightly phytotoxicity most probably due to its low pH. Buffering the supernatant to increase the pH caused strong precipitation. Therefore, we diluted the supernatant with a 1/3 ratio with sterile distilled water before use. Phytotoxicity was prevented by this way but we could not observe any inhibitory effect on plants infected with *Xap* or *Psl*. It can be speculated that inhibitory compounds of the supernatant might have been degraded by plants itself or natural microflora on them. The other possible explanation is that dilution of the supernatant might have prevented the inhibitory compounds to exert their activities on the pathogens due to their low concentration.

Future studies should be followed to determine whether a single compound is responsible for antimicrobial properties or this was a result of antagonistic effects of various metabolites in kefir. Also, it should be created more knowledge about the key antimicrobial compounds against each pathogenic bacterium and their mechanisms. By this way, they can be used more effectively as a single compound against plant bacterial diseases without affected by undesirable conditions like low pH or dilution effect. In addition, after the isolation and identification of each microorganism in the microflora of kefir, they should be investigated as biological agents against plant pathogens.

4. Conclusions

In conclusion, our results revealed different antibacterial potencies according to the type of kefir and the fermentation time. Although we could have not shown this effect in planta studies, some plant growth parameters increased significantly. There is an urgent need for the development of novel antimicrobial agents against pathogenic strains in agriculture. In parallel with the recent developments in the new generation technologies, natural product research has also gained positive momentum. With these technologies, the potential of organisms to produce natural products can be revealed. In principle, such compounds may also find applications in medicine where the antibiotic-resistance problem is also increasing. New narrow-spectrum antimicrobials like bacteriocins may contribute to agriculture's need for more sustainable and effective strategies for plant disease control.

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Dry Period in Cattle: I. Influence on Milk Yield and Reproductive Performance

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ABSTRACT
Purpose of this study was to investigate the relations of dry period lengths (DPL) with subsequent lactation (305-d) milk yield (MY) and reproductive performance of Holstein cattle. Data were obtained from 800 Holstein cows raised in a private dairy operation and which were in different parity (2nd, 3rd and ≥4th). DPL was classified in 5 categories as; ≤40, 41-50, 51-60, 61-70 and ≥71 days. The differences in lactation milk yields of experimental DPL groups were not significant. The highest MY (7808.6±135.1 Lt) was obtained from ≥71 days DPL group and the lowest MY (7529.4±159.8 Lt) was obtained from ≤40 days DPL group. DPL had significant effect on the number of inseminations resulted in pregnancy (P<0.01). The greatest pregnancy ratio (53.0%) in the first insemination was obtained from ≤40 days DPL group and the lowest pregnancy ratio (30.8%) was obtained from 61-70 days dry period group. There was a positive correlation (0.056) between DPL and 305-d MY and a highly significant positive correlation (0.141) between DPL and the number of insemination resulted in pregnancy. Present findings revealed that longer DPL might have positive effects on lactation MY, but shorter DPL practices might have better outcomes for pregnancy ratios of the first insemination for this farm.

Keywords: Dairy cattle; Dry period length; Herd management; Milk yield; Reproductive performance

1. Introduction

Herd management practices together with recent developments in dairy cattle genetics increased milk yield (MY) per cattle (Bachman & Schairer 2003). Dry Period Length (DPL) has significant effects on MY. DPL plays an important role in regeneration of old mammary epithelium cells and increases mammary gland epithelium cell components for the next lactation (Capuco et al 1997). As a common herd management practice in several dairy operations, DPL is usually applied as between 51 and 60 days (Bachman & Schairer 2003). This duration is sometimes practiced as between 40 and 60 days. However, previous researches indicated that DPL of less than 40 days reduced MY of subsequent lactation by 10-30% (Annen et al 2004). In case MY was sustained after a shorter dry period than the current standard dry period, then such a standard period may loss the validity (Bachman & Schairer 2003). While no yield losses were reported for dairy cattle with dry periods shorter than 30 days in a study (Annen et al 2004), lower lactation performance values were reported for the cows with shorter dry periods than the cows with a normal standard (conventional) dry periods in another study (Atashi et al 2013).

Reproductive performance (RP) of dairy cattle is also influenced by DPL. However, genetic factors, management and feeding practices are the primary factors influencing RP of cattle (Beever 2006). Dairy facilities can develop alternative management strategies to improve reproductive efficiency of the dairy cattle. Elimination of long dry periods or shortening these periods may improve RP and thus improve energy balance of the organism (Grummer 2007). Following the parturition, a negative energy balance is developed in dairy cows and losses are observed in body condition to support the milk synthesis. With the alternative management systems, such losses can be prevented in

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prenatal dry period and early postnatal period (de Feu et al 2009). Shortened dry periods have recently attracted the attentions of dairy operations. However, effects of this management practice on RP of the cattle haven not been well elucidated, yet (Gumen et al 2005). The studies carried out to determine minimum DPL were mostly included visual data and retrospective analyses (Bachman & Schairer 2003).

In the present study, the effects of different DPL on subsequent lactation MY and RP of 800 Holstein cow at different parities were investigated.

2. Material and Methods

Data obtained from a commercial dairy farm were used in the study thus measurement of phenotypic characteristics was performed under the routine management and breeding procedure for cattle at farm, no animal experiment and additional handling was involved in the study. Therefore, no ethics approval was necessary.

In the study, data obtained from dairy cattle raised in an intensive commercial dairy breeding operation located in Central Anatolia region of Turkey (Latitude:38°.34'66.79, Longitude: 35°.47'84.66) were used. Data were gathered for 800 heads Holstein cow at different parity (2^{nd}, 3^{rd} and $\geq 4^{th}$). The cattle used in the present study did not have clinical mastitis symptoms and milk from these cattle had somatic cell counts (SCC) of<250.000 cell mL^{-1}. Parities and calving body weights (kg) of multiparous cattle used in this study are provided based on DPL in Table 1.

Table 1- Parities and calving body weights (kg) of multiparous cattle based on dry period length

Lactation	*Dry period length (days)*											
	≤40 (days)		*41-50 (days)*		*51-60 (days)*		*61-70 (days)*		*≥71 (days)*		*General*	
	n	*BW (kg)*	*n*	*BW (kg)*	*n*	*BW (kg)*	*n*	*BW (kg)*	*n*	*BW (kg)*	*n*	*BW (kg)*
2	30	613	47	610	106	597	33	579	15	614	231	603
3	34	609	58	627	156	613	84	623	72	609	404	616
≥4	19	617	23	616	65	600	29	604	29	617	165	611
General	83	613	128	618	327	603	146	602	116	613	800	610

BW, body weight (kg)

The procedure for drying off the cows were carried out by reducing the number of daily milking frequency of 3 gradually to 2 and 1 when the daily milk yield of the cattle decreased to 10 liters (10 Lt/cow) or below. The time between full termination of milking and parturition was monitored as dry period. Mastitis checkups (California Mastitis Test) were conducted and dry period antibiotics (Benzathine Cloxacilin) were administered to the cows. A special feeding was not practiced, all cattle were fed with rations suitable for their physiological stages. Cattle were fed after each milking and 3 times in a day with TMR (Total Mixed Ration) to meet their pregnancy, yield and maintenance requirements (NRC 2001). Composition of TMR given to the cows at dry period and different stages of lactation, is provided in Table 2.

Water was provided *ad libitum*. Cows were housed in partially open sheltered barns from drying off to parturition. Then, the ones showing parturition symptoms were taken into the parturition rooms and the parturition was performed. Lactation started right after parturition. Cows were milked 3 times in a day (at 07:00, 15:00 and 23:00). Milk yields of the individual cows were recorded with computerized herd management system and the total lactation period was considered as 305 days.

Animal health was inspected daily by the veterinarian of the dairy operation and health monitoring records were kept for each animal.

Following the parturition, vaginal secretion-mucous, uneasiness, hyperactivity, frequent roaring and etc. estrous symptoms were observed in every 12 hours (morning/evening) and monitored through pedometer data in a computer. Almost of all 800 cows presented estrous symptoms (visual symptoms+pedometer data) on average 50 days after parturition (45-55 days) and their first insemination (artificial insemination) was performed. Pregnancy tests were carried out 70 days after insemination through rectal palpation. Health inspections were performed for non-pregnant cows and insemination was repeated at next estrous.

Table 2- Composition and nutrient contents of TMR given to cows at dry periods and different stages of lactation

Physiological stage	*Dry period 1*	*Dry period 2*	*Early lactation*	*Milk yield*	
	Drying of 20 days ahead of parturition	*20 days to parturition-parturition*	*Initial 30 days of lactation*	*> 25 Lt*	*< 25 Lt*
Concentrate feed[1], kg	1.5	3	0	0	0
Concentrate feed[2], kg	0	0	8	8	4.5
Maize silage, kg	5	7	18	20	20
Dry alfalfa fodder, kg	2	2.5	4	2	2
Dry vetch fodder, kg	1	2.5	0	0	0
Kernel corn, kg	1	2	4	5	4
Vetch silage, kg	3	0	0	0	0
By-pass fat, kg	0	0	0.4	0.5	0.1
Malt pulp, kg	0	0	3	4	4
Propylene Glycol, kg	0	0.2	0.4	0	0
Alfalfa silage, kg	2.5	0	0	1	2
Hay, kg	2	0	0	0	0
Total[3], kg	18	17.2	37.7	40.5	36.6
Composition[4]	*Dry period 1*	*Dry period 2*	*Early lactation*	*MY > 25 Lt*	*MY < 25 Lt*
Roughage, DM%	22.4	39.5	46.2	48.1	37.3
Concentrate feed, DM%	776	60.5	35.8	51.9	62.7
Dry matter, kg	9.9	11.2	23.1	23.9	20.1
Crude protein, DM%	10.3	12.2	13.5	13.4	13.2
ME, Mcal kg^{-1} DM	2.1	2.4	2.5	2.5	2.4

[1], 2750 Kcal kg^{-1} ME and 16% CP; [2], 2750 Kcal kg^{-1} ME and 16% 25; [3], Ca, P, K, NaCl, trace elements and vitamins A, D, and E were supplemented at concentrations to meet or exceed NRC recommendations; [4], contents calculated based on analysis results; DM, dry matter; ME, metabolic energy; CP, crude protein; MY, milk yield

Statistical analyses were performed with IBM SPSS Statistics 22.0 software. The 305-day milk yield was tested by taking the Least Square Means into consideration in accordance with General Linear Model procedure and results were presented in means ± standard error. The fallowing model is used;

$$Y_{ijklm} = \mu + a_i + b_j + c_k + d_l + e_{ijklm}$$

Where; Y_{ijklm}, milk yield; μ, overal mean; a_i, treatment effects (DPL); b_j, parity (2, 3, ≥4); c_k, calving year (1-2); d_l, calving season (1, 2, 3, 4); e_{ijklm}, residual random errors.

In the model, the dependent variable was MY and independent variables (fixed effect) were DPL, parity, calving year and calving season.

The relationships between DPL and categorical variables (number of inseminations resulted in pregnancy) were tested with Pearson Chi-Square Test and the results were presented in number and ratio (n, %). Differences among the group means were assumed to be significant at $P<0.05$. Also, the correlations among DPL, lactation (305 days) milk yield and number of insemination ended up with pregnancy (1^{st}, 2^{nd} and $\geq 3^{rd}$) parameters were calculated with SPSS (2013) software.

3. Results and Discussion

The relationships between DPL and subsequent lactation (305-d) milk yield (MY) based on parities are provided in Table 3. Lactation MY of the dry period groups did not differ ($P>0.05$). Considering the general results (Table 3), the greatest milk yield (7808.6±135.1 Lt) was obtained from ≥71-day dry period group and the lowest milk yield (7529.4±159.8 Lt) was obtained from ≤40-day dry period group. The cows with ≤40-day dry period had an average of 279 Lt less milk yield than the cows with ≥71-day dry period.

Table 3- The relations of DPL with lactation MY (305-d)

Parity	*Dry period length (days)*						*Fixed Factors*	
	≤40 (n= 83)	*41-50 (n= 128)*	*51-60 (n= 327)*	*61-70 (n= 146)*	*≥71 (n= 116)*	*Total*	*Calving year season*	*Parity*
	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$		
	Lactation milk yield (Lt)							
2	7380.6±257.3	7558.4±205.6	7422.2±136.9	7745.5±245.3	7822.5±363.9	7585.7±113.1		
3	7543.6±251.7	7732.1±192.7	7782.9±117.5	7694.6±160.1	7885.6±173.0	7727.8±82.4		
≥4	7741.3±342.6	7577.2±311.4	7331.3±185.2	8019.5±277.3	7609.9±277.3	7655.8±126.9		
General	7529.4±159.8	7640.5±128.7	7576.2±80.5	7770.6±120.5	7808.6±135.1	7665.9±57.0	**	** ns

**, P<0.01; ns, not significant

The reason for low milk yield in cows with short DPL may be due to less number of breast epithelial cells in these cows. Because, the DPL provides an opportunity to repair the damage to the mammary gland of the cow, the cells of both the alveolar and canal system, and the damage to the lactation period. Cows store mineral and vitamin for the next lactation during dry period, mammary epithelial cells are rested and prepared for lactation period. Dry period was necessary to replace mammary epithelial cells, thus providing one biological basis for the lower milk yield that has been observed with shortened dry periods.

It is commonly estimated that a two month dry period provides a complete regeneration of udder glandular tissue and is favorable for the high production in the forthcoming lactation (Annen et al 2004; Andersen et al 2005). However, more controlled studies are warranted to examine cellular mechanisms that are involved in this process.

In a similar study, van Knegsel et al (2014) reported that the cows with 0 and 30-day dry periods had less milk yield than the cows with 60-day dry period. Similarly, Pezeshki et al (2008) indicated the reasons for less milk yield of the cows with shorter dry periods (≤40 days) as the differences in endocrine systems, decrease in number of mammary epithelium cells and recession in mammary epithelium functions. However, there is still a need for further studies to investigate mammary cell mechanisms in detail. Some other previous researchers also assessed the negative effects of short dry periods on lactation MY (Pezeshki et al 2007; Pezeshki et al 2008; Watters et al 2008; Bernier-Dodier et al 2011). However, different from these studies, Gulay et al (2003) and Jolicoeur et al (2009) investigated the effects of shorter and longer dry periods on MY and indicated that short duration of dry periods could reliably be practiced without significant losses in subsequent lactation MY. In present study, DPL had no effect on milk yield of the cows in their 2nd, 3rd and ≥4th lactations, were not found to be significant. Santschi et al (2011a) in a previous study with Holstein cows applied different DPL (35 and 60 days) and reported that while the effects of DPL on lactation MY were not significant in ≤3rd lactation cows, effects of dry periods on MY of 2nd lactation cows were significant. Researchers (Santschi et al 2011a), also reported significantly reduced milk yield for 35-day dry period.

There is limited published research on the effect of DPL on reproduction and fertility. In the present study, the relationships between DPL and number of inseminations resulted in pregnancy were also investigated. The results for such relations are provided in Table 4. The results were not significant in the 2nd and ≥4th lactations, but significant in the 3rd lactation (P<0.01). The general results including together assessment of 2nd, 3rd and ≥4th lactations were also significant (P<0.01) and such significance was mainly resulted from the 3rd lactation. According to general results, the highest pregnancy ratio in the first insemination (53.0%) was obtained from ≤40-day dry period group and the lowest value (30.8%) was obtained from 61-70-day dry period group.

The shortening or complete removal of the dry time will probably reduce the energy spent on milk production with a decrease in milk yield and may lead to an increase in reproductive efficiency. Generally, the ration is changed in dry time of cows and dry matter consumption is reduced. However, the consumption of dry matter consumed increases when dry time is shortened. As a result, this situation is positively reflected on reproductive activities.

The highest pregnancy ratios in the 1st insemination for on 2., 3. and ≥4 lactation cows were obtained from ≤40-day (56.7%), 41-50 (43.1%) and ≤40-day (68.4%) dry periods cows, respectively. However the lowest pregnancy ratios in the 1st insemination for on 2., 3. and ≥4 lactation cows were obtained from ≥71-day (40.0%), 61-70-day (55.2%) and ≥71-day (55.2%) dry periods cows, respectively.

Table 4- The relations of DPL with number of insemination ended up with pregnancy

Parity	*Number of Insemination*	*Dry period length (days)*						
		≤40 (n= 83) n (%)	*41-50 (n= 128) n (%)*	*51-60 (n= 327) n (%)*	*61-70 (n= 146) n (%)*	*≥71 (n= 116) n (%)*	*Total n (%)*	
		Number and ratio of pregnant animals (n (%))						
2	1	17 (56.7)	24 (51.1)	52 (49.1)	14 (42.4)	6 (40.0)	113 (48.9)	
	2	7 (23.3)	11 (23.4)	22 (20.8)	7 (21.2)	5 (33.3)	52 (22.5)	
	≥3	6 (20.0)	12 (25.5)	32 (30.2)	12 (36.4)	4 (26.7)	66 (28.6)	
Total		30 (100)	47 (100)	106 (100)	33 (100)	15 (100)	231 (100)	
3	1	14 (41.2)	25 (43.1)	37 (23.7)	15 (17.9)	18 (25.0)	109 (27.0)	**
	2	12 (35.3)	15 (25.9)	73 (46.8)	38 (45.2)	22 (30.6)	160 (39.6)	
	≥3	8 (23.5)	18 (31.0)	46 (29.5)	31 (36.9)	32 (44.4)	135 (33.4)	
Total		34 (100)	58 (100)	156 (100)	84 (100)	72 (100)	404 (100)	
≥4	1	13 (68.4)	14 (60.9)	39 (60.0)	16 (55.2)	16 (55.2)	98 (59.4)	
	2	5 (26.3)	7 (30.4)	13 (20.0)	9 (31.0)	6 (20.7)	40 (24.2)	
	≥3	1 (5.3)	2 (8.7)	13 (20.0)	4 (13.8)	7 (24.1)	27 (16.4)	
Total		19 (100)	23 (100)	65 (100)	29 (100)	29 (100)	165 (100)	
General	1	44(53.0)	63(49.2)	128(39.1)	45(30.8)	40(34.5)	320(40.0)	
	2	24(28.9)	33(25.8)	108(33.0)	54(37.0)	33(28.4)	252(31.5)	**
	≥3	15(18.1)	32(25.0)	91(27.8)	47(32.2)	43(37.1)	228(28.5)	
Total		83 (100)	128 (100)	327 (100)	146 (100)	116 (100)	800 (100)	

**, P<0.01

It can be recommended based on these results that ≤40-day dry period should be practiced for cows in their 2nd and ≥4th lactations and 41-50 days dry period should be practiced for the cows in their 2nd lactation. There are quite a few studies investigating the relations between DPL and number of inseminations. Different from the present findings, in a study investigating the effects of DPL on RP of dairy cattle, Pezeshki et al (2008) reported that there were no significant differences in RP of different dry periods. Researchers reported pregnancy ratios of 28 and 49-d dry periods respectively as 66.91 and 62.34%. In a similar study, Santschi et al (2011b) indicated that dry period treatments did not have significant effects on pregnancy ratios of the 1st and 2nd inseminations. Hossein-Zadeh & Mohit (2013) reported that the cows with short dry periods had shorter calving interval than the cows with long dry periods. Similar to the results of this study, Kuhn et al (2007) and Watters et al (2009) indicated an improvement in reproductive performance of Holstein dairy cattle when dry period length was reduced. Re´mond et al (1992) reported similar results for number of cows resulted in pregnancy after the 2nd insemination for two different groups (0 days dry period and 60 days dry period), but the researchers also pointed out quite a few number of animals included in their study.

The traits considered in this study were DPL, lactation (305 days) milk yield and number of insemination ended up with pregnancy (1st, 2nd and ≥3rd). Among these traits correlation coefficients are provided in Table 5.

Table 5- Correlations among DPL, 305-d MY and number of inseminations ended up with pregnancy

	DPL	305-d MY
DPL	1	
305-d MY	0.056	1
Number of Insemination	0.141**	0.024

**, P<0.01

There was a positive correlation between DPL and 305-d MY (0.056) and there was a highly significant positive correlation between DPL and number of inseminations ended up with pregnancy (0.141). There was also a positive correlation between 305-d MY and number of inseminations ended up with pregnancy (0.024).

4. Conclusions

As conclusions, the present findings revealed that DPL did not have significant effects on subsequent lactation MY. However, quantitative decreases were observed in lactation MY with shortening DPL. Dry period lengths on the other hand had significant effects on the number of inseminations ended up with pregnancy. Decreasing number of inseminations ended up with pregnancy were observed with shortening dry period durations for this farm. This research

is one of the few studies to examine DPL effects on subsequent lactation, for traits other than MY, and in particular percentage traits and reproduction.

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Abbreviations and Symbols	
DPL	Dry period lengths
BW	Body weight
DM	Dry matter
HP	Crude protein
kcal	Kilocalories
kg	Kilogram
Lt	Liter
ME	Metabolic energy
mL	Mililiter
MY	Milk yield
SA	Siyah Alaca
SCC	Somatic cell counts
TMR	Total mixed ration

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Appraisal of Linear Type Traits in Simmental Cows Reared on High Altitude of Eastern Turkey

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ABSTRACT
The study was carried out to investigate the magnitude of non-genetic factors influencing linear type traits and to estimate phenotypic correlations among these traits in Simmental cows. The 16 linear type traits were recorded for 148 Simmental cows reared in a private farm in Eastern Turkey. A statistical model used in this research included fixed effects of stage of lactation, parity, season at time of classification and classifier. The age at time of classification was included to the statistical model as co-variable. Parity, stage of lactation, season at time of classification and classifier effect was significant (P<0.01-0.05) for chest width, angularity, teat placement side view, body depth. Parity, stage of lactation, season at time of classification effect was significant (P<0.01-0.05) for rear leg rear view, rump width, suspensory ligament and udder depth. Linear and quadratic effects of age at time of classification were also significant (P<0.01-0.05) for rump width, rump angle, teat placement rear view, foot angle, suspensory ligament and udder depth. Phenotypic correlations among linear type traits were in low to medium range.

Keywords: Linear type traits; Non-genetic factors; Phenotypic correlations; Simmental

1. Introduction

The linear type traits are defined as the body parts of a dairy cow, which make her capable to produce milk and those traits which are directly or indirectly related with each other (Dubey 2010). Linear type traits are considered as the basis of all systems for describing the dairy cattle, and serve as the basis of all modern type classification systems. These traits allow us to describe the cow in detail based on a defined scale for each trait. Linear evaluation is based on measurements of individual type traits in place of opinions, and defines the degree of trait not the desirability (ICAR 2014). The system allows more objective and accurate assessment of dairy cattle than traditional desirability-based schemes (Alphonsus et al 2011).

In recent years, emphasis has altered from subjective grading methods for assessing dairy cows to more objective methods such as linear type traits (Essien & Adesope 2003). Linear type traits especially become important when making reproduction and selection decisions in dairy cows (Schneider et al 2003). They may also have influences directly and indirectly on milk production, longevity and culling decisions (Zavadilova & Stipkova 2012), and might be utilized to define the dairyness of a cow (Dubey et al 2014). Since the linear type traits can be determined in early life of cattle and could have moderate genetic correlations with milk yield and longevity traits, they might be used as indirect predictors of these production traits (Bohlouli et al 2015). In addition, results of several research indicated

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usefulness of linear type traits as predictors of body weight (Berry et al 2004), health (Juozaitiene et al 2006), and fertility (Harris 2015) in dairy cows.

Environmental factors might be defined as factors with measurable effects for example, stage of lactation, age of cow, calving year, season, parity, etc., and factors with non-measurable effects such as parasitic infestations, infectious diseases etc. The measurable effects can be used in preparing the livestock improvement programs in future (Javed et al 2013). In these programs, performance records of animals have to be standardized for the non-genetic sources of variation to decrease known environmental differences among animals (Tuzemen et al 2013).

Parity, classifier, season at time of classification, stage of lactation and age of the cow at time of classification are considered as the most significant environmental factors, although several non-genetic factors affecting linear type traits were pointed out by Esteves et al (2004), Mazza et al (2013), Dubey et al (2014). These factors have to be adjusted in the model, or by pre-adjustment of these records for breeding value estimation of linear type traits (Veerkamp et al 2002). Therefore, the magnitude of the non-genetic factors is required for more accurate appraisal of the type traits (Khan & Khan 2015). The linear type trait scoring and determination of the effects of non-genetic factors on these traits of Simmental cows reared on the mountainous region of Eastern Turkey has not been made in the past. Therefore, the current study was conducted in order to investigate the quantification of non-genetic factors influencing linear type traits of Simmental cows.

2. Material and Methods

In this research, a total of 897 linear type scores obtained from 148 Simmental cows reared at a private farm in Erzurum, Eastern Turkey were used. Erzurum province has mountainous geographical conditions, and its altitude is about 2000 meter above sea level. Especially temperature in winter season is around minus 5-10 °C and it snows a lot.

In this private farm, Simmental cattle were housed in a free-stall closed barn. They were milked twice a day and average lactation length of the cows is 305 days. Artificial insemination is practised in this farm, and the cows give birth on winter, spring and fall seasons. Calves are weaned about 2 months of age, and they are offered 4 kg day^{-1} whole milk in 2 times in a day (in the morning and in the evening).

Linear scoring was made according to the guidelines of International Committee for Animal Recording by 3 classifiers (ICAR 2014). Sixteen type traits were scored on a scale of 1-9, and the definitions of the linear type traits are tabulated in Table 1. Only lactating cows were scored in the afternoon prior to evening milking. Data concerning age of cow at time of classification, parity, days in milk were provided from records available in the farm. Days in milk were classified into 6 stages of lactation that were 1 (<2 months), 2 (2-<4 months), 3 (4-<6 months), 4 (6-<8 months), 5 (8-<10 months), 6 (>10 months). Seasons at time of classification were divided into 4 classes [1: Winter (December, January, February), 2: Spring (March, April, May), 3: Summer (June, July, August), 4: Fall (September, October, November)].

Table 1- Definition of the Linear Type Traits

Traits	*Scores*		
	1-3	*4-6*	*7-9*
Chest width	Narrow	Intermediate	Wide
Body depth	Shallow	Intermediate	Deep
Angularity	Lacks angularity	Intermediate	Very angular
Rump angle	High pins	Intermediate	Extreme slope
Rump width	Narrow	Intermediate	Wide
Rear legs side view	Straight	Intermediate	Sickle
Rear legs rear view	Extreme toe out	Intermediate	Parallel feet
Foot angle	Very low angle	Intermediate	Very steep
Fore udder attachment	Weak and loose	Intermediate	Extremely strong and tight
Rear udder attachment height	Very low	Intermediate	High
Central ligament	Outside of quarter	Intermediate	Inside of quarter
Udder depth	Below hock	Intermediate	Shallow
Teat placement rear view	Outside of quarter	Middle of quarter	Inside of quarter
Teat placement side view	Close	Intermediate	Apart
Teat length	Short	Intermediate	Long
Rear udder attachment width	Narrow	Intermediate	Wide

Statistical analyses were carried out by using the GLM of SPSS statistics program, and all data were analysed by using univariate analysis of variance in general linear model of SPSS (SPSS 2004). Different combination of fixed effects and interactions were included into the mathematical model. Since interaction effects were not significant in the initial analysis, they were excluded from the ultimate model. Fixed effects included in the final model are scorer, parity, season at time of classification and stage of lactation. The linear and quadratic effects of age at time of classification were also added on the statistical model as covariant. The method of LSD multiple range test was used for comparison among subclass means. Correlations among the linear type traits were also calculated by using SPSS program (SPSS 2004).

3. Results and Discussion

Least-squares means with their standard errors and level of significance of type traits of the dairy cows for different parities are presented in Table 2 and 3. The analysis of variance demonstrated that the influence of parity was significant (P<0.01) on all linear type traits except for foot angle, fore udder attachment, rear udder attachment height, teat placement rear view. This finding was compatible with results of Parveen (2008), Marinov et al (2015) who also observed significant effect of parity on large number of type traits in Sahiwal and Black-and-White cattle respectively. In the current study, significant parity effects on chest width, body depth, angularity, rear leg rear view, rear udder attachment width, teat length, suspensory ligament and udder depth were also in agreement to results of Viji et al (1990), Khan & Khan (2015). Additionally, while Liu et al (2014) pointed out significant parity differences for udder depth, fore udder attachment, rear udder height and rear udder width of Chinese Holsteins, Petkov & Stoyanova (2006) reported significant effect of various parities on the fore udder attachment, udder depth, teat length, udder balance and rear legs-rear view in Black-and-White cows in Bulgaria.

Table 2- Least-squares means along with their standard errors and level of significance of linearly scored type traits

		Chest width	*Body depth*	*Angularity*	*Foot angle*	*Rear leg side view*	*Rear leg rear view*	*Rump angle*	*Rump width*
	N	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$
General		5.4±0.1	6.4±0.1	5.0±0.2	5.0±0.2	4.6±0.2	3.9±0.1	6.0±0.1	4.9±0.1
Parity		**	**	**	ns	**	**	**	**
1	582	5.7±0.1^{a}	5.8±0.1^{b}	5.7±0.1^{a}	5.0±0.1	4.1±0.1^{b}	3.9±0.1^{b}	5.6±0.1^{a}	4.8±0.1^{b}
2	156	5.0±0.1^{bc}	6.8±0.1^{a}	4.2±0.2^{b}	5.2±0.2	5.2±0.2^{a}	4.2±0.1^{a}	6.1±0.1^{ab}	5.2±0.1^{a}
3	126	5.2±0.1^{bc}	6.4±0.1^{ab}	5.0±0.2^{a}	5.3±0.2	4.4±0.2^{b}	4.1±0.1^{a}	5.9±0.1^{ab}	4.8±0.1^{b}
4	33	5.5±0.5^{ab}	6.5±0.4^{ab}	4.9±0.6^{ab}	4.4±0.6	4.7±0.6^{ab}	3.5±0.4^{b}	6.2±0.4^{a}	4.7±0.4^{c}
Stage of lactation		**	**	**	ns	**	*	**	**
1	69	4.9±0.2^{b}	5.6±0.2^{b}	5.8±0.2^{a}	5.2±0.2	4.7±0.2^{a}	3.7±0.2^{b}	6.0±0.2^{ab}	4.4±0.2^{b}
2	165	5.4±0.2^{a}	6.6±0.1^{a}	4.8±0.2^{bc}	5.0±0.2	4.3±0.2^{b}	4.0±0.1^{ab}	6.0±0.1^{ab}	4.6±0.1^{b}
3	180	5.6±0.1^{a}	6.7±0.1^{a}	4.8±0.2^{bc}	4.9±0.2	4.8±0.2^{a}	4.1±0.1^{a}	5.9±0.1^{b}	4.9±0.1^{b}
4	207	5.5±0.1^{a}	6.5±0.1^{a}	5.0±0.2^{b}	4.8±0.2	4.9±0.2^{a}	4.0±0.1^{a}	5.9±0.1^{b}	5.1±0.1^{ab}
5	180	5.4±0.2^{a}	6.4±0.1^{a}	4.8±0.2^{bc}	5.0±0.2	4.7±0.2^{a}	4.0±0.1^{ab}	6.2±0.1^{a}	5.0±0.1^{b}
6	96	5.4±0.2^{a}	6.6±0.2^{a}	4.8±0.2^{bc}	4.9±0.2	4.2±0.2^{c}	3.7±0.2^{c}	5.8±0.2^{b}	5.3±0.2^{a}
Season at time of classification		**	**	**	**	**	**	ns	**
1	366	4.9±0.1^{b}	6.0±0.1^{a}	5.0±0.2^{b}	5.1±0.2^{a}	5.0±0.2^{a}	4.5±0.1^{a}	5.8±0.1^{b}	5.0±0.1^{b}
2	243	5.5±0.2^{a}	7.1±0.2^{c}	3.7±0.2^{c}	3.9±0.2^{b}	5.1±0.2^{a}	4.2±0.2^{ab}	6.1±0.2^{a}	4.8±0.2^{bc}
3	33	5.4±0.3^{ab}	5.7±0.2^{a}	6.2±0.3^{a}	5.6±0.3^{a}	4.0±0.3^{b}	2.7±0.2^{c}	6.0±0.2^{ab}	4.5±0.2^{c}
4	255	5.7±0.2^{a}	6.8±0.1^{b}	5.0±0.2^{b}	5.3±0.2^{a}	4.3±0.2^{b}	4.3±0.1^{ab}	5.9±0.1^{ab}	5.2±0.1^{a}
Classifier		**	**	**	ns	ns	ns	ns	ns
1	299	5.2±0.1^{b}	6.2±0.1^{b}	4.8±0.2^{a}	4.9±0.2	4.5±0.2	3.9±0.1	6.0±0.1	4.8±0.1
2	299	5.4±0.1^{ab}	6.5±0.1^{a}	4.9±0.2^{a}	5.0±0.2	4.7±0.2	3.9±0.1	6.0±0.1	4.8±0.1
3	299	5.5±0.1^{a}	6.4±0.1^{a}	5.2±0.2^{b}	5.0±0.2	4.6±0.2	4.0±0.1	5.9±0.1	4.9±0.1
Age at time of classification									
Linear		ns	ns	ns	*	ns	ns	**	*
b_1		-0.067	-0.053	0.101	0.090	-0.007	0.120	0.091	0.083
Quadratic		ns	ns	ns	*	ns	ns	**	ns
b_2		0.001	0.000	-0.001	-0.001	0.00007	-0.001	-0.001	-0.001

**, P<0.01; *, P<0.05; ns, non-significant

A significant decrease in linear score for body depth, rear leg side view in second parity and a slight insignificant increase was observed in third parity (Table 2). The linear score also increased along with progress in stage of lactation for body depth, rear leg side view, fore udder attachment, teat length, teat placement rear view (Table 2 and 3). On the other hand, significant parity effect for a decrease in linear type score was observed for rear udder attachment height, suspensory ligament (Table 3). Findings of the present study were consistent with results from previous studies (Yanar 1999; Marinov et al 2015).

Table 3- Least-squares means along with their standard errors and level of significance of linearly scored type traits

		Fore udder attachment	*Rear udder attachment width*	*Rear udder attachment height*	*Teat placement rear view*	*Teat placement side view*	*Teat length*	*Suspensory ligament*	*Udder depth*
	N	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$	$\overline{X} \pm S_{\overline{x}}$
General		4.2±0.2	4.8±0.8	4.6±0.2	5.1±0.2	5.0±0.2	6.0±0.2	5.9±0.1	5.5±0.1
Parity		ns	**	ns	ns	**	**	**	**
1	582	3.7±0.1	5.0±0.1^{a}	4.9±0.1	5.0±0.1	5.6±0.1^{a}	5.5±0.1^{c}	6.4±0.1^{a}	4.9±0.1^{b}
2	156	3.9±0.2	4.1±0.2^{b}	4.8±0.1	5.0±0.2	4.9±0.2^{b}	5.7±0.1^{bc}	6.4±0.1^{a}	5.9±0.1^{a}
3	126	4.4±0.2	4.2±0.2^{b}	4.8±0.2	5.0±0.2	4.6±0.2^{b}	5.9±0.1^{b}	5.8±0.1^{b}	5.4±0.2^{b}
4	33	5.0±0.7	5.8±0.7^{a}	3.9±0.6	5.4±0.6	4.9±0.6^{ab}	6.9±0.1^{a}	5.2±0.5^{b}	5.8±0.5^{ab}
Stage of lactation		**	ns	**	ns	**	ns	**	**
1	69	5.1±0.3^{a}	4.7±0.3	5.1±0.2^{a}	5.5±0.2	4.7±0.2^{c}	5.8±0.2	5.5±0.2^{c}	6.0±0.2^{a}
2	165	3.4±0.2^{b}	4.9±0.2	4.5±0.2^{bc}	5.2±0.2	5.3±0.2^{a}	6.1±0.1	5.6±0.2^{c}	5.2±0.2^{d}
3	180	3.6±0.2^{b}	4.9±0.2	4.8±0.2^{ab}	5.0±0.2	5.1±0.2^{ab}	6.0±0.1	6.2±0.1^{a}	5.1±0.2^{d}
4	207	4.2±0.2^{b}	4.9±0.2	4.5±0.2^{bc}	5.1±0.2	5.1±0.2^{ab}	6.0±0.1	6.1±0.2^{ab}	5.6±0.2^{c}
5	180	4.6±0.2^{ab}	4.5±0.2	4.5±0.2^{bc}	4.9±0.2	4.6±0.2^{c}	6.0±0.1	6.0±0.2^{ab}	5.6±0.2^{bc}
6	96	4.6±0.3^{ab}	4.8±0.3	4.2±0.2^{c}	5.0±0.3	5.2±0.2^{ab}	6.2±0.2	6.2±0.2^{a}	5.7±0.2^{ab}
Season at time of classification		**	ns	**	**	**	ns	**	**
1	366	4.8±0.2^{a}	4.7±0.2	4.7±0.2^{b}	5.5±0.2^{a}	5.2±0.2^{b}	6.0±0.1	6.1±0.1^{a}	6.2±0.1^{a}
2	243	5.1±0.3^{a}	5.3±0.3	5.5±0.2^{a}	5.1±0.3^{ab}	4.5±0.2^{c}	6.3±0.2	5.9±0.2^{a}	5.2±0.2^{b}
3	33	2.7±0.4^{c}	4.6±0.4	3.7±0.3^{c}	5.1±0.3^{ab}	4.7±0.2^{bc}	5.9±0.2	5.5±0.3^{b}	5.2±0.3^{b}
4	255	4.4±0.2^{b}	4.6±0.2	4.5±0.2^{b}	4.7±0.2^{b}	5.7±0.2^{a}	5.9±0.1	6.3±0.2^{a}	5.6±0.2^{b}
Classifier		ns	ns	ns	ns	*	**	ns	ns
1	299	4.3±0.2	4.8±0.2	4.6±0.2	5.1±0.2	4.9±0.2^{b}	5.9±0.1^{b}	5.9±0.1	5.4±0.2
2	299	4.2±0.2	4.8±0.2	4.6±0.2	5.2±0.2	4.9±0.2^{b}	6.2±0.1^{a}	6.0±0.1	5.6±0.2
3	299	4.2±0.2	4.7±0.2	4.6±0.2	5.0±0.2	5.2±0.2^{a}	6.0±0.1^{b}	5.9±0.1	5.6±0.2
Age at time of classification									
Linear		ns	ns	ns	*	ns	ns	**	*
b_1		-0.098	-0.100	0.030	-0.110	0.233	-0.063	-0.117	-0.005
Quadratic		ns	ns	ns	ns	*	ns	**	ns
b_2		0.001	0.001	0.000	0.001	-0.003	0.001	0.001	0.000

**, P<0.01; *, P<0.05; ns, non-significant

Least-squares means with their standard errors and level of significance of type traits of the dairy cows for several stages of the lactation are presented in Table 2 and 3. Results of the statistical analysis revealed that stage of lactation resulted in significant effect on the large number of linear traits, such as chest width, body depth, angularity, rear leg side view, rump angle, rump width, fore udder attachment, rear udder attachment height, teat placement side view, suspensory ligament, udder depth (P<0.01) and rear leg rear view (P<0.05). Significant effects of the stage of lactation for most of traits in current study were in consensus to the results of Esteves et al (2004) in Brazilian Holstein. Similarly, Khan & Khan (2015) also reported that stage of lactation was significant source of variation for several linear traits, for instance, body depth, angularity, rump width and dewlap surface area, rear legs set, rear udder height, udder depth, fore teat length and rear udder width, chest width (P<0.001), central ligament (P<0.05). Stage of lactation effect on rump width, rear udder height and udder depth in current research was in accordance with results of Dahiya (2005a). Additionally, Angelova (2006) reported statistically significant effect of the lactation stage on chest width, angularity, udder depth and central ligament in Bulgarian Brown cattle. As for this study, similar results were pointed out by

Petkov & Stoyanova (2006) who studied the impact of the stage of lactation on udder traits and established significant effects on fore udder attachment and suspensory ligament.

Results of the current study regarding change in linear score with advancement in stage of lactation for chest width was in consensus to findings of Marinov et al (2015) where chest width score increased with advancing stage of lactation. Significant stage of lactation effects for fore udder attachment, teat placement side view, teat placement rear view rump angle, body depth, suspensory ligament, rump width, and rear udder attachment height were compatible with findings of Mazza et al (2013). Significant stage of lactation effects for rump width, rear udder attachment height and udder depth in the present study were in agreement with those reported by Dahiya (2005b) for Sahiwal cows. Significant effect of stage of lactation on udder depth in current study was also in harmony with findings of Viji et al (1990). Stage of lactation effect on chest width, central ligament and udder depth were concordant with results of Dahiya (2005a) for Hariana cattle.

Least-squares means along with their standard errors and level of significance of linear type traits in lactating Simmental cows for different classifiers are given in Table 2 and 3. The analysis of variance revealed that the effects of classifiers on all type traits were not statistically significant except for chest width, body depth, teat length, teat placement side view and angularity. In other words, classifiers did not cause significant variations on most of linear type traits, and the result might be attributed to the employing expert classifiers in the present study for the linear type traits assessment. The finding was also in agreement with result of a research reported by Yanar (1999).

Least-squares means along with standard errors and level of significance of linear type traits for different seasons at time of classification are presented in Table 2 and 3. Significant influence of season at time of classification for chest width, body depth, angularity, foot angle, rear leg side view, rear leg rear view, rump width, fore udder attachment, rear udder attachment height, teat placement rear view, teat placement side view, suspensory ligament and udder depth was observed in present study. Significant effect of season at time of classification for rear legs rear view and foot angle as obtained in for this study was also reported for Nili Ravi Buffaloes by Mirza et al (2015).

Linear effect of age at time of classification was not significant for all type traits except for foot angle, rump angle, rump width, teat placement rear view, suspensory ligament and udder depth (Table 2 and 3). Dubey et al (2014) reported significant effect of age at time of classification on foot angle and udder depth; Khan & Khan (2015) revealed linear effects of age at time of classification on the rump width and udder depth in Sahiwal cattle. Their findings were in consensus to findings of current study. Significant decrease in linear type score of udder depth with age could be attributed to more milk production and development of udder with age.

Estimated phenotypic correlations among linear type traits are given in Table 4. In general, the correlation values were in between lower and medium range, and most of the correlations were less than 0.20. Similar results were already reported by Duru et al (2012) and Khan & Khan (2016). The strongest positive phenotypic correlations (r= 0.43) were estimated between fore udder attachment and udder depth. The result was in harmony with finding of Liu et al (2014) who reported the strongest correlation value (r= 0.40) between same type traits of Holstein Friesian cows in China. Khan et al (2008) also estimated significant phenotypic correlation (r= 0.29) between fore udder attachment and udder depth. Phenotypic correlations with negative sign calculated between body depth and angularity were also reported by Khan et al (2008) which was in accordance with finding of the current study. Correlation between fore udder attachment and teat placement rear view in this study was comparable to findings of Nemcova et al (2011) (r= 0.13) and Tapki & Guzey (2013) (r= 0.22). On the other hand, higher than current study estimates were reported by Berry et al (2004) (r= 0.28), and Khan et al (2008) (r= 0.38). Similar to the finding of Nemcova et al (2011), correlation between rear udder height and rear udder width in the present study was statistically significant and was in positive direction.

Phenotypic correlations between fore teat length and udder depth were found to be negative and lower in magnitude. These results are in agreement with findings of Nemcova et al (2011) (r= -0.01) and Tapki & Guzey (2013) (r= -0.06). Significant negative correlations between chest width and udder depth reported by Yanar (1999), Berry et al (2004) and Nemcova et al (2011) were comparable to results of the present study. Similarly, negative and significant estimates between body depth and udder depth were reported by Berry et al (2004) (r= -0.20), Khan et al (2008) (r= -0.44), Nemcova et al (2011) (r= -0.23), Duru et al (2012) (r= -0.32), and these findings were in harmony with result of the current study.

Table 4- Phenotypic correlations and standard errors among the linear type traits

Traits		*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*
Chest width	(1)		0.24**	-0.17**	-0.02	0.00	-0.00	0.07*	0.05	-0.09*	0.08*	-0.04	-0.05	0.03	-0.03	-0.02	-0.19**
Body depth	(2)	0.03		-0.40**	-0.03	0.10**	0.08*	0.02	0.07*	-0.18**	0.04	-0.14**	-0.05	0.08*	0.09**	0.09*	-0.24**
Angularity	(3)	0.03	0.03		0.02	-0.12**	-0.06	-0.12**	-0.14**	-0.05	-0.06	0.11**	-0.05	0.09**	0.02	0.05	-0.01
Foot angle	(4)	0.03	0.03	0.03		-0.14**	0.06	0.05	0.08*	-0.01	-0.10**	0.09**	-0.08*	0.04	0.01	0.00	0.12**
Rear leg (side view)	(5)	0.03	0.03	0.03	0.03		0.10**	0.09*	0.08*	0.07*	-0.01	0.02	0.10**	-0.05	0.02	0.03	0.14**
Rear leg (rear view)	(6)	0.03	0.03	0.03	0.03	0.03		-0.04	0.11**	-0.05	0.03	0.04	0.03	0.17**	-0.01	0.08*	-0.03
Rump angle	(7)	0.03	0.03	0.03	0.03	0.03	0.03		0.02	-0.11**	-0.13**	0.03	-0.13**	-0.03	-0.02	-0.16**	0.06
Rump width	(8)	0.03	0.03	0.03	0.03	0.03	0.03	0.03		-0.01	0.04	-0.05	0.02	0.13**	-0.01	-0.03	0.02
Fore udder attachment	(9)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03		0.06	0.07*	0.16**	-0.38**	0.04	-0.13**	0.43**
Rear udder attachment width	(10)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03		0.09**	0.01	0.03	0.18**	0.02	-0.23**
Rear udder attachment height	(11)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03		-0.04	-0.07	0.09**	0.07*	0.07*
Teat placement (rear view)	(12)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03		-0.00	-0.19**	0.10**	0.14**
Teat placement (side view)	(13)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03		-0.13**	0.11**	-0.32**
Teat length	(14)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03		-0.02	-0.09**
Suspensory ligament	(15)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03		-0.11**
Udder depth	(16)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	

Note: Phenotypic correlations (above diagonal) and standard errors below diagonal; **, P<0.01; *, P<0.05

4. Conclusions

Overall results of the study revealed considerable effects of the non-genetic factors on linear type traits of Simmental cows. Parity, season at time of classification and stage of lactation were especially important sources of variation for most of type traits. Linear and quadratic effects of age at time of classification were also significant for some type traits. Phenotypic correlations among linear type traits were in low to medium range. Genetic evaluation of Simmental cows for these traits needs to be based on models that take effects of these environmental factors into account in order to obtain unbiased results. Therefore, adjustment for the non-genetic factors will decrease known environmental differences among animals and increase accuracy of the breeding values.

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Non-destructive Estimation of Chlorophyll *a* Content in Red Delicious Apple Cultivar Based on Spectral and Color Data

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ABSTRACT
Non-destructive estimation of the chemical properties of fruit is an important goal of researchers in the food industry, since online operations, such as fruit packaging based on the amount of different chemical properties and determining different stages of handling, are done based on these estimations. In this study, chlorophyll *a* content in Red Delicious apple cultivar is predicted as a chemical property that is altered by apple ripening stage, using non-destructive spectral and color methods combined. Two artificial intelligence methods based on hybrid Multilayer Perceptron Neural Network - Artificial Bee Colony Algorithm (ANN-ABC) and Partial least squares regression (PLSR) were used in order to obtain a non-destructive estimation of chlorophyll *a* content. In application of the PLSR method, various pre-processing algorithms were used. In order to statistically properly validate the hybrid ANN-ABC predictive method, 20 runs were performed. Results showed that the best regression coefficient of the PLSR method in predicting chlorophyll *a* content using spectral data alone was 0.918. At the same time, the average determination coefficient over 20 repetitions in hybrid ANN-ABC in the estimation of chlorophyll *a* content, using spectral data and color features were higher than 0.92±0.040 and 0.89±0.045, respectively, which to our knowledge is a remarkable non-intrusive estimation result.

Keywords: Spectroscopy; Color features; Non-destructive estimation; Artificial neural network; Regression; Metaheuristic algorithms

1. Introduction

The production and consumption of fruit and vegetables are rising due to their flavor and healthy properties (Li et al 2016; Sabzi et al 2018) There are several methods for grading fruit including grading by the shape, volume, weight, and physicochemical properties (Sabzi et al 2015). The method of grading fruit and vegetables based on physicochemical properties provides a wide choice for consumers (Zhang et al 2018). Estimating these characteristics has already been done using destructive methods, but it is almost impossible to package fruit according to the amount of any physicochemical property, due to the destructive nature of these estimation methods. Nondestructive methods, such as light spectroscopy, have the ability to package fruit and vegetables according to the amount of any physicochemical property (Arendse et al 2018). Other possibility of these non-destructive methods is the recognition of different fruit

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varieties, including apple (Eisenstecken et al 2019). Mohammadi et al (2015) used an imaging method to estimate the ripening time of persimmon fruit. In fact, they divide the whole ripening process of this fruit into three stages: unripe, ripe and overripe fruit. Their algorithm performs classification based on the color features of the persimmon skin in two color spaces, RGB and L*a*b*. In order to verify the reliability of the proposed algorithm, they used a number of physical, mechanical, and chemical properties. After several studies, it was found that color features such as R, G, and b* had a significant difference in the various stages of ripening. Finally, two classifications based on Linear Discriminant Analysis and Quadratic Discriminant Analysis (QDA) were used to categorize the ripening stages of persimmon fruit. Results showed that by using QDA, the ripening stages of persimmons were estimated with a precision of 90.24%.

Banana is among most consumed fruit along the globe. Among all food products, banana ranks fourth (Seymour et al 1993). Ripening stage is one of key factors in post-harvest operations and in general fruit processing. This factor determines consumer acceptability and eating quality (Thompson & Burden 1995). Optical methods are a kind of non-destructive methods that have attracted several researcher's interest in recent years (Ali et al 2017; Adebayo et al 2016a). For example, Adebayo et al (2016b) proposed a method for the non-destructive estimation of qualitative characteristics and classification of the banana ripening stages using optical properties. Five wavelengths of 532, 660, 785, 830 and 1060 nm were used in order to predict fruit quality features. Results showed a high correlation between optical properties and banana ripening stages at 532, 660, and 785 nm wavelengths, so that, the different ripening stages of banana fruit were predictable with an accuracy of 53.97%.

Apple is also a very important garden fruit with high demand around the world. For instance, Cardenas-Perez et al (2017) evaluated the estimated physicochemical properties of Malus domestica cv. Golden Delicious apples by a non-destructive method using a computer vision system. 114 apple samples were used to develop the system. Various color spaces were investigated to extract color features, and finally, it was determined that the most suitable color space for measuring the color of the fruit was CIELab. CIELab coordinates include L* (Luminosity), a* (green to red), and b* (blue to yellow). The physicochemical properties extracted from each sample were: titratable acidity, total soluble solids, firmness, and ripening index. Results showed that by combining color features and chemical properties extracted at different stages of apple ripening, a non-destructive evaluation of the fruit would be possible (Cardenas-Perez et al 2017).

Visible/near-infrared hyperspectral imaging (HSI) technique is one of the most effective non-destructive estimation methods for physicochemical properties, followed by an estimation of the fruit ripening stage. In recent years, HSI method has been widely used to assess food quality and safety (Jackman et al 2009; Wu & Sun 2013). Hu et al (2017) investigated the effect of 1-methylcyclopropene (1-MCP), which causes delayed aging of fruit and vegetables, as one of the most effective ethylene reaction inhibitors in preserving the quality of fruit and aggregating the rate of glucose, fructose, and sucrose in different ripening stages of kiwi fruit, in a non-destructive method. In order to test the system, 210 fruit were selected in the range of 0.09-0.13 kg with no visible defects. Vis/NIR laboratory HSI system (Imspector V10E, Spectral Imaging Ltd., Oulu, Finland) was set in reflection mode. The wavelength range of the spectrometer was set at 308-1105 nm, with a resolution of 1002×1004 pixels (spatial spectrum). During the preparation of hyperspectral images, each kiwi fruit was placed on site, and moved by a stepper motor at a speed of 1.3×10^{-3} m s^{-1} and an exposure time of 30×10^{-2} s, under the objective of a CCD camera. Results showed that glucose, fructose, and sucrose values can be properly modeled using spectral data alone.

In recent years, non-destructive methods have been of interest to researchers around the world due to their practical applications in the food industry (predicting the number of physicochemical properties, detection of fruit and vegetables ripening stages, etc.). Therefore, in this study, two non-destructive spectral and color methods combined with Hybrid (ANN-ABC) were used in garden to estimate the amount of chlorophyll *a* as an index of ripening during different growing stages of Red Delicious apple cultivar, due to its skin visible-range color variation in different stages of ripening.

2. Material and Methods

2.1. Sampling

Spectral, color, and chemical data were collected from 56 Red Delicious apple fruit samples that harvested in four different stages of ripening (14 samples of each stage). The first, second, third and fourth stages of harvesting were 135,

145, 155 and 165 days after full blooming. Apples were obtained from Kermanshah, Iran (longitude: 7.03 °E; latitude: 4.22 °N) gardens. Then, they were transferred to Shahid Beheshti University to measure the spectral data, and in order to extract color features and chemical properties of chlorophyll *a*, they were transferred to the Agricultural Engineering Research Institute, Iran.

2.2. Spectroscopy system configuration

Radiation of electromagnetic waves into material causes the absorption and reflection of radiation by material. This interaction of radiation and material leads to the acquisition and presentation of information in spectroscopic studies. In fruit and vegetables, the absorption process of these beams is carried out by the C-H, O-H, and N-H chemical bonds (Nicolaï et al 2007). To do the spectrometry analysis in a proper way, you need to configure the spectroscopy system, for obvious reasons. An Intel Corei3-CFI PC, 330Mb at 2.13GHz, 4GB of RAM and Windows 10 was used, which was also equipped with SpectraWiz software (StellarNet Inc. Tampa, Florida) to store the resulting spectrum in the computer. The measurement mode in this research was reflective mode. The EPP200NIR (StellarNet, Tampa, Florida) model spectrometer with an InGaAs (Indium Gallium Arsenide) detector with operation range of 200 nm to 1100 nm and a resolution of 1 to 3 nm, was used in this study, which was connected to a computer via a USB2 cable. Also, the SLI-CAL (StellarNet, Tampa Florida) model light source made of tungsten halogen with a power of 20 watts was used in this study. A two branch optical fiber was used to guide light from the light source to apple and from apple to the spectrometer. Because of intense noise, the first 200 nm and the last 100 nm wavelengths were eliminated. So, the studied spectral range was 400 nm to 1000 nm. Given the noise in the spectroscopy, 16 samples out of 56 samples were eliminated from the study resulting a total of 40 samples that were used for analysis.

2.3. Extraction of color features

In order to perform a colorimetric study, parameters L*, a* and b* of the skin of apples were measured by a CR-400 (Konika Minolta, Japan) colorimeter and then, the color purity indices (C*) and hue angle (h_a) were calculated using Equations (1) and (2) (Clerici et al 2011):

$$C^* = \left[(a^*)^2 + (b^*)^2 \right]^{1/2} \tag{1}$$

$$h_a = \tan^{-1}\left({}^{b^*}\!/_{a^*} \right) \tag{2}$$

2.4. Measuring true Chlorophyll a *content*

Due to changes in the color of the fruit during the ripening stages as well as the change in the amount of chlorophyll *a* during this period, we believe that the non-destructive estimation of chlorophyll *a* is very useful, since it is possible to estimate the stage of fruit ripening, specifically Red Delicious apple cultivar (Costa et al 2009; Amoriello et al 2018). Destructive measurement of chlorophyll *a* content was done by the method used in Ncama et al (2017).

Based on this method:
1. 1 gr of rind powder was extracted using 8 mL of 80% acetone.
2. This material was transferred to a glass tube that covered with ice.
3. This glass tube stand for 10 min in this condition.
4. Previous materials were homogenized for 1 min.
5. Centrifuge operation for 10 min at 4 °C.
6. The calculation of pigments based on wavelengths required (Equation (3)).

$$Chl_a = 12.25 A_{663.2} - 2.79 A_{646.8} \tag{3}$$

Where; A-absorbance of a sample at a subscript wavelength, e.g. $A_{663.2}$ is a sample absorbance at 663.2 nm.

2.5. Non-destructive estimation of chlorophyll a *content*

For the non-destructive estimation of chlorophyll *a* content, two spectral and color methods were used. For spectral data, Partial Least Squares Regression (PLSR) method using ParLeS Software (ParLeS_v3.1) (Rossel 2008), and hybrid ANN-ABC method were used. In addition, hybrid ANN-ABC method was also used for color data.

2.5.1. ParLeS software

ParLes is a chemometrics software used for multivariate modeling and prediction. In fact, this software is used for chemical training, research and spectroscopy. This software has the ability of transmitting and pre-processing the received spectrum from different samples by various algorithms (Rossel 2008).

2.5.2. Hybrid artificial neural network-artificial bee colony algorithm (ANN-ABC)

Multi-layer perceptron (MLP) neural network is an effective method for nonlinear modeling of various characteristics. This network has adjustable parameters whose performance depends on the optimal setting of these parameters. Parameters include number of neurons and layers, transfer function, back-propagation training function and back-propagation weight/bias function. In this study, an artificial bee colony algorithm was used to set these parameters. The bee algorithm is an optimization algorithm based on the honey bee swarming behavior suggested by Pham et al (2006). This algorithm is based on the behavior of honeybee in search of food sources. The different stages of the bee algorithm are as follows:

1. Generating initial responses and evaluating them.
2. Selecting better sites (responses) and sending worker bees to those sites.
3. Returning the bees to the hive with artificial dance (producing a neighbor response).
4. Comparing all the bees of a site and select the best case.
5. Replacing non-selected bees with random answers.
6. Saving the position of the best answer - returning to Step 2 if the termination conditions are not met (Pham et al 2006).

In this study, the number of neurons in each layer may be selected in the range of 0-25. The number of layers could be minimum 1 and maximum 3. There were 15 different types of transfer functions, such as *tansig*, *logsig*, *hardlim*, *satlins* and *tribas* to be selected. 19 different back-propagation network training functions such as *trainb*, *traingda*, *traincgb*, *traincgb* and *trainlm* were able to be sub-optimally selected by ABC algorithm. Finally, the back-propagation weight/bias learning function was selected from among 15 different functions such as *learnpn*, *learnk*, *learncon* and *learnwh*. The method is summarized as follows: first, the ABC algorithm considers a similar vector with the mentioned number of parameters, namely a vector with a minimum of 4 and a maximum of 8 members. For example, vector x= [13, 17, *logsig*, *tribas*, *trainb*, *learnpn*] implies a two hidden layers network with 13 and 17 neurons each, *logsig* transfer function in first layer, *tribas* transfer function in second layer, *trainb* back-propagation network training algorithm, and *learnpn* back-propagation weight/bias function. Mean square error (MSE) is a parameter that determines the efficiency of the MLP neural network, which is related to each ABC selected sub-optimal vector sent to it. Such way, each vector with the lowest values of MSE is selected as optimal in order to set the neural network parameters.

2.6. Evaluation parameters of chlorophyll a content predictive model

To evaluate the efficiency of chlorophyll a content estimation models by Hybrid ANN-ABC, the coefficient of determination (R^2), Sum Squared Error (SSE), Mean Absolute Error (MAE), MSE, and Root Mean Square Error (RMSE) were used (Sabzi et al 2013; Sabzi & Arribas 2018). In order to evaluate the predicted models by PLSR, regression coefficient (R_p), adjusted regression coefficient ($R_{p.adj}$), RMSE, Standard Deviation of the Error Distribution (SDE), and Relative Percent Deviation (RPD) were used, (Rossel 2008).

3. Results and Discussion

Figure 1 shows flowchart of the type of data and the used methods to estimation chlorophyll a.

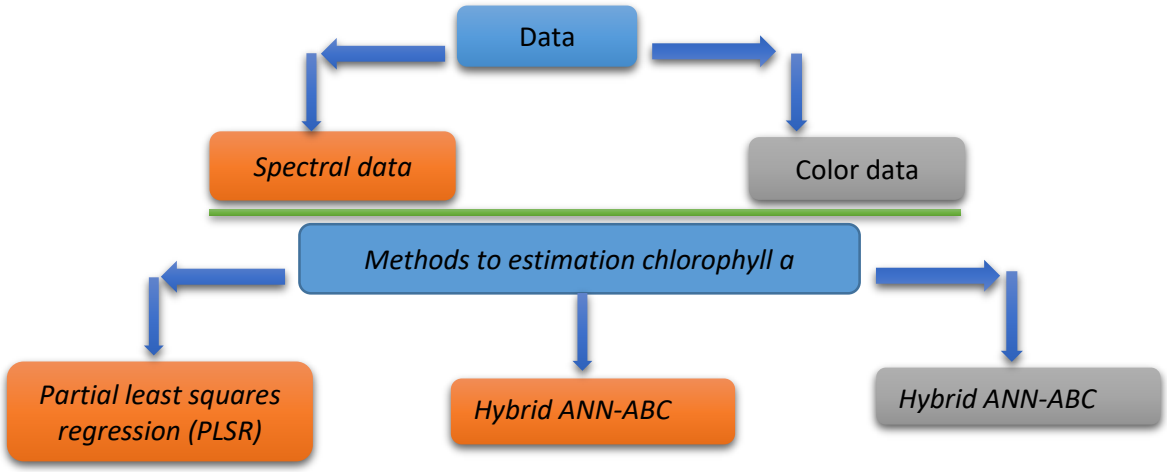


Figure 1- Flowchart of the type of data and the used methods to estimation chlorophyll *a*

3.1. Non-destructive estimation of chlorophyll a content on spectral data using the statistical method

3.1.1. Reflectance and absorption spectra

Nicolaï et al (2007) conducted a study and showed that the reflectance spectra of different fruit such as apples, oranges, nectarines, and pears, were similar. This means that the reasons behind the instance of peaks in the spectral diagrams of fruit are similar. Figure 2 shows the mean reflection and absorption spectra of the visible/near-infrared light. In order to reduce the nonlinearity that may exist in the spectrum, ParLeS software offers the possibility of converting the reflection spectra (R) into the absorption spectra by log (1/R) (Rossel 2008). As you can see, there are peaks near the wavelengths of 490 and 680 nm. A peak near the 490 nm wavelength is related to the absorption of carotenoids and a peak in 680 nm, to the absorption of chlorophyll *a* (Cayuela 2008; Martínez-Valdivieso et al 2014).

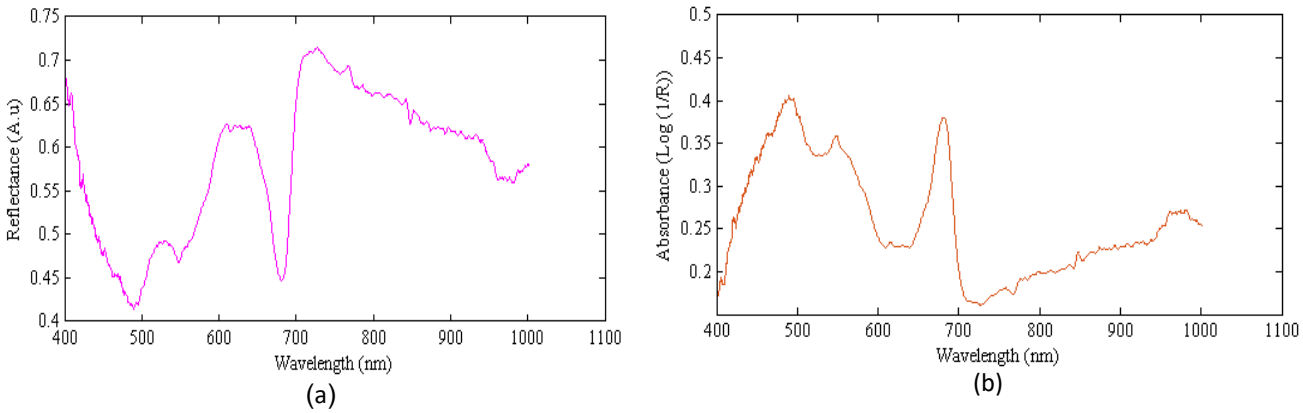


Figure 2- Vis/NIR average spectrum. (a), reflection average spectrum; (b), absorption average spectrum. The unit of reflectance and absorbance is arbitrary unit (A.u)

3.1.2. Pre-processing of spectra

For some reason, such as the effect of light diffusion by changing the detector spacing with the sample, surface roughness in the sample, variation in sample size, noise caused by the increase of the spectrometer temperature, etc., the spectral data, in addition to the sample information, also contains other unwanted information. If one uses ordinary partial least squares regression, this unwanted information reduces the accuracy of the calibration model. On the other hand, ParLeS software, by accessing more stable and reliable models, uses different methods of spectral preprocessing such as Savitzky-Golay (SG), Standard Normal Variate (SNV), Wavelet Filter (WF), SNV with Wavelet Detrending (SNVWWD), Wavelet Detrending (WD), and Median Filter (MF), which are based on various mathematical operations (Rossel 2008).

3.1.3. Partial least squares regression (PLSR) efficiency in predicting the chlorophyll a fruit content

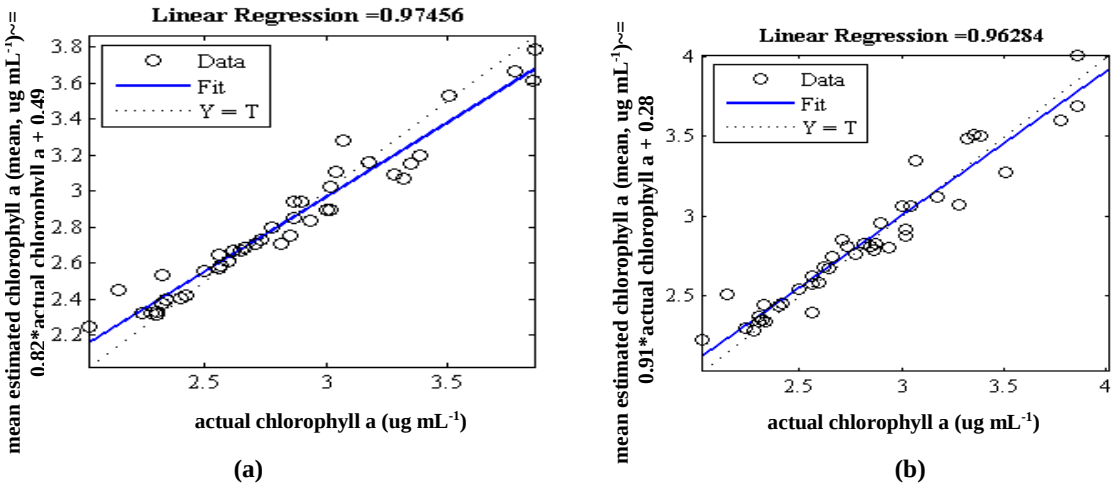
Since the purpose of this study is to estimate the amount of chlorophyll *a* and according to the preceding sections, the light absorption peak near the wavelength of 680 nm is directly related to chlorophyll *a* content, a wavelength window range of 676.282 nm to 686.293 nm was used for estimating Chlorophyll *a* content using PLSR. Table 1 shows the results of the spectral prediction of chlorophyll *a* content using PLSR. In general terms, when Rp, $R_{p.adj}$, and RPD values are large, and at the same time, RMSE Percentage (RMSEP) and SDE values tend to be small, the resulting estimation model will be more efficient and can be used to properly predict the amount of chlorophyll *a*. Based on the explanations given, preprocessing models SNVWW+SG (model 4) and WD+MF (model 5) are better than others and predict chlorophyll *a* content in fruit more accurately, and thus with less error.

Table 1- Calibration results of predictive spectral models of chlorophyll *a* content based on the combination of different preprocessing methods

Model number	*Pre-processing*	R_p	$R_{p.adj}$	*RMSEP (%)*	*SDE*	*RPD*
1	Without preprocessing	0.727	0.700	0.261	0.255	1.882
2	SNV+SG	0.442	0.386	0.367	0.368	1.335
3	SNV+WF	0.438	0.381	0.368	0.369	1.312
4	SNVWW +SG	0.918	0.910	0.151	0.139	3.204
5	WD + MF	0.918	0.909	0.149	0.141	3.253

3.2. Non-destructive estimation of chlorophyll a content on spectral data using Hybrid ANN-ABC

To predict chlorophyll *a* content by Hybrid ANN-ABC, a spectral window of 662.01 to 698.04 nm, which includes the wavelength of 680 nm, was used. As mentioned above, optimal adjustment of the MLP neural network parameters ensures its high performance in predicting the amount of chlorophyll *a* content. The MLP neural network optimal parameters as set by the artificial bee colony algorithm are two-layer structure with number of neurons 5 and 12. Transfer function for first and second layers are tirbas and purelin respectively. Backpropagation network training function is traingda and final backpropagation weight/bias learning function is learnos. After determining the optimal structure of the artificial neural network, in order to validate the predictive method, 20 replications were performed. The mean±std of R, SSE, MAE, MSE and RMSE were 0.962±0.040, 0.441±0.354, 0.129±0.061, 0.034±0.027 and 0.173±0.064 respectively. Also among these 20 replications, the best values of R, SSE, MAE, MSE and RMSE were 0.987, 0.111, 0.062, 0.008 and 0.092 respectively. As it can be seen, the mean determination coefficient of the 20 repetitions is higher than 0.92, and the standard deviation is 0.04. Therefore, it can be concluded that the performance of Hybrid ANN-ABC is much better than partial least squares regression. Figure 3(a) shows the regression analysis scater plot on the estimated values of chlorophyll *a* content and its true measured values, for the test data. Estimated chlorophyll *a* content is related to the average chlorophyll *a* content of the samples in 20 replicates. As can be seen, the regression coefficient is 0.975. This fact implies that the Hybrid ANN-ABC method shows stability on spectral data.



(a) **(b)**

Figure 3- Regression analysis of scatter plot between mean estimated and actual (true measured) apple chlorophyll *a* (ug mL^{-1}) content (test data) using (a), spectral data and (b), color data. Each repetition includes 13 testing samples, so in 20 repetitions 260 samples exist. Since there are 40 input samples in total, there are on average more than 6 different samples over which their average chlorophyll *a* content was measured

3.3. Non-destructive estimation of chlorophyll a content on visible-range color features using Hybrid ANN-ABC

After using different color features as inputs to hybrid ANN-ABC, the results showed that the two properties of the second component of L*a*b* color space (a*) and the hue angle-as input to the artificial neural network-had the higher ability to predict a chlorophyll *a* content as compared to other features. Therefore, these two color properties were used to predict the amount of chlorophyll *a* present in fruit samples. The MLP neural network optimal parameters as set by the artificial bee colony algorithm are two-layer structure with number of neurons 15 and 19. Transfer function for first and second layers are satlin and tirbas respectively. Backpropagation network training function is trainoss and final backpropagation weight/bias learning function is learnwh. In this case, as in the previous section, after determining the optimal amount of artificial neural network parameters, 20 replications were used to determine the stability of the methodology. The mean ± std of R, SSE, MAE, MSE and RMSE were 0.945±0.045, 0.556±0.269, 0.142±0.032, 0.2±0.051 and 0.043±0.021 respectively. Also among these 20 replications, the best values of R, SSE, MAE, MSE and RMSE were 0.982, 0.149, 0.072, 0.107 and 0.011 respectively. As can be seen, the performance of Hybrid ANN-ABC is remarkable in the estimation of chlorophyll *a* content based on color content, but it has a weaker performance than spectral analysis. Figure 3(b) shows the regression analysis of the dispersion plot between the estimated mean and the actual (true measured) value of chlorophyll *a* content in apple (test set) using color data. The regression coefficient of this method over the test data is 0.969, which is an acceptable value in predicting the amount of chlorophyll *a*.

3.4. Comparison of hybrid ANN-ABC method performance in non-destructive estimation of chlorophyll a content using spectral and color data

Figure 4 (a) shows error indices boxplots (MAE, MSE and RMSE) in the Hybrid ANN-ABC method in a non-destructive estimation of chlorophyll *a* content for 20 replicates based on both spectral and color data. As you can see, all the spectral data analysis error boxplot values are lower than the color data counterparts. On the other hand, in general terms the boxplot chart of the mean square error for spectral data is more compact than color data. Latter fact means that the performance of Hybrid ANN-ABC method in the non-destructive estimation of chlorophyll *a* content using spectral data is higher than that based on color data. In a similar fashion as done in Figure 4 (a), Figure 4 (b) also shows the high performance of the Hybrid ANN-ABC method in the non-invasive estimation of chlorophyll *a* content by spectral data. The boxplot charts of regression coefficient and coefficient of determination of the Hybrid ANN-ABC method in the non-destructive estimation of chlorophyll *a* content using spectral data is both tighter and higher than color data counterparts. Partial least squares regression method has poor performance for a state where no pre-processing is performed on the spectral graph. On the other hand, the Hybrid ANN-ABC method on the raw data has a far higher performance than partial least squares regression. This issue is important in real-time applications, since time is a very important factor in real-time applications, and when it is not necessary to preprocess the graph, the estimation of chlorophyll *a* content is possible to be computed in less time. Therefore, the amount of chlorophyll *a* is estimated at a higher speed. The next limiting factor in the non-destructive estimation of chlorophyll *a*, is the cost of building the device. The spectral method is far more expensive than the color method, given that in the color method by only using an algorithm and a typical visible-range camera, it is possible to estimate the non-destructive amount of chlorophyll *a* content. As seen, the performance of the Hybrid ANN-ABC method in the non-destructive estimation of chlorophyll *a* content using color data is close to spectral data. Therefore, considering the cost and performance of the Hybrid ANN-ABC method using color and spectral data, it might be better to use color data in some applications, despite always a trade-off between performance and cost exists. Table 2 shows a comparison of the performance of proposed methods in this study with other non-destructive methods for the estimation of chlorophyll *a* content in the literature. As one can see, the here proposed hybrid ANN-ABC method has better performance than other methods, despite direct comparison is not possible due to the fact that input fruit database is different in each case. It can be concluded that the proposed methodology has proven to have a high-performance and limited cost in the non-destructive estimation of chlorophyll *a* content in Red Delicious apple fruit.

Table 2- Comparison of regression coefficients of different non-destructive estimation methods in estimation of chlorophyll *a* content in various fruit

Method	*Fruit*	*Regression coefficient*
Proposed method using spectrum data	Apple	0.987
Proposed method using color data	Apple	0.982
(Ncama et al 2017)	Grapefruit	0.943
(Adebayo et al 2016b)	Banana	0.978

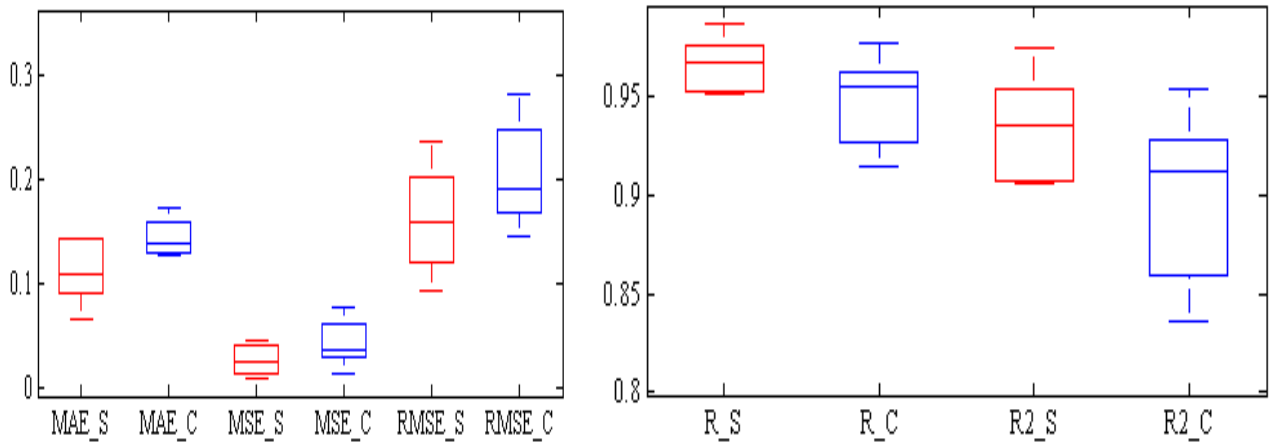


Figure 4- Evaluation criteria; (a), boxplot of hybrid ANN-ABC error indices and (b), Regression (R) and determination (R^2) coefficients in non-destructive estimation chlorophyll *a* over 20 runs (test set). S represents spectral data and C represents color data

4. Conclusions

In this study, two partial least squares regression and the Hybrid Artificial Neural Network-Artificial Bee Colony method were used to estimate the amount of chlorophyll *a* as an important parameter correlated with the stage of fruit ripening (Red Delicious apple cultivar in the present study) using both spectral and color data. The most important results are summarized next to conclude:

1. We believe that the performance of the hybrid Multilayer Perceptron Artificial Neural Network - Artificial Bee Colony Algorithm (ANN-ABC) is better than partial least squares regression because of its random nature in the training phase of the predicting model of chlorophyll *a* content.

2. Since the amount of chlorophyll *a* is predictable with rather high-performance by color features and using hybrid ANN-ABC approach, it constitutes an inexpensive method that can be used in on-line conditions that do not need very high accuracy in the estimation.

3. Using spectral data for the non-intrusive estimation of chlorophyll *a* content does not require spectroscopy over the entire visible/near-infrared range, and only a small window around 680 nm wavelength could be used. This will reduce the cost of the configuration and set-up of the spectroscopy system.

4. When using statistical methods like partial least squares regression for non-intrusive estimation of chlorophyll *a* content, the selection the suitable methods for preprocessing of spectral data is important, and ensures their high performance.

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Development and Analysis of a Belt Picking System for Sesame (*Sesamum indicum* L.) Harvesting

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ABSTRACT
In this study, a picker system with a belt-pulley mechanism, which can be used in sesame harvesting only to aim grabbing the plant stems, was designed and manufactured. In addition, the optimum working criteria were determined in laboratory conditions. To this aim, catching efficiency of the picker was evaluated statistically depending on different pulley diameters (155, 185 and 210 mm), belt speeds (0.55, 0.66 and 0.77 m s^{-1}) and belt gaps (0 and 5 mm). The catching efficiency increased as the pulley diameter, the belt speed and the belt gap increased. The picking system was found to be successful on catching the plant stems. Furthermore, the coefficient of friction between the plant body and the catching belt was determined according to the stem moisture content. As the stem moisture content decreased, a slight decrease was observed in the coefficient of friction between the belt material and the stem.

Keywords: Harvesting machinery; Picker set up; Sesame harvest; Uprooting resistance

1. Introduction

Sesame is one of the oldest annual plants cultivated in many parts of the world with tropical and subtropical climates, including India, Myanmar, Sudan and China (Yol 2011). It is an important industrial crop which contains 50-60% oil and 20-30% protein in its seeds. Sesame oil is widely used in food, cosmetics, insecticides and soap industry. After removing the oil from the sesame seeds, 43% of the crude protein is present in the remaining cusps. For this reason, it has an important role in animal feed industry. Sesame residue is also considered as human food used as a joining material in breads in some countries (Hatipoğlu 2016).

In the world, 6.11 million tons of sesame seeds were produced on 10.57 million hectares of land with an average yield of 578 kg ha^{-1} based on 2016 figures (FAO 2016). Main sesame importing countries in the world are China, Japan, EU countries and Turkey. There has been a decrease in sesame production in Turkey in recent years. The most important reasons for the decline of sesame agriculture in Turkey are the tendency of producers to turn to crops requiring less labor, the preference of crops that can be harvested mechanically, and the increase in labor costs due to the impossibility of mechanic harvesting. In fact, sesame agriculture offers a very profitable production with relatively low inputs, except for the labor costs. Moreover, if the progress in mechanical harvesting of sesame can be achieved, its cultivation as a second crop may be more prevalent (Arslan et al 2014).

Harvesting is still the most important problem in sesame cultivation since its harvest mainly depends on hand labor. Because of this, the harvest cost accounts for about 70% of the total cost of production (Dizdaroğlu & Tan 1995).

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Sesame seeds reaching the maturity condition must be harvested in a timely and rapid manner. The delay in harvest time causes the capsules to lose moisture content and cracks resulting high harvest losses. For this reason, when the lower capsules reach the maturity, the harvesting should be started without waiting the upper capsules to reach full maturity.

In order to prevent seed losses, the plant is harvested by hand while being green. After it is gathered in the form of bundles, it is left to dry for 10 to 15 days. They are then turned back and threshed by hand or with a stick (Ugurluay 2002). Therefore, the mechanical harvesting of sesame is a necessity. As the days go by, most of the producers in Turkey are trying to stay away from labor intensive crops because of the difficulty of finding workers and increasing labor costs.

Harvesting of agricultural crops is generally done by machines which apply cutting-sawing, picking-shaking, shaking-vibrating or picking-collecting methods in many different ways (Ugurluay et al 2010). Vurarak et al (2017) tested a semi-mechanized harvesting method, which may be an alternative to manual harvesting in sesame cultivation. As a harvesting machine, a reaper with re-arranged mowing and binding units according to the technical specifications of sesame seeds was used. It was reported that semi-automatic harvesting can be offered as an alternative to the manual harvesting although it results in 7.5% yield loss. Ugurluay (2008) developed a prototype harvester with belt-pulley mechanism for use in leek production and stated that this harvest head unit can also be used for similar in-row plants. Numerous studies have been carried out for plants such as lentils, peanuts, tubers, garlic, radishes, carrots and green onions that have been handcrafted at the harvest (Jun et al 2005; Geng 2008; Xiaoyan et al 2008; Zhichao et al 2008; Ranbing et al 2009; Deran et al 2010; Wei et al 2011; Jiasheng & Shang 2012; Hong et al 2014).

In Turkey and in many other countries, sesame harvest is still heavily dependent upon hand labor. This study was carried out to determine some basic parameters leading to developing a harvesting machine that can be used to reduce the need for intensive labor in sesame harvesting. Harvesting sesame can be achieved by two methods: cutting or uprooting the stems. In this study, in order to harvest sesame based on uprooting method, an experimental picker set with a belt-pulley mechanism was designed, constructed and tested in laboratory conditions to determine its optimum operating criteria.

2. Material and Methods

In the study, Muganlı-57, which is a local sesame variety was used as plant material. Some properties of this variety were given in Table 1 (Tarimziraat 2017).

Table 1- Some vegetative properties of local variety of Muganlı-57

Vegetative properties	*Values*
Length of the body	80-150 cm
Shape of the stem	four corners
Color of the flower	white
Size of the capsule	3-3.5 cm
Width of the capsule	0.9 cm
Number of capsules in the plant	70-140
Color of the grain	yellow-light brown
Yield	600-1500 kg ha^{-1}

The uprooting force of the plants from the soil was determined. The land on which the experiment was carried out had clayey-loamy soil. In order to determine the uprooting force of the sesame plants, 10 experiments were carried out. Sesame plant is a tap rooted plant but it is attached to the soil mainly by the fibrous roots. For this reason, soil humidity measurements were taken for the first 10 cm depth. In order to find the average uprooting force of the sesame plants, a force gauge (Geratech SH-500) with 500 N capacity and 0.1 N accuracy was used. The force gauge was connected to the plant body with a rope (Figure 1) and then an upward force was applied until the plant was removed from the soil.

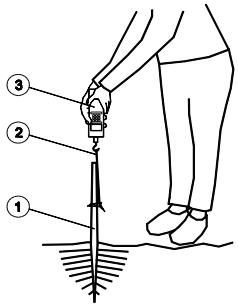

Figure 1- Measurements of the removal of sesame plants from the soil using the force meter (1, plant body; 2, rope; 3, force gauge)

The first step in sesame harvesting is picking of the sesame plants from the field. The belt-pulley mechanism was used in the part of the test set which will perform the picking work. A view of the experimental setup was given in Figure 2. Some of the sections on the experimental set were designed as adjustable to change and study some important parameters. These were the connection of the pulleys to the chassis, the reciprocal states of the belt-pulley pairs in the picker set and the revolutions of the electric motors.

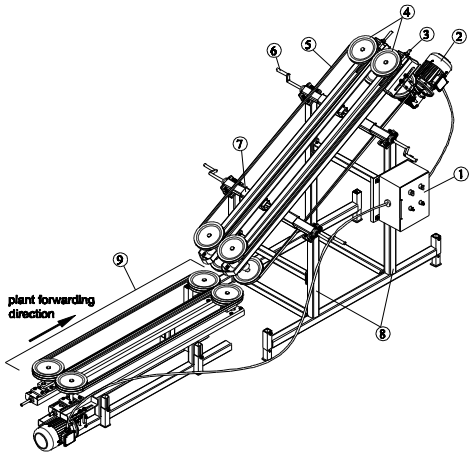

Figure 2- Isometric view of the test set (1, power and control panel; 2, electric motor; 3, belt tensioning setup; 4, pulleys; 5, belts; 6, adjustment lever; 7, gap adjustment setup; 8, frame; 9, plant transmission unit)

A digital speedometer (CEM AT-8) was used to adjust the speeds of the pulleys of the sesame harvest test apparatus. The accuracy of the speedometer was 0.1 min^{-1} and the measurement range was 2 to 10000 min^{-1}.

In order to be able to pick up the plants using the belt-pulley system smoothly without any problems, it is necessary to know the working and design values such as the appropriate pulley diameter, belt speed and the gap between the belts. In the study, the design and operating criteria for the belt-pulley harvest head were determined. Different operating conditions were established in order to determine the appropriate catching criteria of the stems. In the tests, three independent variables namely, pulley diameters, belt speeds and gaps between belts were examined (Table 2 and Figure 3).

Table 2- Independent variables and its levels in the tests

Independent variables	*Levels*
Pulley diameters (mm)	155 (D1)
	185 (D2)
	210 (D3)
Belt speeds (m s^{-1})	0.55 (S1)
	0.66 (S2)
	0.77 (S3)
Belt gaps (mm)	0 (G1)
	5 (G2)

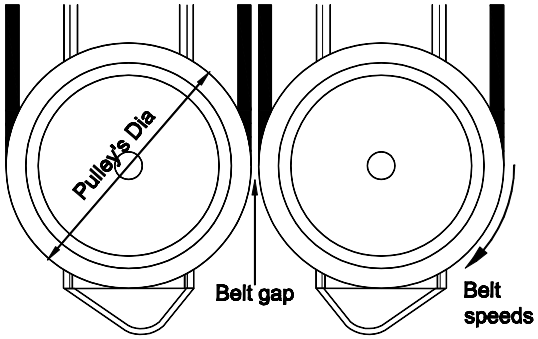

Figure 3- Independent variables in the study

Randomized block design with three replications were used in the tests. As a dependent variable, the catching efficiency (picker's efficiency for grabbing the plants) was evaluated. The data obtained from the trials were analyzed in a statistical package program (SPSS-Statistical Package for the Social Sciences) (Version: 14.0, IBM, Armonk, NY, USA).

For determining the appropriate pulley diameters, the physical dimensions (profile widths, minimum and maximum limits of the range setting mechanism, etc.) of the picker test set were taken into consideration. Belt velocities were chosen according to the machine feed rate, assuming that the harvester experiment set was a harvest head superimposed on the tractor. It was assumed that the speed of progression of the tractor with such a harvest setup would be 2.0-2.5 km h^{-1} (0.55 m s^{-1}) which could be considered as an average speed of a harvesting machinery (For example; sugar beet and potato harvesters). The 1st speed was chosen to be the same as the tractor feed rate, the 2nd speed 20% more than the speed of the tractor (0.66 m s^{-1}), and the 3rd speed 40% more than the tractor speed (0.77 m s^{-1}).

The stems were caught between the belts. The mean body thickness of sesame plants which reached harvesting stage is reported as 10.4 mm (Ugurluay 2002). According to this data, catching tests were performed based on two gaps where the belts were in full contact (0 mm distance) and the belts were slightly spaced (5 mm distance).

One of the parameters affecting the catching efficiency is the friction coefficient between the belt and the stem. The inclined plane method was used to find the static friction coefficient between the plant stem and the v-belts. The inclined plane consisted of two parts; a horizontal and fixed plane and an inclined plane connected by a hinge (Figure 4). The effect of moisture values on friction coefficient was investigated by using One-Way ANOVA test at P= 0.05 significance level.

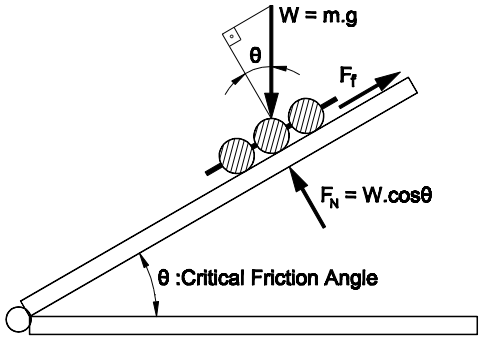

Figure 4- Determination of friction angle by inclined plane method

The v-belt pieces were placed and stuck at regular intervals on the inclined plane. During the tests, an angle meter was used to determine the angles (θ) in the inclined plane. To prevent rolling of the stems on the inclined plane and to allow them to move by sliding, three plants were connected to each other by using wires (Figure 4). Plant stems were placed at the beginning of the v-belts and the inclined plane was slowly moved upwards. At that moment when the stems started to slip, the oblique plane was stopped and the angle made with the horizontal plane was measured. To

obtain the friction coefficient (μ), the tangents of the angles obtained in the experiments were calculated. The experiments were carried out in 3 replicates. The arithmetic mean of the values was taken. Equation 1 was used to obtain the friction coefficient (μ).

$$tan\ \theta = \frac{F_f}{F_N} = \mu \tag{1}$$

Where; F_f, friction force, N; F_N, normal force, N; μ, coefficient of friction

A drying oven was used to determine the moisture content of the plant material. A precision scale with a sensitivity of 0.01 g (Sartorius GP 3202, Gottingen, Germany) is used for material weighing. The relationship between moisture content and friction coefficient in the stem was also investigated. For this aim, twelve plant specimens were divided into groups of two (n= 6). Using a pair of plant stems (Group 1), inclined plane experiments were carried out in 3 replicates for 4 days to study the effect of the moisture content on the friction. The remaining 5 groups of plants were used to measure the change in moisture content during the 4 days. Then, average values were calculated and used in data analysis. In order to determine the moisture content, the wet mass values of the plant samples were recorded using sensitive scales. The weighed samples were dried in a drying oven at 105 °C for 24 hours, after which the masses were again measured. Moisture ratios of plant samples were calculated using Equation 2 (Mohsenin 1970).

$$M_R = \frac{(W_m - D_m)}{W_m} \times 100 \tag{2}$$

Where; M_R, moisture ratio (%); W_m, wet mass (g); D_m, dry mass (g)

The normal force (F_N) that allows the pulleys to clamp and hold the plant stems was measured with a force gauge. Using Equation 3, the frictional force between the belt and the plant stem was calculated.

$$F_f = F_N . \mu \tag{3}$$

The frictional force (F_f) is equal to the maximum holding force (F_h). In Figure 5, the forces that occur on the plant stem during the grabbing was shown.

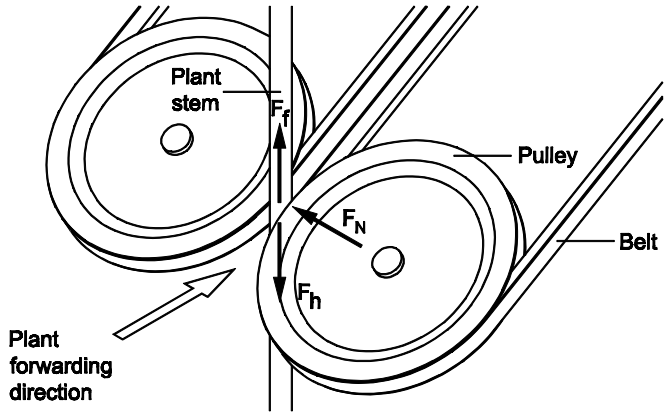

Figure 5- The forces on the plant stem during the grabbing

3. Results and Discussion

As a result of the literature search, no study was found similar to the subject of the current study. The only known and similar work was carried out by Ugurluay (2008) on a prototype harvester that can be used in leek harvesting. In this study, the uprooting conditions were investigated because of the necessity of harvesting the leek plant. A picker unit

with wide belts and pulleys was designed. The plant uprooting forces were measured, found to be quite big, and a knife was used to move under the ground to loosen the root zone.

Another similar study was carried out by Yumak & Evcim (1990). They developed a prototype "two-row cotton stalk pulling machine" to mechanically pull up the cotton stalks after harvesting. It was reported that the machine removed plant stalks from the soil in good conditions up to 95%.

In this study, a double row picking unit with narrow belts and pulleys was designed to suit sesame plant structure. The uprooting conditions have been examined. The plant uprooting strength was measured. The average value of the coefficient of friction between the plant and the picker belts was determined. It has been tried to calculate which values the friction force can reach. The effect of the moisture content on the coefficient of friction between the stem and the belt was shown as a graph in Figure 6.

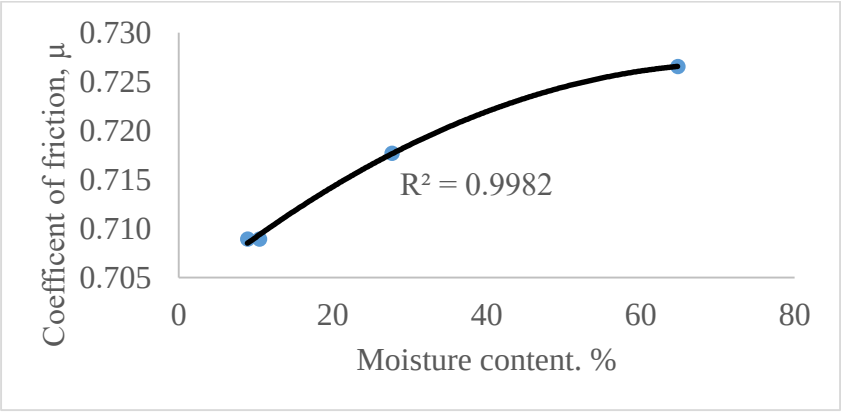

Figure 6- Relation graph between moisture change and coefficient of friction

A slight decrease in friction coefficient values was observed as the moisture content of the stems decreased. Statistically, the effect of moisture values on friction coefficient values was not significant (P>0.05).

Based on the results of the uprooting force test it was found that the stalk diameter was 13.7±1.1 mm, the uprooting force was 309.1±64.9 N, the soil moisture content (%) was 23.56±0.55. The grip strength of the plant stems between the belts was calculated as 200 N on average. It was found that this value was much lower than the plant uprooting force. The finding of the uprooting force as much greater than the grip strength suggested that the plants must be cut from the bottom with a knife or the root zone must be loosened during harvesting.

The picker experimental set was created in two parts as previously mentioned. The first part was used only to transfer the sesame plants to the picker. In the experiments, 30 plants were fed by hand to the conveyor belt to send them to the picker set. Plants conveyed by the conveyor belt were expected to be captured by the picker (Figure 7).

Figure 7- Grabbing the sesame plant of the picker set

The variance analysis results are given in Table 3 and the Duncan Test results are given in Table 4. When the variance analysis table was examined, the pulley diameters, belt speeds and belt gaps were found to be important in

terms of catching the plant stalks. In the catching event, the pulley diameter and the belt speed were found to be insignificant in the dual interactions of variation sources. Belt speed and belt gap and pulley diameter and belt gap interactions were found significant. That is to say, when the belt speed-the belt gap and the belt diameter-the belt gap are evaluated together, there was a difference in the catching efficiency between them. Interaction analysis revealed that 2nd belt gap value (5 mm) gave better results.

Duncan comparison test results (Table 4) revealed that the 1st pulley diameter (155 mm) and the 2nd pulley diameter (185 mm) were similar while the 3rd pulley diameter (210 mm) was found to be different from the other two at the significance level of 0.001 when the pulleys used in the test apparatus were evaluated for the ratio of catching sesame plants. Therefore, the 3rd pulley diameter value (210 mm) was found to be much more successful than the other two pulley diameters in terms of catching ratio. The 3rd belt speed value (0.77 m s^{-1}) was the most successful when the belt speeds used in the test apparatus were evaluated for the ratio of catching sesame plants. The 1st band speed value (0.55 m s^{-1}) gave the lowest catching ratio. The ratio value of the 2nd band speed value (0.66 m s^{-1}) was in the middle. It was found to be similar to both the 1st one and 3rd one. The difference between the 1st (0 mm) and the 2nd gap value (5 mm) was statistically significant and the 2nd gap (5 mm) was found to be much more successful when the band gaps used in the experiment were evaluated for the ratio of catching sesame plants.

Table 3- The variation analysis results

Variation sources	*Sum of squares*	*Degree of freedom*	*Squares average*	*F*
General	149333	53		
Diameter	23111	2	11556	0.000
Speed	9000	2	4500	0.008
Gap	42667	1	42667	0.000
Diameter * Speed	6556	4	1639	0.114
Speed * Gap	9000	2	4500	0.008
Diameter * Gap	23111	2	11556	0.000
Diameter * Speed * Gap	6556	4	11556	0.114
Error	29333	36	1639	

Table 4- The catching success of the belt picking system based on different parameters

Pulley Diameter (D)	*Catching Ratio*	*Belt speed*	*Catching Ratio*	*Belt gap*	*Catching Ratio*
D1 28.4±2.04[a]	94.7%	S1 28.6±2.09[a]	95.6%	G1 28.2±2.02[a]	94.0%
D2 28.9±1.81[a]	96.3%	S2 29.1±1.81[ab]	97.0%	G2 30.0±0.00[b]	100.0%
D3 30.0±0.00[b]	100.0%	S3 29.6±0.78[b]	98.7%		
P<0.001		P<0.01		P<0.001	

Values are mean ± s. e.; a, b with the same superscript indicate no significant difference in the same column

4. Conclusions

The harvesting system with belt pulleys was found to be successful in the grabbing of sesame plants. As a result of the obtained data, such a collecting system can be used on a machine designed for sesame harvesting. The plant's uprooting force from the soil was measured and it was not possible to remove it directly with a harvesting head with belt pulley mechanism. For this reason, it is necessary to either loosen the root zone of the plant by using the underground knives or cut off the plant stem during the catching with a knife mechanism.

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Identification of SSR Markers for Differentiating Rice (*Oryza sativa* L.) Varieties Marketed in Turkey

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ABSTRACT
This study was carried out to identify SSR markers for the differentiation and identification of rice cultivars marketed in Turkey between 2016 and 2017. In this study 60 registered or production permitted, some local and foreign rice varieties were used as a material. DNA was isolated from single polished rice kernels for PCR amplification and rice cultivars were genotyped by 50 SSR markers. We found that 45 out of 50 SSR markers produced reproducible and polymorphic alleles. Thirty-six rice varieties had variety specific alleles among 60 rice varieties analysed in the study. This variety specific alleles belong to only one variety and they can be used to identify rice variety among 60 rice varieties studied. Osmancık-97, Cammeo, Ronaldo and Baldo are the most import 4 varieties in Turkish rice production and milled rice market. In this study, it was found that RM152, RM144, RM259 and RM118 SSR markers can be used to differentiate and identify these most important four varieties with different combinations. Collectively, this study provided some variety specific SSR markers that can be used to differentiate and identify rice varieties sold in Turkey.

Keywords: Polished rice; Rice cultivar; SSR

1. Introduction

The quality criterias of rice vary from country to country, even from city to city within a country. However, the main quality criterias generally accepted by everyone are milling yield, physical, chemical, cooking and eating quality. Even if all quality criterias are very good, marketing mixture of different rice varieties decreases the rice quality sharply. Mixing of the rice varieties during marketing also causes unfair competition among rice industry and misleads the consumers. This is done generally by mixing low quality rice with high quality rice to sell mixed rice for higher price.

In Turkey, polished rice is generally sold under Osmancık-97 or Baldo varieties name. These two rice varieties are well accepted by consumers with their quality characteristics. It is very difficult to identify mixed rice kernels by checking physical characteristics of polished rice inside the package. Although the rice in the package is physically similar, the chemical and cooking properties may be different. Thus, in order to identify and differentiate polished rice kernels in the package, molecular markers need to be discovered.

Because microsatellites (SSRs) are well distributed within the genome in eukaryotic organisms, found in large numbers and show more polymorphism (Morgante & Olivieri 1993), they are more advantageous for identification and purity analysis of vaieties than other molecular markers (Weising et al 1997; Ni et al 2002; Kostova et al 2006). It was reported that the use of SSR markers to study genetic diversity in rice also very appropriate (Powell et al 1996).

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Many SSRs have been developed for rice to use genetic diversity studies (Zhao & Kochert 1993). It was reported that more than 20000 SSR markers were mapped for genome-specific regions in rice (Pervaiz et al 2010). Many genetic diversity and characterization studies were done by using SSRs in rice (Hossain et al 2007; Pervaiz et al 2010; Rahman et al 2012; Choudhary et al 2013; Worede et al 2013; Kumar et al 2014; Li et al 2014). Some studies on the genetic diversity of Turkish rice varieties were also done by using SSRs (Cömertpay et al 2016), RAPDs (Bay 2009) and IRAPs (Yüzbaşıoğlu et al 2016) markers. However, there is not a particular study to identify and differentiate rice cultivars from single polished rice kernel by using molecular markers for Turkish rice market. Thus, there is a need for the development of molecular markers to identify and differentiate rice varieties in polished rice package for a fair competition of industry and protect consumers.

The objective of this study to find out SSR markers which can be used to differentiate and identify rice varieties by using single polished rice kernel. Detected SSRs can also be used for variety identification and purity test studies in seed production.

2. Material and Methods

2.1. Plant material

In Turkey, there were 48 registered and production permitted rice cultivars at the registration list in 2016. In addition to these registered and production permitted rice varieties at registration list, some local varieties and foreign varieties were also chosen as plant material. Totally 60 rice varieties, given in Table 1, were obtained from Trakya Agricultural Research Institute and Trakya Genetics RD Consulting Production Import Export and Marketing Co Ltd. Some rice varieties used in this study are not grown anymore in Turkey, but sometimes there are polished rice with their names at the rice market. The most widely grown rice varieties in Turkey are Osmancık-97, Cammeo and Ronaldo. About 80% of rice growing area in Turkey was planted with these three varieties in 2017.

Table 1- List of rice varieties analysed in this study

Akçeltik	Durağan	Kıral	Mis-2013	Sarıçeltik
Altınyazı	Efe	Kırkpınar	N1-41T-1T-0T	Sürek M711
Aromatik-1	Ergene	Kızılırmak	Neğiş	Sürek-95
Bafra Yıldızı	Europa	Kızıltan	Osmancık-97	Şumnu
Balaban	Gala	Koral	Paşalı	Thainato
Baldo	Gönen	Krasnodarsky-424	Plovdiv	Tosya Güneşi
Beşer	Halilbey	Küplü	Ranballi	Trakya
Biga İncisi	Hamzadere	Manyas Yıldızı	Ribe	Tunca
Cammeo	İpsala	Maratelli	Rocca	Ülfet
Çakmak	Kale	Meco	Rodina	Veneria
Demir	Karadeniz	Meriç	Ronaldo	Yatkın
Diyarbakır Yerli	Kargı	Mevlutbey	Sarhan	Yavuz

2.2. DNA extraction from single polished rice grain

Single polished rice kernel was used for DNA extraction. High quality gDNA was extracted from single polished rice kernel as described by Rajendrakumar et al (2007). Isolated gDNA samples were quantified spectrophotometrically by OPTIZEN NanoQ (Kaia, Panama). Quality of the gDNAs was analysed by agarose gel (0.8%) electrophoresis. The gDNAs were diluted to 25 ng μL^{-1} concentration for PCR.

2.3. PCR amplification of SSR loci

In this study, 50 SSR markers, which are suggested for diversity analysis in the Gramene Database (Gramene 2015) spread across all rice chromosomes, were used. The PCR amplifications of the each SSR loci were carried out in 15 µL reaction mixture containing 50 ng gDNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM, primers and 1 U Taq polimerase (Invitrogen). The amplification was performed by T100 Bio-Rad thermal cycler (CA, USA) following the cycles; 2 min at 94 ºC for initial denaturation, and 35 times 45 secs at 94 ºC, 1 min at 53-61 ºC (depending on the primer Tm), 1 min at 72 ºC and 10 min at 72 ºC for final extension.

2.4. DNA fragment analysis

The amplified PCR products were separated and analysed by AATI Fragment Analyser System (Advance Analytic, IA, USA). The PCR products were prepared for capillary system analysis according to the manufacturer's instructions (DNF-905 dsDNA Reagent, IA, USA). Each reaction was diluted with a 1:5 ratio by dilution buffer and 24 µL of the mixture transferred to the 96-well plate. Each well was covered by mineral oil and electrophoresis was performed by applying 9.0 kV for 80 min. For sizing of the SSR alleles, 1-500 bp DNA ladders were used in each run and DNA fragment sizes were calculated by system software ProSize. Results were analysed by GenAlex 6.5 software program.

3. Results and Discussion

Forty-five SSR markers produced reproducible and polymorphic alleles among 50 SSR markers used in this study. Five of them (RM154, RM124, RM454, RM284 and RM447) did not produce satisfactory quality PCR products, thus they were not used for the later studies. A total of 279 alleles were yielded by the 45 polymorphic SSR markers. The size of allelic DNA fragments varied from 77 bp (marker RM44) to 430 bp (marker RM171). Maximum allele numbers per locus were produced by primers RM25 with 19, and followed by RM259, RR237, RM452, RM287, RM162, RM431 and RM19 with 13, 11, 11,10, 10, 10 and 10 alleles, respectively. Average allele number was 6, 2.

3.1. Variety specific SSR alleles for identification of rice varieties

Identification of the variety specific alleles is one of the significant results of this study. Because variety specific alleles belong to only one variety, and they can be used for identifying desired rice variety. Variety specific alleles found in this study and their SSR locies are given in Table 2. As can be seen in Table 2, 36 rice varieties had variety specific alleles, among 60 rice varieties studied. Aromatik-1 had the highest number of variety specific allele with 17 alleles amplified by 17 SSR markers. This was followed by Akçeltik, Karadeniz and Sürek-95 with 4 variety specific alleles (Table 2).

Sixteen rice varieties had two variety specific alleles ampliphied by different SSRs. These are Rocca Kızıltan, Hamzadere, Negis, Balaban, Şumnu, Sarıçeltik, Krasnodarsky-424, Plovdiv, Sarhan, Diyarbakır yerlisi, Duragan, Meriç, Küplü, Meco and Mevlütbey (Table 2).

On the other hand, 16 varieties, İpsala, Gönen, Yatkın, Mis-2013, Veneria, Yavuz, Biga İncisi, Kral, Kargı, N1-41T-IT-OT, Tahinato, Bafrayıldızı, Kızılırmak and Baldo had only one variety specific allele (Table 2). In this group Baldo, Gönen and Yatkın are important rice varieties in polished rice market in Turkey.

One of the significant result of this study is to find out variety specific alleles for 36 rice varieties including Baldo. Because Baldo is marketed with the highest price in Turkish rice market, polished kernel of some other rice varieties can sometimes be mixed with polished kernel of Baldo variety, and sold as a Baldo variety with higher price. Moreover, some rice varieties with the similar physical characteristics of Baldo polished rice kernel are marketed as a Baldo rice. Baldo specific 150 bp allele amplified by RM118 marker can be used to identify Baldo polished rice kernels among 60 rice cultivars studied. Gönen variety has similar physical characteristics of Baldo polished rice, and these two varieties can be also differentiated by variety specific alleles of Baldo and Gönen. Yatkın variety is one of the newest variety and its planting area is increasing every year. Cultivar specific 138bp allele amplified by RM408 can be used to distinguish Yatkın rice variety from other 60 varieties.

Pal et al (2004) reported that the same allel (147 bp) was amplified by RM255 in traditional Basmati rice varieties, which was different to that in IR 36 (145 bp) and Azucana. They suggested to use RM255 marker to differentiate traditional Basmati and non Basmati rice varieties.

Table 2- Variety specific allels

Variety	*Locus*	*Allele*	*Variety*	*Locus*	*Allele*
Rocca	RM25	186	Aromatik-1	RM507	255
Rocca	RM431	256	Aromatik-1	RM271	98
Kızıltan	RM510	124	Aromatik-1	RM307	131
Kızıltan	RM431	259	Aromatik-1	RM474	233
Sürek-95	RM277	124	Aromatik-1	RM484	300
Sürek-95	RM118	181	Aromatik-1	RM118	163
Sürek-95	RM452	174	Aromatik-1	RM178	116
Sürek-95	RM452	209	Aromatik-1	RM287	121
Hamzadere	RM507	267	Aromatik-1	RM162	219
Hamzadere	RM413	86	Aromatik-1	RM452	211
İpsala	RM25	148	Aromatik-1	RM161	165
Negis	RM5	113	Veneria	RM431	224
Negis	RM452	192	Mis-2013	RM408	354
Gönen	RM431	254	Yavuz	RM19	221
Demir	RM259	175	D.bakır yerlisi	RM25	163
Ulfet	RM452	216	D.bakır yerlisi	RM413	92
Balaban	RM171	422	Duragan	RM552	181
Balaban	RM44	97	Duragan	RM55	233
Sumnu	RM536	246	Biga incisi	RM237	135
Sumnu	RM338	189	Kral	RM133	232
Yatkın	RM408	138	Meric	RM19	224
Sarıceltik	RM259	199	Meric	RM552	231
Sarıceltik	RM452	201	Kuplu	RM178	118
Krasnodorsky	RM215	164	Kuplu	RM452	342
Krasnodorsky	RM162	256	Kargı	RM455	129
Akceltik	RM237	374	Baldo	RM118	150
Akceltik	RM484	295	N1-4IT-IT-OT	RM55	231
Akceltik	RM259	195	Meco	RM105	132
Akceltik	RM162	250	Meco	RM144	248
Plovdiv	RM25	165	Mevlutbey	RM162	240
Plovdiv	RM259	197	Mevlutbey	RM431	252
Sarhan	OSR13	103	Thainato	RM178	132
Sarhan	RM338	270	Bafrayıldızı	RM514	254
Aromatik-1	RM19	247	Kızılırmak	RM316	214
Aromatik-1	RM536	232	Karadeniz	RM237	375
Aromatik-1	RM489	240	Karadeniz	RM474	268
Aromatik-1	RM11	152	Karadeniz	RM178	112
Aromatik-1	RM283	156	Karadeniz	RM125	225
Aromatik-1	RM1	116			

3.2. Differentiating and Identification of rice varieties with using several alleles

Osmancık-97, Cammeo and Ronaldo rice varieties account for about 80 percent of the total planted area of rice in Turkey. Thus it is important to identify and differentiate these three varieties as well as Baldo rice variety from other rice varieties sold in Turkish rice market using molecular markers. Interestingly, any variety specific allele was not found for those 4 rice varieties except Baldo. On the other hand, in order to differentiate these three varieties from each other and Baldo variety, 3 SSR markers (RM152, RM144, RM259) and Baldo specific RM118 (150 bp) allele were identified. Electropherogram results of AATI analyser for RM152, RM144, RM259 SSR markers to differentiate and identify Baldo, Osmancık-97, Ronaldo and Cammeo varieties were given in Figure 1, 2 and 3. As can be seen in Figure 1, 2 and 3, Baldo, Ronaldo, Cammeo and Osmancık-97 varieties can be differentiated each others with their alleles amplified by these 3 SSRs (RM152, RM144, RM259). RM152 amplified 143 bp allele, 146 bp, 152 bp and 152 bp alleles from Baldo, Ronaldo, Osmancık-97 and Cammeo respectively. With the use of RM152 allele, Baldo and Ronaldo varieties can be differentiated from Cammeo, Osmancık-97 and each other. However, it was not possible to differentiate Osmancık-97 and Cammeo with RM152 (Figure 1). On the other hand, RM144 amplified 266 bp allele for Osmancık-97, while it amplified 254 bp, 255 bp and 255 bp alleles for Ronaldo, Baldo and Cammeo respectively. RM144 marker can be used to differentiate Osmancık-97 from Ronaldo, Cammeo and Baldo (Figure 2). In addition, while RM259 amplified 172 bp allele for Baldo and Cammeo, it amplified 175 bp and 178 bp alleles for Osmancık-97 and Ronaldo respectively (Figure 3), indicating that RM259 marker can be used to differentiate Baldo and Cammeo

from Osmancık-97 and Ronaldo. This marker can also be used to differentiate Osamancık-97 and Ronaldo from each other. With the use of only these three SSR markers (RM152, RM144 and RM259), the differentiation and identification of Baldo, Osmancık-97, Ronaldo and Cammeo varieties, which have more than 80% market share in Turkish rice market, can be done. In addition to these three markers, Baldo specific 150 bp allele amplified by RM188 can also be used together with these three markers (RM152, RM144 and RM259) to differentiate and identify these four markets dominating varieties.

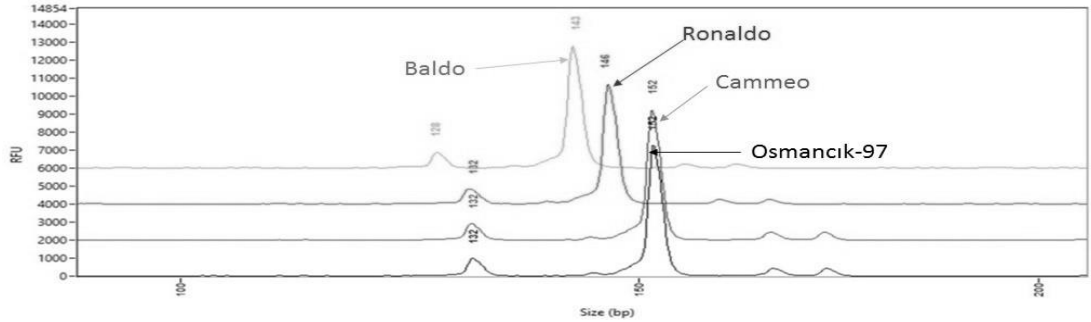

Figure 1- Electropherogram of Baldo, Ronaldo, Cammeo and Osmancık-97 rice varieties for RM152 marker

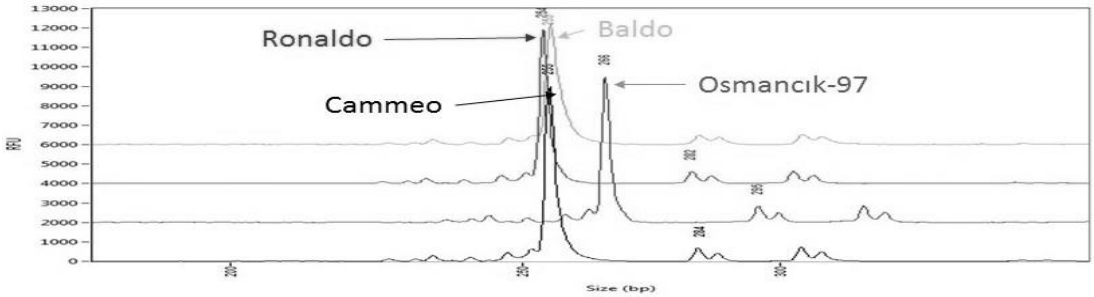

Figure 2- Electropherogram of Baldo, Ronaldo, Osmancık-97 and Cammeo rice varieties for RM144 marker

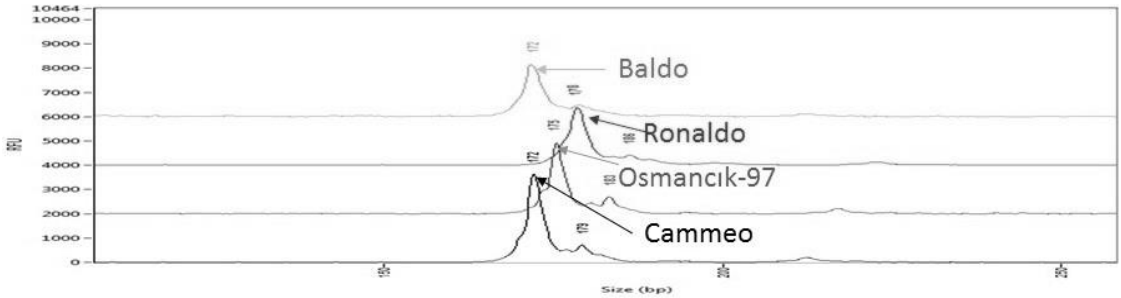

Figure 3- Electropherogram of Baldo, Ronaldo, Osmancık-97 and Cammeo rice varieties for RM259 marker

4. Conclusions

This study was carried out to differentiate and identify rice varieties by means of SSRs analysis with using single polished rice kernel in Turkey. It was found that 36 rice varieties can be differentiated and identified with variety specific alleles, while rest of the varieties can be differentiated and identified using several allele combinations. The most imported rice varieties for Turkish rice market, namely Osmancık-97, Cammeo, Ronaldo and Baldo varieties. These four varieties can be differentiated from each other using RM152, RM144, RM259 and RM188 SSR markers.

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Effects of Race, Gender, Body Condition Score and Pregnancy on Serum Apelin Levels in Ewe

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ABSTRACT
Apelin is an important adipokine hormone that is released from adipose tissue, which is considered as the energy store of the body, which plays a role in many physiological processes in the body, as well as cardiovascular, immune functions and energy, nutrients and fluid metabolism. In this study, it was aimed to determine the effect of lactation, pregnancy and gender on apelin hormone levels in blood serums belonging to different races of ewe. In the present study, the hormone levels of the pregnant, non-pregnant ewe and rams of the Akkaraman Kangal and Morkaraman races were thin, with different body condition scores (<2, 3 to 3.5 and ≥4). Apelin hormone level was determined by ELISA technique in blood serum samples of ewe's Jugular vein. It was determined that there was a difference in body scores between races and the interaction between race and body score was important ($P<0.05$). Apelin level in ewe in lactation and pregnant ewe did not change according to body condition scores ($P>0.05$). In terms of apelin, gender and body condition score, race and gender, and body condition score interactions were found to be significant.

Keywords: Ewe; Apelin; Race; Gender; Pregnancy; ELISA

1. Introduction

Adipose tissue is the source of circulating lipids, a metabolically active endocrine organ that is involved in the production and secretion of adipokines and has a role in energy homeostasis (Medina-Gómez 2012; Pérez-Pérez et al 2015).

Adipose tissue is categorized into three groups based on its structural characteristics: white, brown and beige adipose tissue (Awad & Bradford 2010). White (unilocular) adipose tissue is an endocrine gland from which the adipokines are secreted, has a thin cytoplasmic section and the nuclei in the cells are pushed to the side. Brown adipose tissue (BAT) has numerous large mitochondria in its cytoplasm (Symonds 2013). The role of BAT in thermoregulation is to produce heat for thermogenesis via uncoupling protein-1 (UCP-1) instead of adenosine triphosphate (ATP) (Cinti 2012). UCP1 is only present in small amounts in the fetus and in ewe and humans. In neonatal lambs, it is activated with endocrine stimulating factors during delivery only at the beginning of respiration and to provide protection against severe hypothermia (Symonds et al 2003). Numerous endocrine factors including leptin (Mostyn et al 2002), catecholamines (Symonds et al

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2000), thyroid hormones (Heasman et al 2000), cortisol (Clarke et al 1998), and prolactin (Pearce et al 2005) have potential to activate BAT. In neonatal lambs, major BAT depots are found in the peri-abdominal and inguinal regions of lambs (Everett-Hincks & Duncan 2008). Moreover, the anterior-scapular region and the subcutaneous tissues in the leg regions contain functional BAT (Jackson et al 2001).

Apelin hormone secreted from adipose tissue is a member of the hormone-cytokine family, which was discovered recently, isolated from the bovine gastric juice, originates from preproapelin and has a 77-amino acid precursor (Tatemoto et al 2001).

It has various isoforms such as apelin and APJ receptor, apelin-12, 13, 17, 36. The levels and localizations of these isoforms (APLN-12, 13, 17, 36) in the body vary according to the tissues (Kawamata et al 2001). Apelin/APJ is present in various brain areas (hypothalamic supraoptic nucleus; SON, paraventricular nucleus; PVN), heart, stomach, skeletal muscle, testicle and ovary (Hosoya et al 2000). Apelin-36 isoform is present at higher levels in the lung, testicle and uterus, while apelin-13 is present at higher levels in the mammary glands (Kawamata et al 2001). When the isoforms are analyzed in terms of action, apelin 13 isoform has 8 times more potent biological activity than apelin 17 and 60 times more potent biological activity than apelin 36 (Tatemoto et al 1998). Therefore, most of the recent studies were conducted on apelin-13 with a greater biological activity compared to other apelin isoforms and with N-terminal pyroglutamate residues (Bełtowski 2006).

Apelin/APJ system is effective in important physiological processes including endocrine stress response (Taheri et al 2002; O'Carroll et al 2013), *cardiovascular functions* (Szokodi et al 2002), regulation of blood pressure (Tatemoto et al 2001), angiogenesis (Zhang et al 2016), thermoregulation (Reaux et al 2001) and regulation of energy metabolism (Bertrand et al 2015). One of the potential roles of apelin is the regulation of food intake. Due to the nutritional regimen, apelin and insulin secretion increase together with adipocytes (Boucher et al 2005). While intracerebro-ventricular injection of apelin does not affect food intake in satiated rats, it increases feed intake dose-dependently in hungry animals. This effect arising suggests that apelin has an anorectic effect (Brailoiu et al 2002). Therefore, possible changes in the amount of adipose tissue for a mammalian species result in important metabolic, immunological and endocrine problems.

The plasma level of apeline hormone, a member of the cell signaling protein group secreted by adipose tissue, called adipokines, is anticipated to be a potential serum biomarker that predicts the early diagnosis of diabetes mellitus (Ma et al 2014), cardiovascular diseases, obesity, and various types of cancer (Wysocka et al 2018).

Apelin/APJ is an important molecule and confirmation of its use as a biomarker in different diseases, determination of the potential of therapeutic strategies are a very important step for its clinical use. On the other hand, ewe races in Turkey is a branch of the livestock sector that has an important contribution to both economy and human nutrition (meat and milk production) (Yildiz & Denk 2006). The lactation, reproduction and pregnancy periods are important stages in the production cycle for ewe races sustainability. For an effective reproduction process to ensure sustainability, genetic and endocrine control pathways should be known in detail. Lactation, one of the important stages affecting the production cycle, causes radical changes in the adipose tissue metabolism of ruminants. Apelin hormone, secreted from adipose tissue and found in the mammary gland, regulates the activity of mammary gland in ewe (Mercati et al 2018). In addition, primarily apelin, leptin, and adinopectin hormones in the adipokine group, and hormones such as growth hormone, prolactin and parathyroid hormone are secreted from the mammary gland. Some adipokines such as apelin, adiponectin and leptin have a role in the growth, function and lactogenic regulation of the mammary gland (Habata et al 1999; Palin et al 2017). Adipose tissue has a critical importance in meeting the metabolic energy required for lactation and in the strategies for increasing and improving the milk yield (Vernon & Pond 1997) its ability to use adipose tissue energy (Lau et al 2005). The regulation of reproductive and nutritional behavior is controlled by the central effectors in the brain, the hypothalamus and the endocrine and nerve signals (Traslaviña et al 2014).

Apelin and APJ (G protein-coupled receptor) has a diffuse distribution in the reproductive areas of the brain such as hypothalamus, pituitary gland, and in the ovary and testicle. The role of apelin in the reproductive behavior and regulation of the hormone realizes the release of LH and FSH (Hosoya et al 2000). It has been reported that its regulatory role in male reproductive system suppresses the release of LH and testesterone (O'Carroll et al 2013; Sandal et al 2015).

The issue of infertility in rams is one of the most fundamental issues that affects the sustainability in ewe sector since it affects the reproductive performance in ewe, the number of ewe giving birth and lambs born per ewe. It is reported that apelin/apj agonist might be a useful drug in the treatment of infertility (Valle et al 2008). However, there is a limited number of studies investigating the effect of apelin hormone on male reproductive system. Another important stage in the production cycle is pregnancy. Pregnancy, is a period of intense physiological changes due to fetal growth. Protection of

maternal and fetal health is very important in this period. If the body does not provide sufficient energy support required for the development of fetus or fetuses during pregnancy, other energy sources and body adipose depots are used. This process also corresponds to the lactation period. The energy source used in this period is glycogen reserves in the liver (Ağaoğlu & Akgul 2006; Kaymaz 2006). The energy that cannot be met from carbohydrates is met from the fatty acids accumulated in the liver. If this accumulation is excessive, the liver functions are impaired, resulting in hepatic lipidosis, on the other hand, some fatty acids transform into toxic ketone bodies. Pregnancy toxemia occurs with the increased toxic ketones in blood, milk and urine and due to disorders in carbohydrate and fat metabolism (Rook 2000; Brozos et al 2011). Pregnancy toxemia, a metabolism and herd problem, most commonly results in death, if not diagnosed and treated timely. Therefore, attention should be paid to the feeding program from the 30th day of pregnancy, especially in multiple pregnancies (Scott 2007). In the treatment of pregnancy toxemia, the administration of insulin increases the chance of recovery of animals with severe disease since it stimulates glucose intake (Henze et al 1998; Rook 2000). There is a strong link between apelin and insulin secretion (Guo et al 2009). The concentration of circulating apelin is directly increased by insulin, while it is reduced by glucocorticoids (Boucher et al 2005). Insulin acts directly on adipocytes to stimulate apelin production (Sörhede et al 2005). Apelin hormone modulates fetal angiogenesis and energy homeostasis during pregnancy. However, although its effect on pregnancy metabolism has not been fully clarified, it has an important role in providing a normal fetal development and in the regulation of placental vascularity and blood flow (Cobellis et al 2007; Telejko et al 2010). In a study conducted on experimental animals, it has been shown that the use of glucose was increased in the skeletal muscle and the plasma glucose level significantly decreased after acute apelin injection. With this aspect, apelin is considered as a promising development in the management of insulin resistance (Dray et al 2008).

The presence of apelin isoforms (Apelin 13, 17, 36) in plasma (Bełtowski 2006) allows the effect of apelin hormone to be analyzed, which forms the basis of our study. Therefore, the effect of race, lactation, pregnancy and gender factors on serum levels of apelin-13 isoform, which has been indicated to have a high biological activity in the literature, was investigated in our study. Fat in the tail of fat-tailed ewe is the fat stored in the tails of ewe raceed in regions with long winter season and limited possibility of feeding. It is also a reserve store of food and energy for ewe (Sönmez 1968; Anonymous 2012). Moreover, it is a human food containing quite high amounts of omega acids and the total fat and protein content of 100 g is 86.12 and 2.87 g, respectively (TÜRKOMP 2019).

Clinical studies report that apelin hormone has a therapeutic potential in clinical use as a potential serum biomarker for the early diagnosis of various diseases (cancer, diabetes and cardiovascular problems) and follow-up of metabolic processes. The data obtained as a result of our current study is a preliminary study for the studies that we will conduct to investigate the therapeutic efficacy as a result of the use of synthetic apelin hormone among alternative treatment methods for pregnancy toxemia. Moreover, since this study on elucidating the factors affecting the level of apelin hormone is the first comparative and comprehensive study on domestic ewe, it will serve as a reference for further studies and provide new information to the literature. Furthermore, the studies to be conducted on large numbers of animals and different ewe races are of importance in terms of strategies. The aim of this study was to investigate the effect of gender, body condition score and race on serum apelin hormone levels of domestic ewe races (Akkaraman-Kangal and Morkaraman), during lactation and pregnancy periods.

2. Material and Methods

2.1. Animal selection and creation of groups

In the study, 270 ewes of Morkaraman and Akkaraman-Kangal races aged between two and seven with equal average live weight, gave normal birth in the previous reproductive season and 90 adult rams of Morkaraman and Akkaraman-Kangal-races were used.

The study was conducted in 6 ewe farms in Bayburt Province center and two districts (Demirozu and Aydıntepe) (40.16 N, 39.89-K; 40.22 N, 40.26-K; 40.3 N, 40.14-K) which ewe production is performed under intensive conditions and records are regularly followed up. The animals used in the study were divided into three groups as Group I (lactation group), ewes in the early lactation period (n= 90); Group II (pregnancy group), ewes in the first period of pregnancy (on the 100th day of first pregnancy) (n= 90) and Group III (rams) by randomization so that the average total live weight of the groups was equal. During the study period, the environment and feed factor (Table 1) were taken into account. The content of feeds used in this study was analyzed according to the standard AOAC methods.

Table 1- Nutrient composition of diets used in the study (%)

Ration composition	*Morkaraman*	*Kangal Akkaraman*
Barley	65.00	65.00
Wheat bran	10.50	7.00
Soybean meal	22.00	20.50
Dicalciumphosphate	1.00	1.00
Salt	0.50	0.50
Premix	0.50	0.50
Chemical composition, %		
Dry Matter	90.39	90.60
Crude protein	17.37	16.99
Crude ash	5.59	5.78
ADF	8.84	11.26
NDF	34.44	32.72
ME Kcal kg^{-1}	2642	2620

1 kg vit.-min. Premix contains vitamin A, 7,000,000 IU; vitamin D3, 1,000,000; vitamin E, 30,000; Mn, 50,000 mg; Zn, 50,000 mg; Fe, 50,000; Cu, 10,000 mg; I, 8,000 mg; Co, 200 mg; Se, 150 mg; and Mg, 100 mg

2.2. Collection of serum samples

Blood sample of 10 mL was collected from the jugular vein of the ewe into the tubes not containing anticoagulant (VACUETTE® TUBE 9 mL Serum Clot Activator). The blood samples collected were centrifuged at 3000 rpm/min for 10 minutes on a refrigerated centrifuge (NF 1200, NUVE, Ankara, Turkey) in the laboratory, then resulting blood serums were separated. The separated serums were transferred into sterile tubes and stored in deep freezers (-80 °C) until the laboratory analyses were carried out.

2.3. Measurement of apelin hormone levels in serum

The basic principle of the Elisa method is based on the use of enzyme to determine the antigen-antibody combination in the sample. The enzyme used converts the colorless layer (chromogen) into a colored product, indicating the presence of antigen-antibody, and the intensity of the resulting color is read by the elisa plate reader at the recommended wavelength so the relevant concentration is determined (Marai et al 2001).

The minimum detectable concentration of the apelin hormone kit used in the measurement of apelin levels in the blood serums obtained as a result of the study is reported as <18.75 pg mL^{-1}. The race-specific ewe APLN ELISA Kit (Apelin, FineTest, Product code: ESH0081, CHINA) was studied in accordance with the procedure described in the manufacturer's catalog using the determination of 31.25-2000 pg mL^{-1}, the intra assay coefficient of 8.0% and the inter-assay coefficient of 10.0%.

2.4. Statistical analysis

In this study, the serum apelin hormone levels of Kangal Akkaraman and Morkaraman ewe races in the lactation and pregnancy period were analyzed as dependent variables and gender, body score and race factors were analyzed as fixed effects. In the analysis of the effects of gender, body score and race on the serum apelin hormone levels of Kangal Akkaraman and Morkaraman ewe races in the lactation and pregnancy period, normality test was applied to the apelin measurements. As a result of significant Shapiro Wilk test, logarithmic transformation was applied to the data. After the transformation process, Levene's test (P= 0.33) was used to determine whether the data was normally distributed and the group variances were homogenous. Then, the univariate procedure was used in the generalized linear models for the logarithmic apelin measurements. The analyses were performed in the full factorial setting with 3 factors (2x3x3) according to completely randomized design, and the interaction effects of the body score and race, body score and gender, race and gender, and body score, gender and race were also investigated. Tukey's multiple comparison test was used to compare the differences for the means of apelin hormone. IBM SPSS v25 statistical package software was used in all analyses in this study. All significant differences were evaluated by testing at P<0.05 level.

3. Results

Kangal Akkaraman and Morkaraman ewe with a body condition score of ≤2 (low), 3- 3,5 (optimal), and ≥4 (high). The treatment groups were: Group 1; non-pregnant (n= 90); Group 2 pregnant (n= 90) and Group 3, rams (n= 90). The experimental material was composed of animals of similar ages. At the end of the research, when the apelin level of the coaches was examined according to the races and body condition score (Table 2), it was determined that there was a bodyscore difference according to the races, while it was found to be important in the apelin-related interaction between race and body score (Table 3). In the study conducted on the ewe, it was found that the apelin level in the lactation and pregnancy period was similar in terms of body condition score (Table 3 and 4), but it was determined that there was a difference between races. As a result of the statistical analysis, it was found that the effect of the apple's gender on the body condition score, the race's gender and race's body condition score interaction (P<0.05) (Table 4).

Table 2- Effect of apelin on ram races and body condition score (ng mL^{-1})

	N	*Morkaraman*	*Kangal Akkaraman*
2 Bodyscore	30	5.60±0.54^{a}	4.43±0.34^{ab}
3 Bodyscore	30	3.31±0.54^{bc}	4.85±0.39^{ab}
4 Bodyscore	30	4.04±0.34^{abc}	3.22±0.22^{c}
Source of variation (P-values)			
Race		0.66	
Bodyscore		0.00**	
Race × Bodyscore		0.01**	
Main effect means diet			
2 Bodyscore		3.99±0.18^{a}	
3 Bodyscore		3.86±0.18^{ab}	
4 Bodyscore		3.74±0.14^{b}	
Race			
Morkaraman		4.32±0.28	
Kangal Akkaraman		4.17±0.19	

Table 3- Effect of apelin on pregnancy and body condition score in pregnancy (ng mL^{-1})

	N	*Morkaraman*	*Kangal Akkaraman*
2 Bodyscore	30	4.55±0.58	4.32±0.47
3 Bodyscore	30	4.75±0.58	5.47±0.37
4 Bodyscore	30	4.45±0.52	5.61±0.36
Source of variation (P-values)			
Race		0.18	
Bodyscore		0.35	
Race × Bodyscore		0.37	
Main effect means diet			
2 Bodyscore		4.43±0.37	
3 Bodyscore		5.11±0.34	
4 Bodyscore		5.03±0.31	
Race			
Morkaraman		4.58±0.32	
Kangal Akkaraman		5.14±0.23	

Table 4- Simultaneous comparison of apelin in ewe and rams Interaction of gender, races and body condition score (ng mL^{-1})

		Ewe in Lactation		*Ewe in Pregnancy*		*Ram*	
	N	*Morkaraman*	*Kangal*	*Morkaraman*	*Kangal*	*Morkaraman*	*Kangal*
2 Bodyscore	90	2.33±0.39	2.71±0.38	4.55±0.43	4.32±0.35	5.60±0.61	4.43±0.39
3 Bodyscore	90	1.91±0.35	2.87±.39	4.75±0.43	5.47±0.28	3.31±0.61	4.85±0.43
4 Bodyscore	90	2.32±0.35	2.81±0.35	4.45±0.38	5.61±0.27	4.04±0.39	3.22±0.25

Source of variation (P-values)	
Gender	0.000
Body Condition Score	0.492
Race	0.009
Gender X Body Condition Score	0.013
Gender X Race	0.029
Body Condition Score X Race	0.014
Gender X Body Condition Score X Race	0.131

Main effect	
Lactation Ewe	2.49±0.15^{c}
Ewe in Pregnancy	4.86±0.15^{a}
Coaches	4.24±0.19^{b}

	Morkaraman	
Lactation Ewe	2.19±0.21b	2.80±0.22c
Ewe in Pregnancy	4.58±0.24a	5.14±0.18a
Rams	4.32±0.32a	4.17±0.21b

	Ewe in Lactation	*Ewe in Pregnancy*	*Rams*
2 Bodyscore	2.52±0.27^{bc}	4.43±0.28^{a}	5.01±0.36^{a}
3 Bodyscore	2.38±0.26^{c}	5.11±0.26^{a}	4.08±0.37^{ab}
4 Bodyscore	2.56±0.25^{bc}	5.03±0.24^{a}	3.63±0.23^{a}
	2 Bodyscore	*3 Bodyscore*	*4 Bodyscore*
Morkaraman	4.15±0.28^{ab}	3.32±0.27^{b}	3.60±0.21^{ab}
Kangal	3.82±0.21^{a}	4.39±0.21^{a}	3.87±0.17^{a}

Races	
Morkaraman	3.70±0.15^{b}
Kangal	4.03±0.12^{a}

4. Discussion and Conclusions

Pregnancy and lactation are important processes involving physiological and hormonal changes. The mammary gland is subject to physiological changes both during pregnancy and postpartum period lactation. Endocrine gland mammary gland, especially apelin, leptin, adiponectin hormones, especially growth hormone, prolactin, hormones such as parathyroid are secreted (Habata et al 1999; Palin et al 2017). Apelin hormone originating from adipose tissue; body mass index, depending on the secretion of adipose tissue, varies according to many physiological periods such as pregnancy, lactation (Lérias et al 2014; Hughes & Watson 2018). In our current study, it was found that the relationship of race, lactation, pregnancy and gender with apelin hormone level in ewes was not investigated in the literature and there was no study in this regard.

As a result of clinical studies, it has been reported that apelin hormone has a therapeutic potential in clinical use as a potential serum biomarker in the early diagnosis of various diseases (cancer, diabetes, cardiovascular) and follow-up of metabolic processes (Ma et al 2014; Mutlak et al 2018; Wysocka et al 2018; Diakowska et al 2019). In the studies on rats, it was reported that the level of apelin in breast tissue increased during pregnancy and lactation, but reached the maximum level during the delivery period and decreased during lactation (Habata et al 1999; Clarkson et al 2004; Mercati et al 2018). In the studies on ruminants, it was stated that apelin levels of ewes with a body condition score of 2 were higher in the blooming period of range land compared to the period of dry grass, and that apelin levels decreased in the postpartum period (Mercati et al 2018). In our study, serum apelin levels of ewes of different races (Morkaraman and Kangal Akkaraman) with different body condition scores (<2, 3, 3.5, and ≥4) in the lactation and pregnancy period and serum apelin levels of rams were investigated. In our study, it was determined that the apelin level of those with a body

condition score of <2 in rams of Morkaraman race was high, whereas there was no difference in Kangal Akkaraman race; when the interaction between them was analyzed, it was found that there was difference between rams on the basis of race according to the body condition score, and that the interaction of race x body condition score was significant ($P<0.05$), and this difference did not exhibit the same tendency between the ram races in the groups with the same body condition scores. According to the result of the analysis on pregnancy, it was determined that pregnancy did not have any effect on body condition score in animal races. Whereas in the lactation period, apelin levels varied according to the races, and it was determined that apelin was higher in Kangal Akkaraman race. In the lactation period, the apelin levels were found to be similar in the body condition score groups. The apelin levels in the body condition score groups of races had the similar tendency and no interaction effect was found (Table 5). In addition to that, apelin levels were simultaneously compared between the ewes in lactation and pregnancy period and rams. A statistically significant difference was determined in terms of different gender groups and races created according to this. On the other hand, the effect of apellin on the interaction of race-sex, body condition scores- sex and body condition score on race was found to be statistically significant. According to these results, apelin levels in the same race but in different gender groups or at different body condition score groups did not change.

Therefore, when we examined similar studies, the same results were obtained (Mercati et al 2018), however, studies on different species things have shown different results (Habata et al 1999; Clarkson et al 2004). This dissimilarity can be attributed by species differency and it can also be explained by the continuation of secretory activation from breast tissue in the dry period in ruminants, unlike other species (Holst et al 1987;Sordillo and Nickerson 1988; Mercati et al 2018). In the light of the results obtained from our current study, we can predict early diagnosis and prevention of metabolic diseases such as pregnancy toxemia. However, it is thought that the continuation of studies on large numbers of animals and different sheep races will benefit in understanding the physiological mechanisms and the strategies that can be developed for this mechanism.

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Genetic Variability of Indels in the Prolactin and Dopamine Receptor D2 Genes and Their Association with the Yield of Allanto-Amniotic Fluid in Russian White Laying Hens

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ABSTRACT
Currently, there is virtually no information on genetic factors affecting the yield of allanto-amniotic fluid, which is the raw material for the production of human and animal vaccines. Association studies including this trait are beneficial for increasing productivity of a biotechnological line of chickens used for the production of 'Clean Eggs'. We examined here a population of the Russian White breed for the effects of indels in the prolactin (*PRL*) and dopamine receptor D2 (*DRD2*) genes on the yield of extraembryonic fluid (YEF) and embryo weight at 12.5 days of development. A 24-bp insertion in the 5' flanking region of the *PRL* gene significantly (P<0.01) increases YEF in the embryos. The heterozygous embryos contained the highest YEF (9.6 mL) than that of the homozygous insertion (9.4 mL) and deletion embryos (8.4 mL). We also found a significant association (P<0.001) between the *PRL* genotypes and egg weight (EW). The results of the present study suggest a significant association between the *PRL* gene variation and quantitative traits in the Russian White chickens, contributing to a long-term programme on the effective use of the genetic potential of Russian gene pool breeds and populations of chickens.

Keywords: Chicken population; Biotechnological line; Candidate genes; Insertion-deletion polymorphism; Economically important traits

1. Introduction

Extraembryonic fluid obtained from chicken eggs is beneficial in the production of human and animal vaccines against viruses, and in pharmaceutical studies. Currently, a complex epizootic situation and the need for ever-increasing production of embryonic viral vaccines for humans, farm animals and poultry, using the developing chick embryos (DCEs), require search for new genetic approaches to create specialized poultry lines to produce eggs with increased volumes of extraembryonic fluid, which will not only reduce the number of embryos used, but also lower the cost of the vaccine itself.

One of the perspective directions in breeding is the marker-assisted selection that capitalises on studies of the polymorphisms of different target genes or genome regions and their relationship with production traits and is widely

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used in animal husbandry (Fulton 2008). Variant detection and studies of association between candidate genes or genetic markers and economically important traits have been broadly attempted in commercial poultry populations (Dunn et al 2004) and native breeds (Li et al 2008; Guo et al 2016). However, application of this approach in non-commercial, germplasm poultry populations has been complicated by their small size and the need to maintain their genetic diversity.

The prolactin (*PRL*) and dopamine receptor D2 (*DRD2*) genes have been suggested among the most promising candidate genes, allelic variants of which could be associated with the production traits in poultry. The biological activity of PRL manifests via interaction with membrane receptors found in numerous target tissues (Fleenor et al 2006). PRL is considered to be more associated with egg production traits, as it is believed that this hormone determines the broody instinct in the body of laying hens (Romanov 2001; Reddy et al 2002, 2007; Wang et al 2009; Wang et al 2011). Broodiness has a negative effect on egg production, which is crucial in egg industry. Genetic changes in the regulatory system comprising *PRL* and its receptor, and entailing inhibition of their activity may be used as genetic markers for breeding laying hens with the reduced instinct of broodiness, and hence, with increased egg production (Bagheri Sarvestani et al 2013). Sequencing of the promoter region of the chicken *PRL* gene resulted in detection of a 24-bp insertion located at nucleotide position 377 to 354 (GenBank Accession No. AB011438). In several studies, it was observed that the presence of the insertion at this site of the promoter region in the *PRL* gene was correlated positively with the intensity of lay in hens and negatively with incubation behaviour (Reddy et al 2002, 2007; Cui et al 2006; Jiang et al 2009; Wang et al 2009; Wang et al 2011; Kulibaba & Podstreshnyi 2012). It was also found that individuals with the heterozygous genotype for insertion and deletion (*In/Del*) had the greatest expression level of PRL mRNA (Cui et al 2006). This may suggest that this polymorphic locus could be associated with the chick hatchability characteristics through modulation of the *PRL* gene at the transcriptional level. Also, there has been an attempt to identify association between this mutation and egg production traits. There were reports of a significant correlation ($P<0.05$) found between the indel locus polymorphism and egg production (Cui et al 2006). On the other hand, an efficiency of using the *PRL* gene was also studied as a candidate gene and a marker in the selection programme of Silkie chicken, but no significant associations between genetic variation at this gene locus and performance traits were identified (Wada et al 2008; Rowshan et al 2012).

Dopamine functionally interacts with PRL, which determines its indirect effect on the instinct of incubation. In some studies (Xu et al 2010), there was a suggestion that mutations in the chicken *DRD2* gene might also affect the broody instinct. Involvement of dopamine has been demonstrated in the processes of stimulation and inhibition of the PRL secretion in the brain. In addition, the inhibitory effect of dopamine on the PRL secretion was mediated by DRD2 in the pituitary (Youngren et al 1995; Al Kahtane et al 2003). In chickens, which were treated with antagonists or blockers of dopamine receptors, termination of incubation behaviour was identified due to the inhibition of PRL secretion (Hall & Chadwick 1984). An indel mutation was found in the 5'-flanking region of the *DRD2* gene, and effect of the *DRD2* indel polymorphisms on body weight was examined in Chinese and Japanese chicken breeds (Zhou et al 2010). Yet, the influence of variability in the genes of dopamine and its receptors on performance of chickens remains insufficiently clarified.

The Russian White breed was produced in the Soviet period (in 1929 to 1953) by crossing different White Leghorn populations with local chickens of Russian Central regions (Paronyan & Yurchenko 1989). More recently, a population under the name of 'Russian Snow White' was developed at the Russian Research Institute of Farm Animal Genetics and Breeding (RRIFAGB) Branch 'Genofond' (Tyshchenko 2002). The population is unique and characterized by tolerance of chicks to cold growing conditions, and is of particular interest for use in genetic research. It was created by selection for tolerance to low temperatures in the first days of life and for high egg production. Chicks of this breed can safely bear temperature regime of 8-10 degrees below normal and are also resistant to certain avian leukosis sarcoma complex diseases. Furthermore, possibly due to the recessive gene *sw* (Hutt 1951), 25% day old chicks in the population have completely snow-white (instead of yellow) down. This breed was also selected for increased resistance to leukemia, Marek's disease and carcinomas of the internal organs.

On the basis of this population, a specialized biotechnological line is bred at the RRIFAGB for the yield of extraembryonic fluid (YEF) to be used in the production of 'Clean Eggs' and vaccines in the bioindustry. For this purpose, we assessed 30-35-week females for extraembryonic fluid volume in 12.5-day DCEs (Lapa et al 2015).

The present study has been undertaken to test indel polymorphisms in the *PRL* and *DRD2* genes in the biotechnological line of Russian White chickens and search for their associations with YEF and egg laying traits as a part of a broader survey on genetic features of the Russian gene pool chicken breeds.

2. Material and Methods

2.1. Experimental population and traits

A population of the egg-type Russian White chickens was chosen for the study. It represents a biotechnological line of chickens used for the production of 'Clean Eggs'. The experimental group consisted of 160 females and 24 males. All selected roosters met the sperm quality requirements for artificial insemination.

To examine YEF, three to six consecutively laid eggs were collected for evaluation from each chicken at 34 weeks of age, weighed and labeled with the mother's pedigree number, egg weight, and date of lay. Then, the eggs were placed in an incubator. The age of embryos was chosen to be 12.5 days to assess the genetic potential of chickens in terms of YEF of their descendants, as found in the previous study (Lapa et al 2015). To determine YEF at 12.5 days of development, the eggs were taken out of the incubator and placed for two days in a refrigerator, and after that the volume of extraembryonic fluid was measured. To do this, the egg was weighed (to determine shrinkage), and the shell over the air pocket was broken and removed. Puncturing the exposed membrane and pushing the embryo aside with tweezers, the allantoic fluid was collected with a pipette and the volume of the allantoic fluid was measured. A total of 451 eggs were evaluated for YEF in 12.5-day-old embryos.

A number of performance traits were also recorded during the experiment time. In particular, chickens were assessed for egg production (by months for the entire laying period), body weight, age at first egg and egg weight at 30 weeks.

2.2. DNA sampling and genotyping

One hundred to 500 μL of whole blood per sample was collected as a DNA source from the wing vein into a tube containing anticoagulant (30 μL 200 mM EDTA). The whole procedure for collection of the blood samples of all animals was carried out in strict accordance following the standard protocols approved by the RRIFAGB. Samples were frozen and stored at -20 ºC.

DNA was isolated by standard phenol procedure using proteinase K (SibEnzyme, Russia) and diluted in TE buffer. The concentration and purity of the samples was determined using a NanoDrop 2000 instrument.

Polymerase chain reaction (PCR) was performed using Thermal Cycler T1000 (Bio-Rad, USA) as follows: initial denaturation for 5 minutes at 95 ºC, followed by 40 cycles of denaturation for 30 seconds at 95 ºC, annealing for 30 seconds at 60 ºC (for *PRL* gene) or 63 ºC (for *DRD2* gene) and extension for 30 seconds at 72 ºC, and final elongation for 5 minutes at 72 ºC.

The *PRL* and *DRD2* genes were tested for the presence of indel polymorphisms. The following primers were used for the PCR (Rahman 2014): *PRL* gene, forward primer: 5'-GGTGGGTGAAGAGACAAGGA-3'; reverse primer: 5'-TGCTGAGTATGGCTGGATGT-3'; *DRD2* gene, forward primer: 5'-TGCACTTCAATCCTTCCCAGCTT-3'; reverse primer: 5'-TTGCGCTGCCCATTGACCA-3'. Amplification products obtained for the *PRL* and *DRD2* genes were separated on 2% agarose gel. In case of *PRL* gene, the amplified fragment size was 130 and 154 bp depending on the 24-bp indel. For the *DRD2* gene, size of the PCR fragments was 165 and 187 bp due to the 22-bp indel (Figure 1 and 2).

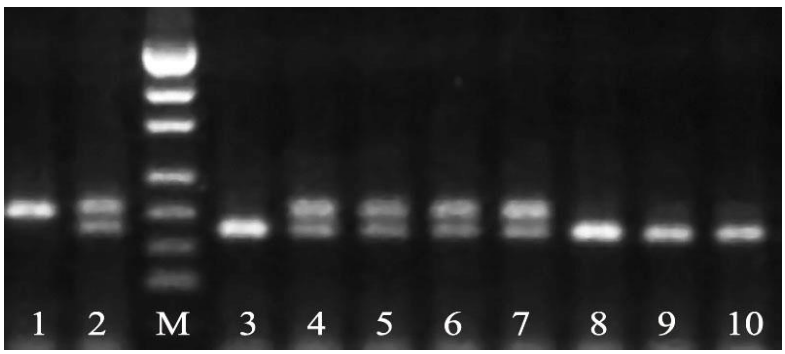

Figure 1- Electrophoregramme of the amplified fragments of the chicken *PRL* gene for genotypes *In/In* (154 bp, lane 1), *Del/Del* (130 bp, lanes 3, 8, 9 and 10), and *In/Del* (lanes 2, 4, 5, 6 and 7). Lane M= DNA size marker pUC19 DNA *Hae*III Digest (Sigma)

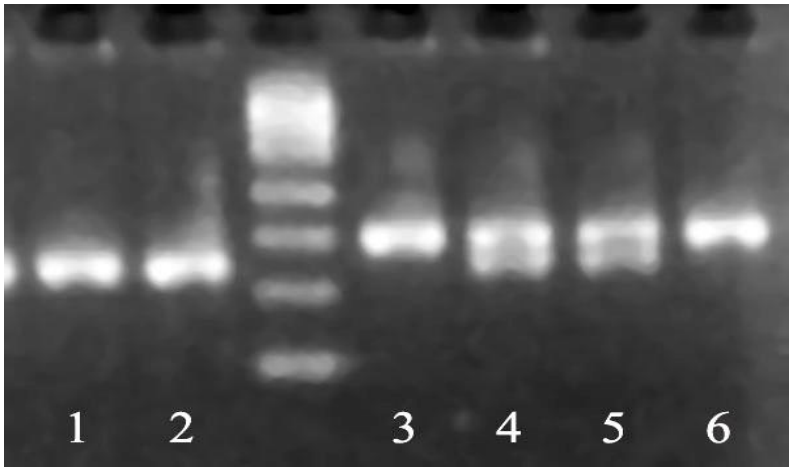

Figure 2- Electrophoregramme of the amplified fragments of the chicken *DRD2* gene for genotypes *In/In* (187 bp, lanes 3 and 6), *Del/Del* (165 bp, lanes 1 and 2), and *In/Del* (lanes 4 and 5). Lane M= DNA size marker pUC19 DNA *Hae*III Digest (Sigma)

2.3. Statistical analysis

Frequencies of genotypes and alleles were calculated based on the PCR genotyping data obtained. Deviation of frequencies from Hardy-Weinberg equilibrium was evaluated using the $\chi 2$ test.

Differences between genotypes and production traits were explored in SigmaPlot software package (version 12.0.5) using analysis of variance correction for multiple comparisons (All Pairwise Multiple Comparison Procedures).

3. Results and Discussion

To study genetic variability for indel polymorphisms in the *PRL* and *DRD2* genes, we genotyped respectively 155 and 140 laying hens of the Russian White breed. At the *PRL* locus, predominance of individuals homozygous for the 24-bp insertion (*In/In*) was observed. In the case of the *DRD2* gene, heterozygotes for the 22-bp indel (*In/Del*) prevailed (Table 1). Such a genotype distribution appears to be characteristic for egg-type breeds with a decent egg production, as also confirmed by other authors (Cui et al 2006; Kulibaba & Podstreshnyi 2012).

Table 1- The frequency of alleles and genotypes at the *PRL* and *DRD2* loci in the chicken population of the Russian White breed

Genes	*No. of observations*	*Alleles*		*Genotypes*			*H*	*PIC*	χ^2
		Del	*In*	*Del/Del*	*In/Del*	*In/In*			
PRL	152	0.26	0.74	0.07	0.37	0.56	0.37	0.298	0.081
DRD2	152	0.56	0.44	0.33	0.46	0.21	0.46	0.371	0.239

The analysis of the observed and theoretical distribution of genotypes for the *PRL* and *DRD2* gene did not reveal any deviations from Hardy-Weinberg equilibrium (with χ^2 being equal to 0.0808 and 0.1411, respectively; Table 1).

To examine potential associations and improve further the selection efficiency, we studied the effect of indel polymorphisms on basic performance traits in the Russian White chicken population. In particular, we looked for associations between polymorphic variants of the two genes and the main selected traits such as body weight at 52-week age, age at first egg, egg production for 360 days of life, and egg weight at 30-week age. The performance data was collected in 117 individuals genotyped for the *PRL* gene and in 104 birds genotyped for the *DRD2* gene.

According to the ANOVA results (Table 2), a significant difference ($P<0.001$) was found between the *PRL* indel genotypes relative to egg weight. Hens heterozygous for the insertion in the *PRL* gene promoter demonstrated an earlier

sexual maturity as well as a tendency for a greater egg production and egg weight. As for the *DRD2* gene, there were no significant associations between its indel genotypes and the characteristics studied including age at fist egg, egg production for 360 days of life and egg weight at 30-week age (Table 3).

Table 2- Genotype variants at the *PRL* locus as associated with economically important traits

Trait	*Genotype*			*P*
	Del/Del (N= 11)	*In/Del (N= 56)*	*In/In (N= 85)*	
Age at fist egg, days	168.3±2.0	166.4±0.6	167.4±0.5	0.336
Egg production for 360 days of life	136.7±7.9	145.2±2.2	141.7±2.4	0.391
Egg weight at 30 weeks of age, g	48.2±0.7	50.7±0.5	50.0±0.3	0.066
Body weight, g	1685±75	1677±25	1693±25	0.910

Del, deletion; In, insertion; P, significance value

Table 3- Genotype variants at the *DRD2* locus as tested for association with economically important traits

Trait	*Genotype*			*P*
	Del/Del (N=50)	*In/Del (N=70)*	*In/In (N=32)*	
Age at fist egg, days	166.9±0.8	167.5±0.5	166.5±0.9	0.610
Egg production for 360 days of life	143.9±3.1	141.49±2.4	143.3±3.8	0.804
Body weight, g	1667±32	1719±25	1662±39	0.326

Del, deletion; In, insertion; P, significance value

Using ANOVA, we analysed main quantitative estimates of extraembryonic fluids in the groups of polymorphic variants in the *PRL* and *DRD2* genes. Table 4 summarises the ANOVA results concerning effect of the hens' genotype on embryo weight and YEF in the 12.5-day-old embryos. The insertion in the *PRL* gene in the dams was associated with a YEF increase by 1.3 mL in the embryos produced from the heterozygous hens (*In/Del*) and by 1.1 mL from the homozygotes (*In/In*) as compared with the homozygotes having the deletion (*Del/Del*) in the *PRL* gene (P<0.01).

Table 4: Genotype variants at the *PRL* and *DRD2* loci as associated with yield of extraembryonic fluids (YEF) and embryo weight

Trait	*Genotype*			*P*
	Del/Del	*In/Del*	*In/In*	
PRL				
No. of eggs	32	173	246	
Egg weight, g	47.94±0.48[a]	50.52±0.31[b]	49.78±0.22	0.003
YEF, mL	8.391±0.327[c]	9.622±0.165[d]	9.423±0.121[e]	0.006[*]
Embryo weight, g	9.769±0.161	9.302±0.009	9.473±0.331	0.795
DRD2				
No. of eggs	149	205	97	
Egg weight , g	50.01±0.29	50.19±0.26	49.50±0.41	0.333
YEF, mL	9.19±0.17	9.64±0.150	9.42±0.19	0.116
Embryo weight, g	9.41±0.08	9.17±0.08	9.19±0.12	0.109

Del, deletion; In, insertion; P, significance value; YEF, yield of extraembryonic fluids; Significant difference; [a, b], P<0.001; [c, d], P<0.001; [c, e], P<0.01; [*], significant association, P<0.01

A variety of current poultry breeds can be divided into two groups, old breeds and novel lines and populations. Certain new populations have been or are being created on the base of old breeds and contemporary commercial lines. Breeding purpose in such new populations is often to solve problems of commercial strains including disease resistance, and to handle insistence to growing conditions and specific production technologies. Within the current breeding programme that focuses on the Russian White chicken breed kept at the RRIFAGB, one of the selection objectives is to create a population with high biotechnological qualities of eggs. Such a population is an ideal material for conducting effective selection and creating poultry lines that can be used in the production of embryonic viral vaccines.

At present, efforts are undertaken to develop a procedure of breeding chicken lines with an increased volume of allantoic/amniotic fluid for producing further a biotechnological virus-containing material (Lapa et al 2015). VALO Biomedia GmbH (http://www.valobiomedia.com/) is the world's largest supplier of specific pathogen-free (SPF) and 'Clean Eggs' used for vaccine manufacture. In Russia, there are no specialized poultry populations for vaccine production, with eggs from commercial crosses kept at large poultry farms being utilised for that purpose. The main feature of the SPF eggs is the absence of any vaccinations of hens, and that of 'Clean Eggs' is a gentle vaccination against diseases. There are also no enterprises for the production of 'Clean' and SPF eggs in Russia, and SPF eggs have to be imported from other countries, while 'Clean Eggs' are substituted for chicken eggs of commercial crosses exposed to a rigid schedule of vaccination, which significantly reduces the quality of the vaccines. There are no reports about genetic factors that may influence the amount of allantoic/amniotic fluid, which is the raw material for the production of vaccines, and the titer of the vaccine virus in it. Therefore, search for genomic associations with the increased amount of amniotic fluid is one of the areas in our research. Accordingly, we evaluated 30-35-week parents in terms of extraembryonic fluid from 12.5-day-old DCEs and genotypes in the *PRL* and *DRD2* genes and observed a significant association with the *PRL* indel variation.

4. Conclusions

In summary, our search for possible candidate genes including the *PRL* and *DRD2* genes associated with the volume of extraembryonic fluid in 12.5-day-old embryos has suggested that YEF may be affected by the genes that control the growth and development of the embryo. As found in the current study, one of such candidate genes is the *PRL* gene showing the indel polymorphism in its promoter region associated with YEF and egg weight in the Russian White chicken population. The results can be used within a long-term programme on the effective use of the genetic potential of Russian chicken breeds and populations, and specifically for breeding poultry lines with performance characteristics of interest (YEF, egg and meat production, egg quality, etc.) and producing progeny with desirable genotypes.

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Abbreviations and Symbols	
PRL	Prolactin
DRD2	Dopamine receptor D2
DCEs	Developing chick embryos
YEF	Yield of extraembryonic fluids
RRIFAGB	Russian research institute of farm animal genetics and breeding
SPF	Specific pathogen-free

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