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Kastamonu Üniversitesi Su Ürünleri Fakültesi - KASTAMONU
Tlf: 0366 280 23 07 Fax: 0366 280 23 13
e-mail: alinteridergisi@hotmail.com www.alinteridergisi.com

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CONTENTS / İÇİNDEKİLER

RESEARCH / ARAŞTIRMA

SHORT COMMUNICATION / KISA BİLDİRİ

Alinteri J. of Agr. Sci. (2019) 34(2): 103-110
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RESEARCH ARTICLE

Effect of Swirl Plates on Volumetric Discharge Rate and Spray Characteristics of Hollow Cone Nozzles

Bahadır Sayıncı[1*] • Ruçhan Çömlek[2] • Bünyamin Demir[3] • Mustafa Çomaklı[4]

[1]*Mersin University, Faculty of Engineering, Department of Mechanical Engineering, Mersin/Turkey*
[2]*Atatürk University, Graduate School of Natural and Applied Science, Department of Agricultural Machinery, Erzurum/Turkey*
[3]*Mersin University, Vocational School of Technical Sciences, Department of Mechanical and Metal Technologies, Mersin/Turkey*
[4]*Atatürk University, Faculty of Agriculture, Department of Agricultural Machinery and Technologies Engineering, Erzurum/Turkey*

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ABSTRACT

The aim of the study is to determine the effect of orifice diameter, swirl plate and spray pressure on the volumetric flow, discharge coefficient and some spray characteristics of hollow cone nozzles. In the trials, five nozzle discs with 1,0, 1,2, 1,6, 2,0 and 2,4 millimetres orifice diameters and three polyacetal materials with 2 or 3 slots and one stainless steel with 2-slots swirl plate were used. Spray application were made at spray pressures of 2, 4, 6, 8 and 12 bar. The highest discharge rate at constant pressure was obtained with stainless steel and the lowest blue swirl plates. Although the number of slots was different, the effect of brown and yellow swirl plates on volumetric discharge rate variation was statistically insignificant. The discharge coefficient decreased as the diameter of the nozzle orifice increased. Accordingly, the average discharge coefficient for the 1,0 mm, 1,2, 1,6, 2,0 and 2,4 mm diameter nozzle discs was 0,411, 0,362, 0,285, 0,236 and 0.201, respectively. It was estimated that the droplet diameter in the range of 2-12 bar in the hollow cone nozzles varied between 76,3-219,0 µm and it was determined that mostly very fine and partially fine and medium sized droplets were produced.

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Introduction

Spray characteristics in pesticide application have a significant impact on biological activity of harmful agents. Laboratory experiments have been demonstrated that droplets smaller than 100 µm are more useful in insecticides and fungicides (Matthews et al., 2014).

The transport potential of pesticides to the target in droplets, spray deposition, droplet penetration, spray coverage and drift potential depends on the droplet diameter in spray application (Bode et al., 1983; Nuyttens et al., 2007). The large or small droplets in pesticide application can limit the success of the application. The drift problem can be minimized (Bode et al., 1983) in applications with large droplets, but there may be a problem of deposition or spray coverage on the target surface (Smith et al., 2000). Because the energy of the small droplets is low, the droplets can be suspended in the air, they can evaporate before reaching the target (Bayat and Bozdoğan, 2005) or they can transport out of the target due to wind (Bode et al., 1983). For these reasons, the optimum droplet size in spray application is important in terms of the efficiency of pesticide application and environmental pollution caused by drift.

* Corresponding author
E-mail address: bsayinci@mersin.edu.tr

In order to reduce pesticide losses due to drift, new spray technologies have been developing, some R&D studies on existing equipment have been carrying out and various improvements on spray technologies have been made. Some of the current developments; new generation nozzles (low drift potential, pneumatic, rotary disc, variable displacement, double slotted and multi-head nozzles, etc.), auxiliary airflow spray technologies, electrostatic charging technique, boom arm protection curtains, tunnel type atomizers, sprayers that detect plant canopy, variable-rate herbicide application technology can be listed as GPS detection of sprayer transitions, direct injection system, product tilting system and spray boom balancing systems (Dursun et al., 2000).

Despite advances in spray technologies, operators do not abandon conventional methods and prefer standard nozzles since they are cheap and easy to procure rather than new generation hydraulic nozzles. Commonly used hollow cone nozzles are in the form of disc and there is orifice with circle geometry in the centre. When the nozzle discs are used together with the swirl plate, atomization takes place. Swirl plates may affect the nozzle discharge rate and may cause a change in the spray characteristics (Sayıncı et al., 2017).

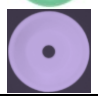
The aim of this study is to determine the effects on discharge coefficient and volumetric discharge rate variation of swirl plates slot number of which are made from different manufacturing materials, and reveal spray characteristics by estimating the droplet diameter at various operational pressure levels.

Materials and Methods

Hollow Cone Nozzles and Swirl Plates

In the experiments, hollow cone nozzle discs made of polyacetal (POM) material with orifice diameters of 1,0 mm, 1,2 mm, 1,6 mm, 2,0 mm and 2,4 mm were used. Each orifice diameter group were replicated five times. Four swirl plates having different slot number or manufacturing material were used in each nozzle disc. To ensure discrimination among the swirl plates, each plate was named according to colour codes or material, and some features were given in Table 1.

Table 1. Hollow cone nozzle discs and swirl plates

Nozzle discs		Swirl plates		
Orifice diameter (Ø*d*)	Nozzle discs	Colour code/Material (Slot number, material)	Manufacturing	Swirl plates
1.0 mm		Blue (2-slot, POM*)	Imported (C23)	
1.2 mm		Brown (3-slot, POM)	Local	
1.6 mm		Yellow (2-slot, POM)	Local	
2.0 mm		Stainless steel (2 slot, SS**)	Local	
2.4 mm				

*: polyacetal; **: stainless steel

Hydraulic Pressure Unit

The hydraulic pressure was provided by a conventional sprayer with a tank capacity of 200 litres. The pressure regulator (max. 40 bar, 90 L min^{-1}, RG-7 Model) connected on the pressure line provided to be controlled of the operating pressure by a manometer (Pakkens® Model, TR) with a glycerine filling with a maximum of 25 bar display. The self-pump of the sprayer (TAR30 piston-membrane, double piston, rated nominal pressure of 40 kg cm^{-2}, rated discharge rate of 30 L min^{-1}, 67% efficiency, Taral®, TR) was used to transmit the fluid in the polyethylene tank to the spray line. In the study, the pump shaft was mounted on a belt-pulley driven mechanism which takes action of the electric motor (2,2 kW, 1405 rpm, AGM 100L 4a type, Gamak, TR). The pump shaft revolution was measured as 500 rpm using an optical tachometer (Testo 465, KGaA).

Determination of Volumetric Discharge Rate

The discharge rate was measured with a flowmeter (Sprayer Calibrator, SpotOn®, Model: SC-1, IL, measurement accuracy: ±2,5%; measuring range: 0,08-3,79 L min^{-1}) without using any filter. The measurements were replicated five times at five different spray pressures including 2 bar, 4 bar, 6 bar, 8 bar and 12 bar. Sayıncı and Kara (2015) found differences between the operational pressure measured on the regulator and the spray pressure measured from a close point to the nozzle due to the pipe loss. Therefore, the fluid pressure was controlled by a digital manometer (Ref D2, 0,1%, 0-400 bar, SICA GmbH & Co. KG) mounted at a point close to the nozzle and the measured value was referred to as the spray pressure.

In the combinations of the nozzle disc and the swirl plate, the linear variation among the discharge rate and square root of the pressure was given in the form of a [$y = ax + b$] equation. In order to test the effect of the swirl plates on the discharge

rate with a common variable, the slope (a) of the line was determined from the [$y = ax$] equation and subjected to the analysis of variance (ANOVA). The differences between the significant averages were determined by the Tukey multiple comparison test at 95% significance level.

Sayıncı et al. (2013) stated that there was no any reference standard for the operating characteristics of the locally used hollow cone nozzles. In this study, a demonstration indicating the operational characteristics of nozzle orifices used with different swirl plates was made. Hypro® (2014) catalogue prepared according to BCPC was taken as reference and "nozzle type / discharge rate (L min^{-1}) / pressure (bar)" notation layout was used.

Determination of Discharge Coefficient

The discharge coefficient, which expressed the energy loss caused by friction in the nozzle disc and swirl plate, was calculated using Equation (1) (Srivastava et al., 1993; Ballester and Dopazo, 1994; Rashid et al., 2012; Yu et al., 2013; Sayıncı, 2016).

$$C_D = \frac{Q}{\sqrt{\Delta P}} \cdot \sqrt{\left(\frac{\rho_L}{2 \cdot A}\right)} \quad (1)$$

C_D: discharge coefficient
Q: discharge rate (m^3 s^{-1})
ΔP: pressure (Pa)
ρ_L: liquid density (999.1 kg m^{-3}, @15 ºC liquid temperature)
A: orifice area (m^2)

One-way analysis of variance (ANOVA) was performed to test the effect of the swirl plate on the discharge coefficient in the nozzle orifice groups. The difference between the significant averages was determined by the Tukey test at 95% significance level.

Droplet Size ($D_{V0.50}$)

The droplet diameter was estimated using Equation (2) in nozzle disc and swirl plate combinations with different spray pressures (Iqbal et al., 2005).

$$D_{V0.50} = 437 \cdot \sqrt[3]{\frac{k}{\Delta P}} \quad (2)$$

$D_{V0.50}$: droplet diameter (µm)
k: orifice coefficient ($k=q/\sqrt{\Delta}P$)
ΔP: pressure (psi)
q: discharge rate (gal h^{-1})

The droplet diameter classes have been classified into eight categories according to ASABE S572.1 standard (ASABE, 2009) as shown in Table 2, and the diameter categories have been standardized according to their colours respectively in purple, red, orange, yellow, blue, green, white and black. According to this standard, many researchers used different reference ranges for droplet diameter in spray categories. In this study, the droplet diameter colour category of nozzle orifices determined according to the diameter ranges specified Kruger et al. (2013) and Arag® (2017) catalogue.

Table 2. Droplet size ($D_{V0.50}$, µm) categories (classification according to ASABE S572.1 standard) (ASABE, 2009)

Droplet sizes categories	$D_{V0.50}$ (µm) ranges							Droplet sizes colour categories
	Hypro® (2014)	Hipkins and Grisso (2014)	Hypropumps (2006)	Spandl (2010)	Wolf (2017)	Kruger et al.(2013); Arag® (2004)	Matthews et al. (2014)	
Extremely fine (XF)	60 <	60 <	-	50 <	-	~ 50	50 <	Purple
Very fine (VF)	61-105	60-145	100 <	51-145	150 <	136 <	51-100	Red
Fine (F)	106-235	145-225	100-175	145-225	151-250	136-177	101-200	Orange
Medium (M)	236-340	226-325	175-250	226-325	251-350	177-218	201-300	Yellow
Coarse (C)	341-403	326-400	250-375	326-400	351-450	218-349	> 300	Blue
Very coarse (VC)	404-502	401-500	375-450	401-500	451-550	349-428	-	Green
Extremely coarse (XC)	503-665	501-650	> 450	501-660	> 551	428-622	-	White
Ultra coarse (UC)	> 665	> 650	-	> 661	-	> 622	-	Black

Results

Effect of Swirl Plate on Discharge Rate Variation

Linear equations between discharge rate and pressure variables were given in Table 3. In reference to variance analysis, the effect of the swirl plate on discharge rate was found to be very significant. In the same orifice, the highest discharge rate was obtained by stainless steel and the lowest blue swirl plate. Although the number of slot on swirl plate was different, no significant difference was found between brown and yellow plates. The effect of brown, yellow and stainless steel swirl plates on the nozzle disc with an orifice diameter of 2.4 mm was found insignificant and the lowest discharge rate was obtained with a blue plate. The BCPC reference display was taken notice for the presentation of discharge rates of the nozzle discs with different swirl plates at 3 bar pressure.

Factors Affecting Discharge Coefficient

The discharge coefficient of the nozzle discs with an orifice diameter of 1,0 mm, 1,2 mm, 1,6 mm, 2,0 mm and 2,4 mm was determined as 0,411, 0,362, 0,285, 0,236 and 0,201, respectively (Figure 1). According to this result, the discharge coefficient decreased as the orifice diameter of the hollow cone nozzles increased. The swirl plates changed significantly the discharge coefficient of the nozzle (Table 4). The blue swirl plate had the lowest discharge coefficient. The highest coefficient was found at stainless steel swirl plate. The difference between the average discharge coefficients of the brown and yellow plates was mostly insignificant.

Table 3. Effect of swirl plates on nozzle discharge rate (q, L min^{-1})

Orifice diameter (mm)	Swirl plates	[1]Linear equations ($y = ax + b$)	R^2 (Corrected)	[2]Slope ($y = ax$)	F value (p, sigma)	[3]BCPC code
Ø1.0	Blue (2-slot, C23)	$y = 0{,}214x + 0{,}036$	0,997	0,228±0,003 c*	42,91	KH/0.41/3
	Brown (3-slot)	$y = 0{,}273x + 0{,}009$	0,961	0,276±0,016 b	(0,000)**	KH/0.48/3
	Yellow (2-slot)	$y = 0{,}268x + 0{,}032$	0,973	0,280±0,013 b		KH/0.50/3
	S. steel (2-slot)	$y = 0{,}296x + 0{,}022$	0,988	0,304±0,006 a		KH/0.53/3
Ø1.2	Blue (2-slot, C23)	$y = 0{,}262x + 0{,}045$	0,999	0,279±0,001 c	61,79	KH/0.50/3
	Brown (3-slot)	$y = 0{,}332x + 0{,}034$	0,961	0,345±0,019 b	(0,000)	KH/0.61/3
	Yellow (2-slot)	$y = 0{,}344x + 0{,}027$	0,988	0,354±0,011 b		KH/0.62/3
	S. steel (2-slot)	$y = 0{,}386x + 0{,}039$	0,969	0,401±0,018 a		KH/0.71/3
Ø1.6	Blue (2-slot, C23)	$y = 0{,}346x + 0{,}068$	0,979	0,372±0,014 c	55,46	KH/0.67/3
	Brown (3-slot)	$y = 0{,}464x + 0{,}065$	0,969	0,489±0,024 b	(0,000)	KH/0.87/3
	Yellow (2-slot)	$y = 0{,}486x + 0{,}066$	0,969	0,511±0,024 b		KH/0.91/3
	S. steel (2-slot)	$y = 0{,}602x - 0{,}094$	0,950	0,566±0,033 a		KH/0.95/3
Ø2.0	Blue (2-slot, C23)	$y = 0{,}448x + 0{,}051$	0,980	0,468±0,018 c	65,76	KH/0.83/3
	Brown (3-slot)	$y = 0{,}635x + 0{,}034$	0,983	0,648±0,024 b	(0,000)	KH/1.13/3
	Yellow (2-slot)	$y = 0{,}641x + 0{,}058$	0,938	0,663±0,029 b		KH/1.17/3
	S. steel (2-slot)	$y = 0{,}765x - 0{,}075$	0,949	0,736±0,047 a		KH/1.25/3
Ø2.4	Blue (2-slot, C23)	$y = 0{,}526x + 0{,}040$	0,987	0,541±0,016 b	58,16	KH/0.95/3
	Brown (3-slot)	$y = 0{,}827x - 0{,}012$	0,939	0,823±0,063 a	(0,000)	KH/1.42/3
	Yellow (2-slot)	$y = 0{,}821x + 0{,}046$	0,959	0,839±0,046 a		KH/1.47/3
	S. steel (2-slot)	$y = 0{,}869x + 0{,}022$	0,957	0,877±0,043 a		KH/1.53/3

[1] y: nozzle discharge rate (q, L min^{-1}); a: slope of the line; x: square root of pressure ($\sqrt{P}$, bar); b: intercept
[2] In order to test the effect of swirl plates on the nozzle discharge rate, the intercept (b) was accepted as zero (0) and linear equations are obtained in the form of [$y = ax$]. y: nozzle discharge rate (q, L min^{-1}); a: slope of the line; x: square root of pressure ($\sqrt{P}$, bar) (mean±SD)
[3] The coding according to BCPC reference shows the nozzle discharge rate (L min^{-1}) at 3 bar pressure. KH: hollow cone spray nozzle (Hypro®, 2017)
* According to the Tukey multiple comparison test results, the averages shown different letters in the same column for each orifice diameter group are different at 95%; **: $p<0{,}01$ very important

Table 4. The effect of swirl plates on discharge coefficient (C_D) (mean±SD)

Orifice diameter (mm)	Swirl plates	Discharge coefficient (C_D)	F value (p, sigma)
Ø1.0	Blue (2-slot, C23)	0,346±0,008 c*	193,44
	Brown (3-slot)	0,415±0,023 b	(0,000)**
	Yellow (2-slot)	0,424±0,019 b	
	S. steel (2-slot)	0,459±0,014 a	
Ø1.2	Blue (2-slot, C23)	0,294±0,008 c	265,99
	Brown (3-slot)	0,362±0,020 b	(0,000)
	Yellow (2-slot)	0,371±0,012 b	
	S. steel (2-slot)	0,421±0,021 a	
Ø1.6	Blue (2-slot, C23)	0,221±0,009 d	240,30
	Brown (3-slot)	0,289±0,014 c	(0,000)
	Yellow (2-slot)	0,302±0,015 b	
	S. steel (2-slot)	0,329±0,019 a	
Ø2.0	Blue (2-slot, C23)	0,177±0,007 c	227,33
	Brown (3-slot)	0,244±0,009 b	(0,000)
	Yellow (2-slot)	0,250±0,017 b	
	S. steel (2-slot)	0,274±0,019 a	
Ø2.4	Blue (2-slot, C23)	0,142±0,004 c	235,63
	Brown (3-slot)	0,214±0,015 b	(0,000)
	Yellow (2-slot)	0,219±0,013 b	
	S. steel (2-slot)	0,229±0,016 a	

* According to the Tukey multiple comparison test results, the averages shown different letters in the same column for each orifice diameter group are different at 95%; ** $p<0{,}01$ very important

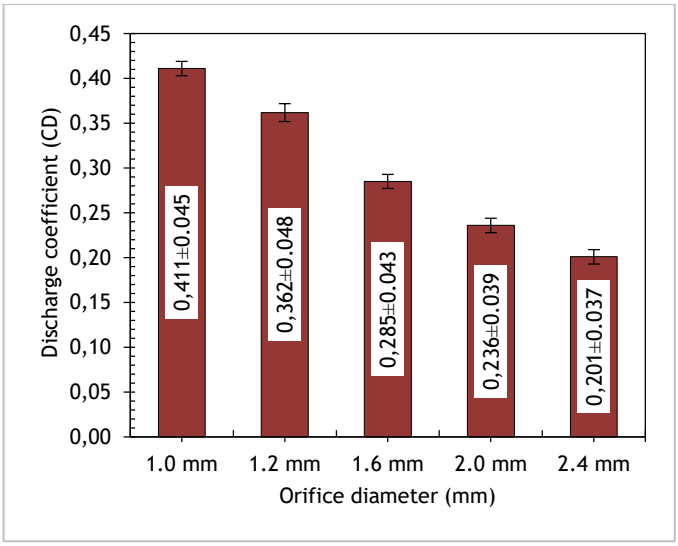


Figure 1. Discharge coefficient (mean±SD)

Droplet Size ($D_{V0.50}$)

As shown in Figure 2, the droplet diameter decreased as the spray pressure increased. While the droplet diameter averages at 2 bar spray pressure varied between 141,5-219,0 µm, the averages decreased at 12 bar and the averages ranged from 76,3 to 120,8 µm. In reference to the nozzle groups, the largest droplet diameter was obtained at 2 bar spray pressure level with the nozzle of 2.4 mm orifice diameter. As the orifice diameter increased, the droplet diameter increased. The droplet diameters obtained from the nozzles with 1,0 mm and 2,4 mm orifice diameters varied between 76,3-154,8 µm and 102,3-219,0 µm, respectively.

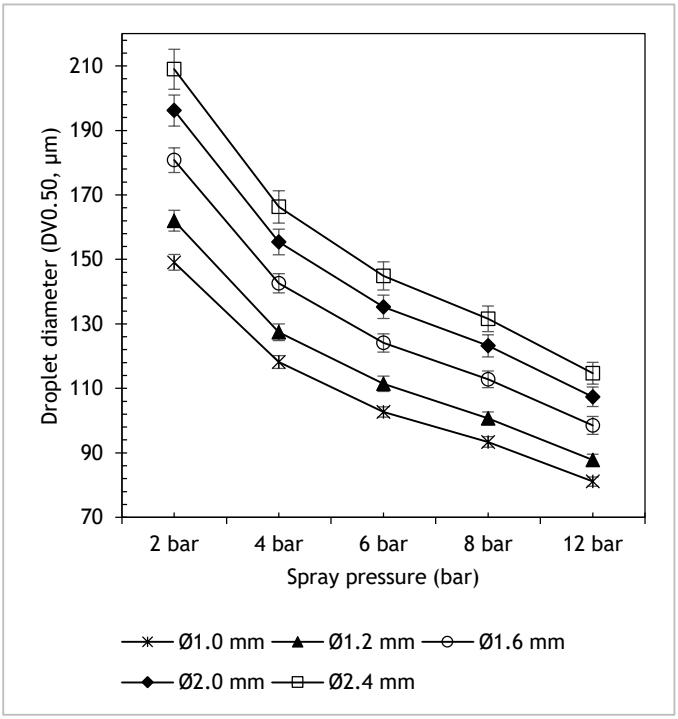


Figure 2. The variation of droplet diameter ($D_{V0.50}$, µm) according to the spray pressures for different orifice groups (mean±2·SE)

Table 5. Effect of swirl plates and spray pressures on droplet diameter ($D_{V0.50}$, µm) for the nozzle discs (mean±SD)

Orifice diameter (mm)	Swirl plates	Spray pressure				
		2 bar	4 bar	6 bar	8 bar	12 bar
Ø1.0	Blue (2-slot, C23)	141,5±0,8 c	111,9±0,7 c	97,1±0,5 c	88,2±0,4 c	76,3±0,3 c
	Brown (3-slot)	149,1±2,8 b	118,5±2,6 b	103,4±2,2 b	94,0±2,0 b	81,8±1,5 b
	Yellow (2-slot)	151,2±2,1 b	119,8±2,0 ab	103,9±1,5 ab	94,3±1,5 b	82,1±1,5 b
	S. steel (2-slot)	154,8±1,3 a	122,8±1,4 a	106,4±1,2 a	97,3±1,0 a	84,4±0,8 a
	F value (*p*, sigma)	44,64 (0,000)**	32,16 (0,000)	35,51 (0,000)	39,72 (0,000)	43,12 (0,000)
Ø1.2	Blue (2-slot, C23)	152,2±0,7 c	119,4±0,3 c	103,8±0,6 c	94,0±0,2 c	81,9±0,1 c
	Brown (3-slot)	162,0±3,3 b	127,8±2,3 b	111,7±1,9 b	100,9±2,2 b	88,0±1,7 b
	Yellow (2-slot)	163,3±1,2 b	128,5±1,7 b	112,6±1,4 b	101,9±1,0 b	88,8±0,8 b
	S. steel (2-slot)	170,7±2,3 a	133,9±2,6 a	117,4±2,1 a	106,1±1,7 a	92,5±1,6 a
	F value (*p*, sigma)	64,95 (0,000)	47,06 (0,000)	61,54 (0,000)	58,16 (0,000)	61,69 (0,000)
Ø1.6	Blue (2-slot, C23)	167,2±1,8 c	132,0±1,3 c	114,2±1,3 c	103,5±1,4 c	89,9±1,4 c
	Brown (3-slot)	182,1±3,2 b	144,0±2,3 b	125,1±1,9 b	113,7±1,8 b	98,5±1,9 b
	Yellow (2-slot)	185,1±2,9 ab	145,9±2,4 ab	126,7±2,2 b	115,7±2,1 ab	100,0±1,7 b
	S. steel (2-slot)	188,6±2,4 a	148,6±2,2 a	130,4±1,9 a	118,3±1,8 a	105,4±3,3 a
	F value (*p*, sigma)	65,85 (0,000)	62,27 (0,000)	70,24 (0,000)	64,12 (0,000)	43,06 (0,000)
Ø2.0	Blue (2-slot, C23)	179,6±1,9 b	142,0±2,3 b	123,1±1,4 c	111,7±1,4 c	97,5±1,4 c
	Brown (3-slot)	198,9±2,5 a	157,4±2,0 a	137,5±1,7 b	124,8±1,6 b	108,7±1,5 b
	Yellow (2-slot)	201,4±4,6 a	159,2±3,6 a	137,9±2,5 b	125,7±3,1 b	109,5±3,2 b
	S. steel (2-slot)	204,9±4,5 a	162,9±4,5 a	142,8±3,2 a	130,4±3,4 a	114,1±2,5 a
	F value (*p*, sigma)	49,14 (0,000)	39,35 (0,000)	67,60 (0,000)	49,55 (0,000)	48,08 (0,000)
Ø2.4	Blue (2-slot, C23)	187,6±1,7 b	148,6±1,5 b	129,3±1,3 b	117,5±1,5 b	102,3±1,1 b
	Brown (3-slot)	213,6±5,4 a	169,5±4,5 a	148,9±4,1 a	135,5±3,5 a	117,7±3,0 a
	Yellow (2-slot)	215,7±5,2 a	171,5±3,9 a	150,4±3,8 a	136,2±2,7 a	118,1±1,6 a
	S. steel (2-slot)	219,0±8,4 a	175,6±4,3 a	151,1±2,3 a	137,1±3,0 a	120,8±1,8 a
	F value (*p*, sigma)	32,23 (0,000)	51,56 (0,000)	57,83 (0,000)	56,75 (0,000)	90,83 (0,000)

* According to the Tukey multiple comparison test results, the averages shown different letters in the same column for each orifice diameter group are different at 95%; **: *p<0,01* very important

Table 6. The results of the regression analysis between droplet diameter ($D_{V0.50}$, µm) and spray pressure (P, bar) variables, and exponential functions

Orifice diameter (mm)	Swirl plates	[1]Exponential functions	R^2 (Corrected)	Mean error squares	F value	p (sigma)
1.0	Blue (2-slot, C23)	$D_{V0,50} = 179{,}947 \cdot P^{(-0{,}344)}$	1,000	7,4E-06	30260,7	0,000**
	Brown (3-slot)	$D_{V0,50} = 188{,}254 \cdot P^{(-0{,}335)}$	1,000	2,8E-06	75060,5	0,000
	Yellow (2-slot)	$D_{V0,50} = 191{,}777 \cdot P^{(-0{,}341)}$	1,000	3,4E-06	64100,2	0,000
	S. steel (2-slot)	$D_{V0,50} = 195{,}812 \cdot P^{(-0{,}338)}$	1,000	1,3E-05	17014,0	0,000
1.2	Blue (2-slot, C23)	$D_{V0,50} = 193{,}176 \cdot P^{(-0{,}346)}$	1,000	3,0E-06	74828,9	0,000
	Brown (3-slot)	$D_{V0,50} = 205{,}129 \cdot P^{(-0{,}341)}$	1,000	2,9E-06	76421,1	0,000
	Yellow (2-slot)	$D_{V0,50} = 206{,}358 \cdot P^{(-0{,}339)}$	1,000	5,7E-06	38054,0	0,000
	S. steel (2-slot)	$D_{V0,50} = 215{,}849 \cdot P^{(-0{,}341)}$	1,000	9,9E-06	22156,5	0,000
1.6	Blue (2-slot, C23)	$D_{V0,50} = 212{,}894 \cdot P^{(-0{,}347)}$	1,000	3,8E-06	59436,6	0,000
	Brown (3-slot)	$D_{V0,50} = 231{,}167 \cdot P^{(-0{,}342)}$	1,000	4,9E-06	45389,7	0,000
	Yellow (2-slot)	$D_{V0,50} = 234{,}638 \cdot P^{(-0{,}342)}$	1,000	1,2E-05	18163,2	0,000
	S. steel (2-slot)	$D_{V0,50} = 235{,}016 \cdot P^{(-0{,}327)}$	0,998	7,8E-05	2576,0	0,000
2.0	Blue (2-slot, C23)	$D_{V0,50} = 227{,}631 \cdot P^{(-0{,}342)}$	1,000	4,2E-06	52470,7	0,000
	Brown (3-slot)	$D_{V0,50} = 251{,}223 \cdot P^{(-0{,}337)}$	1,000	7,4E-07	290127,4	0,000
	Yellow (2-slot)	$D_{V0,50} = 254{,}871 \cdot P^{(-0{,}340)}$	1,000	7,5E-06	29064,3	0,000
	S. steel (2-slot)	$D_{V0,50} = 256{,}533 \cdot P^{(-0{,}326)}$	1,000	3,4E-06	58590,3	0,000
2.4	Blue (2-slot, C23)	$D_{V0,50} = 237{,}314 \cdot P^{(-0{,}338)}$	1,000	9,9E-07	218236,0	0,000
	Brown (3-slot)	$D_{V0,50} = 268{,}783 \cdot P^{(-0{,}331)}$	1,000	1,1E-05	18819,0	0,000
	Yellow (2-slot)	$D_{V0,50} = 272{,}771 \cdot P^{(-0{,}335)}$	1,000	1,9E-05	11150,2	0,000
	S. steel (2-slot)	$D_{V0,50} = 277{,}035 \cdot P^{(-0{,}336)}$	0,999	5,4E-05	3897,2	0,000

[1] $D_{V0.50}$: droplet diameter (µm); P: spray pressure (bar); ** $p<0,01$ very important

Table 7. Droplet diameter ($D_{V0.50}$, µm) categories for each combination of the nozzle discs with different orifice diameter and swirl plate according to the spray pressures

Orifice diameter (mm)	Swirl plates	Spray pressure (bar)										
		2	3	4	5	6	7	8	9	10	11	12
1.0	Blue (2-slot, C23)	F[a]	VF[b]	VF	VF	VF	VF	VF	VF	VF	VF	VF
	Brown (3-slot)	F	VF	VF	VF	VF	VF	VF	VF	VF	VF	VF
	Yellow (2-slot)	F	VF	VF	VF	VF	VF	VF	VF	VF	VF	VF
	S. steel (2-slot)	F	VF	VF	VF	VF	VF	VF	VF	VF	VF	VF
1.2	Blue (2-slot, C23)	F	VF	VF	VF	VF	VF	VF	VF	VF	VF	VF
	Brown (3-slot)	F	F	VF	VF	VF	VF	VF	VF	VF	VF	VF
	Yellow (2-slot)	F	F	VF	VF	VF	VF	VF	VF	VF	VF	VF
	S. steel (2-slot)	F	F	VF	VF	VF	VF	VF	VF	VF	VF	VF
1.6	Blue (2-slot, C23)	F	F	VF	VF	VF	VF	VF	VF	VF	VF	VF
	Brown (3-slot)	M[c]	F	F	VF	VF	VF	VF	VF	VF	VF	VF
	Yellow (2-slot)	M	F	F	VF	VF	VF	VF	VF	VF	VF	VF
	S. steel (2-slot)	M	F	F	F	VF	VF	VF	VF	VF	VF	VF
2.0	Blue (2-slot, C23)	M	F	F	VF	VF	VF	VF	VF	VF	VF	VF
	Brown (3-slot)	M	F	F	F	F	VF	VF	VF	VF	VF	VF
	Yellow (2-slot)	M	F	F	F	F	VF	VF	VF	VF	VF	VF
	S. steel (2-slot)	M	M	F	F	F	F	VF	VF	VF	VF	VF
2.4	Blue (2-slot, C23)	M	F	F	F	VF	VF	VF	VF	VF	VF	VF
	Brown (3-slot)	M	M	F	F	F	F	VF	VF	VF	VF	VF
	Yellow (2-slot)	M	M	F	F	F	F	F	VF	VF	VF	VF
	S. steel (2-slot)	C[d]	M	F	F	F	F	F	VF	VF	VF	VF

[a] F, fine; [b] VF, very fine; [c] M, medium; [d] C, coarse

At the nozzle discs of 1,0 mm, 1,2 mm and 1,6 mm orifice diameters, the largest droplet diameter was obtained in the stainless steel swirl plate, the lowest blue swirl plate (Table 5). At low spray pressures (2 bar and 4 bar), the impact on the droplet diameter of the swirl plates at the 2,0 mm and 2,4 mm nozzle discs reduced. The effect of brown, yellow and stainless steel plates on droplet diameter was found insignificant for the nozzle discs of large orifice diameters.

Table 6 showed the results of the regression analysis between droplet diameter and spray pressure, and the exponential functions for each of the nozzle orifice diameter and swirl plate combinations. Using the exponential functions, the droplet diameter of any orifice diameter and swirl plate combination can be estimated in reference to the spray pressure.

In Table 7, the droplet diameter spray categories were given in the spray pressure range of 2-12 bar in the nozzle disc and swirl plate combinations. Accordingly, the hollow cone nozzles produced mostly thin and medium-sized droplets. Medium-sized droplets produced in nozzle groups with orifice diameter greater than 1,6 mm were obtained at the low spray pressures (2 and 3 bar).

Discussion

The Effect of Swirl Plates on Discharge Rate Variation

It is known that the discharge rates at the hollow cone nozzles alters in reference to the swirl plates. However, there was no any information about the operational characteristics of the and nozzle discs and the swirl plates produced or used locally (Arag®, 2004; Albuz®, 2009; Teejet®, 2014; Hypro®, 2017). Sayıncı et al. (2013) determined that 2-slotted swirl plates varied the flow characteristics and discharge rates of the spray nozzles used together with the 50-mesh size strainer. In the present study, all measurements were performed without using a strainer. As the strainers were known to alter the flow characteristics of the spray nozzles (Sayıncı and Kara, 2015; Sayıncı, 2014; Sayıncı, 2015; Sayıncı, 2016), the nozzle discharge rate and other measurements were performed specific to this study. In conclusion, the swirl plates on the nozzle discs with small orifice diameter have a significant impact, and the effect of the swirl plates on the discharge rate variation gradually decreased as the orifice diameter increased.

Discharge Coefficient

Sayıncı et al. (2013) determined that the discharge coefficients of the hollow cone nozzles with 1,0 mm, 1,2 mm, 1,5 mm, 2,0 mm and 2,5 mm orifice diameters, and the averages was found as 0,402, 0,361, 0,337, 0,232 and 0,184, respectively. Wilkinson et al. (1999) reported that the flow coefficient depends on the orifice geometry of the nozzle and ranged from 0,15 to 0,65. Maniarasan and Nicholas (2006), Chu et al. (2008) and Hussein et al. (2012) stated that the flow coefficient is higher in small orifice nozzles than the larger ones. All literature findings have been consistent with the results of this study. In terms of the nozzle material, Sayıncı et al. (2013) found that the discharge coefficient of polyacetal (POM) nozzle discs was lower than those of ceramic and stainless steel. In terms of the nozzle type, the discharge coefficient ranged between 0.85-0,98 for standard flat fan nozzles (Sayıncı and Kara 2014; Sayıncı, 2015; Zhou et al., 1996; Cloeter et al., 2010; Dorr et al., 2013); 0,67-0,77 for pre-orifice chamber flat fan nozzles (Sayıncı and Kara, 2015); 0,38-0,43 for air-induction flat fan nozzles (Cloeter et al., 2010; Dorr et al., 2013).

Droplet Size

It was determined that the droplet diameter in all nozzle disc and swirl plate combinations in the range of 2-12 bar spray pressure ranged between 76,3-219,0 µm. In this range, the droplets were very fine and fine according to the spray classification indicated in the Hypro® (2014) catalogue, while the droplets were very fine, fine, medium and coarse in reference to Hypropumps® (2006), Kruger et al. (2013), Matthews et al. (2014) and Arag® (2017) literatures. In a study conducted by Serim and Özdemir (2012), droplet diameter measurements were performed at the constant spray pressure of 6 bar for hollow cone nozzles obtained from local manufacturer. The nozzle discs with orifice diameters of 1,0 mm, 1,2 mm and 1,5 mm were separately analysed in five groups and the volumetric median diameters ($D_{V0.50}$) of the four groups were determined in the 115,1-132,7 µm range. In this study, droplet diameters obtained from similar orifice diameter nozzles at 6 bar spray pressure ranged between 97,1-130,4 µm and these results were found compatible with the literature findings.

Conclusion

Spray nozzles, which are one of the most important parts of sprayer equipment, are manufactured from different materials in different types and sizes. The hollow cone nozzles choosing due to their cheap and easy supply are mostly operated at high pressures in the application area and produced very fine droplets sensitive to drift. In this respect, it is of great importance that pesticide applications are carried out under low wind speed conditions. The nozzle discharge coefficient is an important parameter in terms of flow dynamics and nozzle design. In this study, it was determined that the discharge coefficient of hollow cone nozzles was significantly lower than the standard flat fan nozzles. The differences among the discharge coefficients varies considerably depending on the swirl plate used behind the nozzle disc. This result is a reference for new design swirl plates. There is no standardization and quality standardization for the nozzle discs and swirl plates manufactured locally. It is predicted that the nominal size standardization for the hollow cone nozzles will increase the production quality.

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RESEARCH ARTICLE

A New Financing Model for Carbon Emission Reduction Projects: The Use of Carbon Emission Reduction Purchase Agreements (ERPA) in the Private Pension System

Gökhan Şen[1] • Mehmet Yunus Çelik[2*] • Tolga Ulusoy[3]

[1]*Kastamonu University, Faculty of Forestry, Department of Forest Engineering, Kastamonu/Turkey*
[2]*Kastamonu University, Faculty of Economics and Administrative Sciences, Department of Economics, Kastamonu/Turkey*
[3]*Kastamonu University, Faculty of Economics and Administrative Sciences, Department of Banking and Finance, Kastamonu/Turkey*

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ABSTRACT

As a result of the awareness of global climate change, many studies have been carried out to avoid this situation. These studies have been carried out intensely since the protocol signed in Kyoto in 2005. Reduction of carbon dioxide emissions from engines operating on fossil fuels, the promotion of using the renewable natural sources such as wind, solar, etc. which are called clean energy, methane gas decompositions, and similar studies are some of these. As the studies increased, funding difficulties began to be seen in supporting the projects. In this context, especially project selection has started to gain importance. When the carbon emission reduction projects are examined, it is seen that forestry and especially forestation projects are very important. Especially, afforestation investments are very important in terms of increasing carbon sinks and many ecosystem services they provide are of great value. The aim of this study is to develop a new mechanism to create a source of financing for afforestation investments. In this context, the Private Pension System Funds was used as a fund-raising tool for the financing of afforestation investments. in this new mechanism, it was aimed that preventing climate change by making all actors gain income. With the implementation of this system, it is thought that the problems in the existing carbon markets will be prevented, the investments on afforestation will increase and also the environmental funds will become widespread.

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Introduction

It is very important to ensure the continuity of natural resources, which are the basic elements of sustainable living, in line with the principles of conservation-use. The cliché of humankind, understanding the loss of their possessions only when they lose them has also been observed in the process of realizing climate change. After the industrial revolution with the increasing carbon-based gas emissions and as a result of the greenhouse effect caused by the increase in the ratio of these gases in the atmosphere, there has been a rapid change in the world climate. This phenomenon called Global Climate Change caused warming in some places and cooling in some places. Especially, these changes, which cause extreme and sudden climatic events, have caused various natural disasters.

As a result of the awareness of global climate change, many studies have been carried out to avoid this situation. As a result

* Corresponding author
E-mail address: mycelik@kastamonu.edu.tr

of international meetings and agreements, efforts to reduce carbon dioxide emission have been accelerated. In this context, it was decided to conduct a series of studies to reduce the rate of greenhouse gases in the atmosphere. These studies have been carried out intensely since the protocol signed in Kyoto in 2005. Reduction of carbon dioxide emissions from engines operating on fossil fuels, the promotion of using the renewable natural sources such as wind, solar, etc. which are called clean energy, methane gas decompositions, and similar studies are some of these.

Although there are many reasons for the formation of greenhouse gas, the necessity of forest protection has become more important, especially after the great damage caused by excessive human pressure and intensive use of the forests, which is the largest terrestrial ecosystem that keeps these gases. In this context, there are still many things to achieve to protect and take care of the forests despite the efforts put forth so far. In addition to the studies carried out in different sectors and especially in the energy sector, various studies are carried out in the forestry sector in order to reduce the carbon-based gases in the atmosphere and these works are generally aimed at creating carbon sink and increasing the amount of it. The most important studies in this context are REDD+ and LULUCF programs. Whereas REDD+ (Reducing Emissions from Deforestation and Forest Degradation) tries to reduce carbon emissions from deforestation and forest degradation (UNDP, 2015), LULUCF (Land Use, Land Use Change and Forestry) tries to determine changes in carbon footprint due to changes in land use and greenhouse and g their effects on emissions (UN, 2015).

It is seen that some of these studies are based on economic considerations. One of these is the so-called carbon finance, which is focused on reducing greenhouse gas emissions. This method is briefly defined as providing funding for a project to purchase carbon. (The National Experience of Carbon Markets, 2011).

In the framework of the studies carried out for the purpose of reducing greenhouse gases, although carbon financing is obtained for the projects carried out on many different subjects, it doesn't seem that potential value of forestry projects is fully realized, but in recent years an increase has been observed. 17% of gas emissions result from deforestation and forest degradation according to the Intergovernmental Panel on Climate Change. Although this amount is higher than the emissions from the entire transportation sector, the reduction of carbon dioxide emissions of automobiles seems more popular and important.

When the causes of greenhouse gas emissions are analyzed, it is seen that the amount of emission due to the forest destruction comes second after the energy sector. In this context, the role of reclamation and expanding of forests is very important in greenhouse gas emissions. Forests have four main roles in climate change. Forests are carbon sinks along with the forest soil and forest products. Sustainable forest management removes more CO2 from the atmosphere. Forests are a source of clean energy alternative to fossil fuels. Forest destruction leads to an increase in emissions (ASAN, 2010).

In addition to the contributions of forests to the climate, there are many different service and products offered to the ecosystem as well. Although wood-based products are the most widely known, non-wood products and services are more useful by both quantity and benefit than wood-based products.

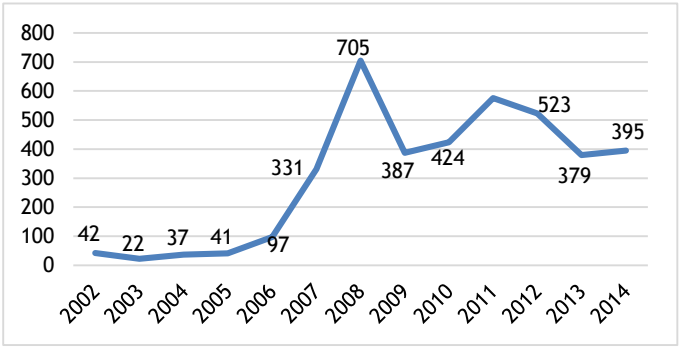

Figure 1. The total value of the voluntary carbon markets figure

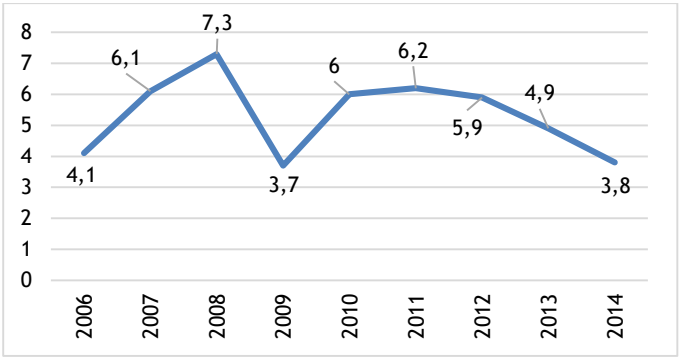

Figure 2. Average price of carbon credits being traded in the voluntary carbon market ($/Tons)

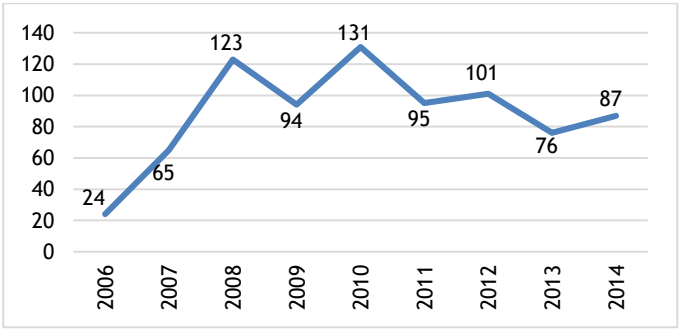

Figure 3. Traded on Voluntary Carbon Markets carbon volume (Million tons of CO_2)

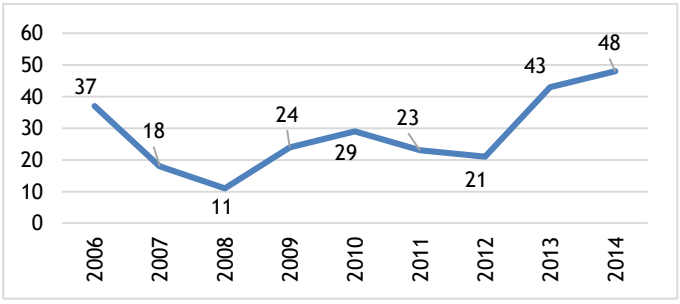

Figure 4. In the Voluntary Carbon Markets, the importance of forestry projects (%)

Forests, with wood and non-wood forest products, are a must for the economy and they are one of the main pillars of sustainable development, and these products and services are among the wheels of the economy. Services such as recreation, clean drinking water production, hunting animal provision etc. are some of them. In the last decade, they have entered the carbon market within the framework of the carbon sink feature in greenhouse gas reduction studies based on economic considerations. Figure 1, 2, 3, and 4 indicates the process in the voluntary carbon market and the status of the forestry projects (Hamilton and et al., 2007; Hamilton and et al., 2008; Hamilton et al., 2009; Hamilton et al., 2010; Peters-Stanley and et al., 2011; Peters-Stanley and vd., 2012; Peters-Stanley and et al., 2013 Peters-Stanley and vd., 2014; Hamrick and Goldstein, 2015).

Increasing the number of projects in the forestry sector developing them and increasing the share of financing allocated to these projects are more important than other projects due to the diversity of outputs and characteristics of forestry projects. Forestry sector projects to be performed in order to reduce greenhouse gas are of great importance in two aspects. First of all, the forests have a high percentage of carbon capture for many years through their above-ground, litter and subsoil structures, then with the use of particularly wood-based materials, it can preserve carbon in its structure for many years. The second is that these projects will also help to increase and preserve forested lands and provide many products and services as well as carbon capture.

As in all other areas, the most important problem in projects intended to reduce carbon emission is to get funding. In particular, the problem of getting funding in the voluntary carbon market including Turkey is felt even more. In addition to the support provided by the private sector companies to reduce their carbon emissions, it remains limited to get funding for projects to reduce carbon emissions. Therefore, the main question to be answered is how the funds circulating in the investment market can be directed to forestry projects.

In this study, “Private Pension Funds” as a new financing source that can be used in afforestation projects which are considered important in reducing greenhouse gas emissions and the usability of "Carbon Bonds" as a new tool in the portfolio of these funds are discussed. The study was exemplified in the scale of Turkey, were discussed and evaluated.

Why Afforestation Projects?

Turkey's surface area is about 78 million hectares (OGM, 2014). When the area of the stream and lakes of (1 million ha.) is taken out from this area, the area of 77 million ha. remains and when high mountainous areas (areas over 1500m) is taken out, an area of 55.6 million ha. remains. In addition, when arable lands of 26,5 million ha. with land capability classifications I, II, III and IV (Anonymous, 2014) and other forested lands (17.5 million ha.) are taken out (Haktanır et al., 2000, Anonymous, 2014) the amount of lands available for carbon reduction projects is approximately 11.6 million ha. In this context, it is seen that there is great potential for afforestation activities within the scope of both forestation of non-forest areas and improvement of degraded forest areas.

Due to the high potential availability of the lands to be afforested together with the fact that the afforestation projects produce many outputs of different types and economic characteristics, for carbon bonds applications afforestation investment projects within the forestry sector have been selected as implementation projects. In this context, the fact that the labor-intensive industry is high in Turkey because of its geographical features emerges as a factor in strengthening the social dimension of these studies.

However, another important point here is the selection of species to be used in afforestation. Industrial afforestation to be conducted with species that are growing fast and with shorter management time plantation works to be performed with tree species relevant to target diameter with longer time period? The answer to this question will lead to differences in contributing these investments to the economy and will lead to the use of different ways of transferring the revenues obtained to the market. Since the fast-growing species requires special areas and favorable climatic conditions and needs to be raised in areas with low slope and not too high above sea level and as the ultimate goal is to reduce greenhouse gas emissions expanding carbon sinks, the plantations in the industrial plantation forestry were excluded.

Why Private Pension System?

Today, one of the most effective methods used to fund investments is to create funds. The funds collected by investment companies and banks from individuals or corporate customers are combined into the funds created and invested. In private pension systems, money is collected in the same way and it turns into investment through funds. In recent years, the imbalance in the financial structure of the Social Security Institution and the increase in the debt ratios have brought into question supporting individual pension by the state. In this context, in addition to the money invested, putting into practice the state contribution of 25% of this invested money led to the growth of the private pension funds both in quantity and in popularity. According to April 2015 data, the size of private pension funds reached 40.3 billion TL with 5.3 million participants. In the 2015 election manifesto of the current government, the reduction of individual pension cut-off rates and the introduction of automatic participation in individual pension system can be shown as a proof that this system will grow even more in the following years.

What is a Private Pension System?

The private pension system was established to increase the savings of individuals for retirement and to provide additional income for their golden years. The private pension is a premium-based system managed by the private sector. Private pension can be considered as retirement insurance, based on the assessment of the financial contributions that employees regularly invest in their personal accounts depending on a predefined contract.

Retirement funds benefit from many financial assets in respect of their structure. Social security systems are the cornerstones of a country. The problems experienced in these foundation stones do not only affect individuals, but also lead to an increase in interest rates in the economies where individuals live, and thus, the slowdown in the accumulation of capital and the economic slowdown and welfare loss. When we look at many developed countries, it is obvious that carrying out private pension funds through companies with their investor characteristics as well indicates that they are a source of hot money flow for the markets. With these characteristics, private pension funds can be seen as a means of eliminating the problems that may arise in financial systems. When we look at the industrialized countries, it is obvious that the balance of working and non-working age is getting worse. To prevent this deterioration, while the young population contributes to production, they at the same time invest in private pension funds to make them comfortable and prosperous during their retirement period. When we look at the individual pension systems, we see that private pension systems have emerged with the collapse of the old pension systems. Especially the financial crises experienced in the last 10 years and the decrease in the life welfare of people together caused loss of income. Citizens or individuals who have lost their income want to contribute to the economy together with the private pension system and to live a comfortable life. In addition to the innovations made in September 1999 through the regulations made in the Law No. 5510, the private pension system aims to establish an individual pension system that will increase the savings for the elderly, to restructure the health services in a more efficient way and to establish a comprehensive social assistance system. For this purpose, the private pension savings and investment system law was implemented on October 7, 2001, and the individual pension program was completed in order to complement the pension plans in the social security system for the first time in our country. It is possible to summarize the basic elements of the individual pension system as follows.

- The system is fully voluntary
- Is open to everyone who has the right to use civil rights
- It is also open to employees in public or private organizations together with not working people and retired ones.
- It is possible for employers to be included in the system as a legal entity when they want to contribute to their employees.
- The system also allows anyone who participates in the system and who is an insured person to monitor their investments and the dividend shares from these investments in their own accounts.

Thus, the main purpose of the individual pension is not to replace the existing pension systems. It is to provide complementary effects on the peace and prosperity of individuals or system participants.

Overall Process of Private Pension System

The process starts with the individual account opening in pension companies in order to benefit from a private pension. Employers can contribute voluntarily to the accounts. The company that manages the retirement accounts will operate exclusively n the pension system market with all its legal personality characteristics having a complete specialization and competitive advantage. Companies that currently carry out insurance activities and who have fulfilled the requirements related to the corporate activities in the law can turn into a pension company whenever they wish. Companies that wish to continue their activities as pension companies will create different mutual funds according to different risk alternatives. The management of these funds will be carried out by portfolio management companies. while the funds will be monitored and supervised by the private pension company and the portfolio consultancy company, they will be under the supervision and control of the state. Individuals participating in the insurance system will be able to receive risk cover. In addition, they will be able to diversify across different fund alternatives in a way to reduce their risks without abiding by any investment fund and also have the right to make a transfer to another pension company within a certain period. When the age limit stated in the contract is reached, the contributions shall be repaid together with the dividend shares, whether as bulk or in monthly installments. There may be differences in applications such as tax-deduction, as there may be early departures from the pension system without waiting for the end of the contract.

Process of Individual Pension Savings and Investment System

Participation in private pension system

Persons who have the right to use civil rights can participate in the private pension system. People participating in the system must sign a contract with a private pension company.

Individuals who are over the age of 18 years can participate in the private pension system. After determining the pension companies with their own free will, the participants sign the pension contract of the company. After this stage, preparing the best-suited retirement plan, the company presents the participants these plans. After the dividend shares are decided, signatures shall be taken, and the pension contract shall be initiated upon the payment of the first contribution. The Pension Contract is mainly composed of the participant and the company covering the principles regarding involving of the participants in the system, retiring or jumping out of the system, payment of the contributions, monitoring of these contributions in the private pension accounts, the investments in the funds, principles concerning the payments to the participants or beneficiaries and regulating other rights and obligations of the parties.

The financing of the private pension system

Funding in the private pension system consists of the contribution fees and entry fees together with other deductions requested from the participants. The costs and deductions received from the participants during the individual pension contracts are shown in the diagram below. (Figure 5)

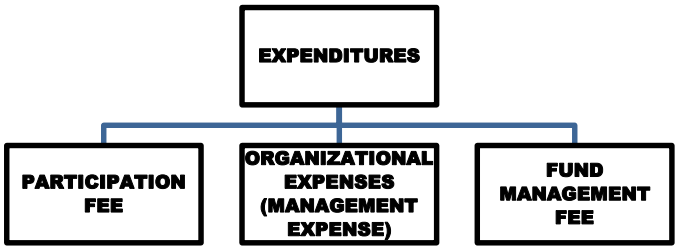

Figure 5. The costs of PPS participants

Pension company may receive entry fees from the participants or people acting on behalf of the participants in case of joining the private pension system for the first time or opening a new private pension account, provided that it does not exceed the monthly amount of the minimum wage valid on the date of signing the pension contract. The pension company may deduct an administrative expenses fee not exceeding the rate of maximum eight percent of the contribution of the participant to the individual pension account and a fund management fee calculated on the basis of the net asset value at a rate of maximum one hundred thousand to ten percent. Companies are obliged to clearly show any deductions from individual pension accounts in the pension contracts and announcements. In order to make changes in the entry fee, administrative expenses fee and fund management fee included in the pension contracts, it is essential that there is no contrary provision in the contract and that the change is approved by the Undersecretariat.

The participant contributes to the individual pension account to be opened in the company in accordance with the principles specified in the individual pension contract; he may decide on the allocation of the contribution share among multiple funds of the same company and the change of pension plan within the framework of the conditions in the pension contract and request the transfer of their savings in the individual pension account to another pension company. In order to request to be transferred to another company, the participant must be at least one-year member of the company. In this case, the company is obliged to fulfill the request within seven business days from the notification and transfer the information and documents related to this account together with the holdings. The participant may change the pension plans or the distribution of the contribution fees to the funds at most four times a year. The participant may stop paying contributions to the private pension system before qualifying for retirement. Within the term of retirement contract, the participant may withdraw from the private pension system requesting his holdings at any time or in case of permanent disability. In case the participant requests a withdrawal, the holdings in the individual retirement account are paid according to the provisions of the pension contract. Participants have the right to choose between the same company's products.

Overview of funds available

Private pension mutual funds enable us to protect our holdings from the corrosive effect of inflation, to gain profit in line with interest rates in the money markets and to benefit from the possible opportunities of market trends.

If we look at the existing funds in general, investments can be made in government bonds and short-term treasury bonds. Savings at short-term treasury bonds or government bonds are protected from corrosive effects of inflation. Their return percentage is at a similar rate of Stock Exchange İstanbul interest rates. The money market makes use of the opportunity gains generated by interest movements and achieves high-profit targets without high fluctuation. Investors seeking to invest in long-term treasury bonds or government bonds benefit from medium and long-term treasury bonds and government bond returns. These types of funds ensure that savings are protected from the long-term corrosive effect of inflation, creating regular and continuous income flows in the future. Investors who want to diversify their investment tools in stock funds, which are riskier, keep their portfolios in balance by investing in different investment tools. The investor, who wants to take the fund return rate to a reasonable and stable level, can invest in stock funds and get riskier but high-yield returns. Those who prefer exchange funds benefit from returns in foreign currency or exchange-indexed government borrowing tools. From a medium and long-term perspective; from foreign exchange, they try to get regular and continuous income flow in the future. It constitutes an alternative for investors who do not want to bear exchange and country risk. This type of funding can turn the real return of foreign stock and debt instruments in foreign currency into fund revenue. Apart from these, investors can invest in precious metal funds. Precious metals funds can be financed through gold and gold-based capital market instruments. In standard funds, investors who do not want to take a high risk may also have a balanced investment.

Methodology from PPS'S to ERPA'S

The Carbon Trading Agreement is a contract between the buyer and the buyer of the carbon emission rights (ER). ERPA provides a written legal framework that regulates the acquisition, sale, acquisition, and transfer of Carbon Emissions rights. The purpose of ERPAs consists of four elements: the creation of a contract by writing the provisions between the parties, determination of responsibilities, identification of rights and risk management. The parties of ERPA are buyers and sellers. The buyer receives emission reductions from the project and pays for the reduction. Thereafter, initial and periodic verification, verification and certification operations are carried out. Finally, emission reductions are delegated. Parties, definitions, sale and purchase related articles, delivery, documents or other evidence proving the validity of the ERs, articles related to the basic evaluation, articles related to risk management, contract price and payment

terms, declarations and commitments, responsibilities and indemnities, delinquency, rescission and legal solutions, progress reports and auditor's rights, privacy statement, provisions relating to the settlement of arbitration and disputes, taxes, duties and fees, force majeure and articles relating to third parties and other matters matters should be included in Carbon Emission Reduction Contracts. The four types of contracts can be arranged in the form of ERPAS: futures contracts, spot contract, option contract or a mixed contract (Cob. gov. tr. Access date: 19.10.2015)

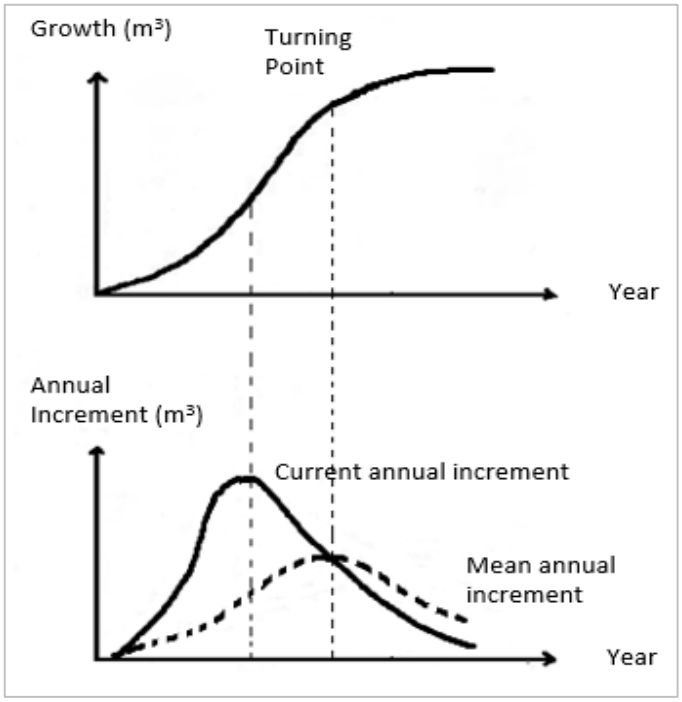

Figure 6. Single-tree volume-age and increment-age graph (Saraçoğlu, 2002)

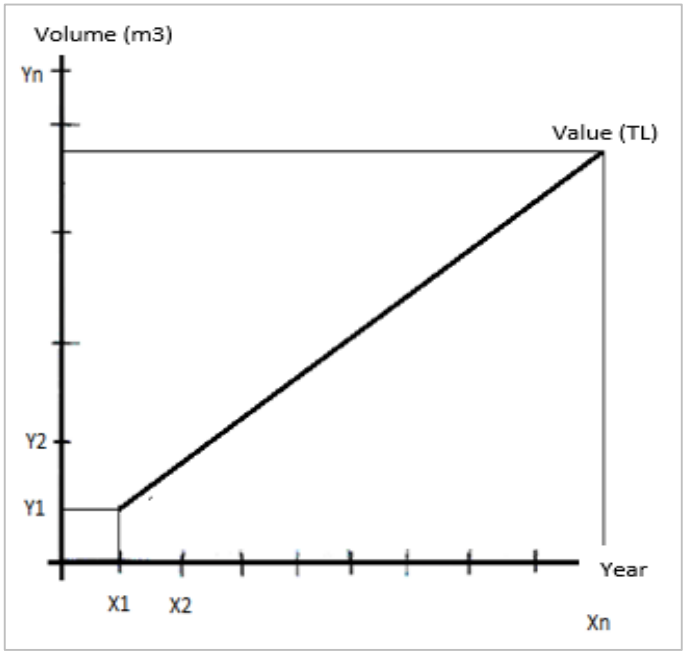

Figure 7. Bond value change chart

In this context, it is foreseen to make use of the carbon accumulation from the afforestation projects and to use bonds as instruments to be used in private pension funds. This is because the bonds and a tree or forest have the same overall structure (Figs. 6 and 7).

Bonds are debt instruments issued by corporations, state or public institutions with the same parity and nominal values in order to provide medium- and long-term debt. While bonds issued by corporations are subject to Turkey Commercial Code provisions, those issued by public agencies and state are subject to special laws in Turkey. Corporations can meet their cash needs in various ways. One of them is a capital increase, that is, taking new partners to the enterprise by issuing shares and the other is borrowing. Companies can also obtain the loans they need by either borrowing from banks or issuing bonds to a public offering. Both bonds and stocks are investment instruments bought and sold at the Stock Exchange Markets. However, there are some fundamental differences between them. Bonds are debentures and do not give ownership to the company. They are usually limited to a specific term and provide a bearer with interest income during this period. However, stocks are instruments that represent ownership over the enterprise (ensuing partnership), which do not have a certain payback period. They do not bring in a fixed income, such as interest rate. However, they give the right to share the profits. The capital of the bonds can be paid when due or reimbursed before the due date if its number comes out of draws being performed according to a redemption plan. A premium can be paid to the bonds remaining in circulation until the due date in the redemption plan or to the bonds winning the draw when the due date is close taking into account the length of time. Bonds may be issued by name or bearer. Bonds can be sold at the price written on the bill, or at a price above or below this price (Issued Value). There is an inverse correlation between the selling price of a bond and the true interest rate it provides. For example, if a bond with a nominal value of TL 1,000 (the written price), which has an interest rate of 8%, is marketed at a price of TL 800, the true interest rate is 10%. Corporations can issue bonds of various types. For example, premium, bonus, profit participation, collateralized, stock exchange and interest rate adjustable bonds are some of them (What is Bond? Access Date 10.08.2015).

The process of the system will be as follows (Figure 8). Afforestation investments to be conducted by individual or legal persons are approved by independent certification institutions and the main framework in the system is directing the money source collected in PPS to forestry investments. For this purpose, it is necessary to establish a fund in the name of an environmental fund/climate change fund/carbon reduction fund.

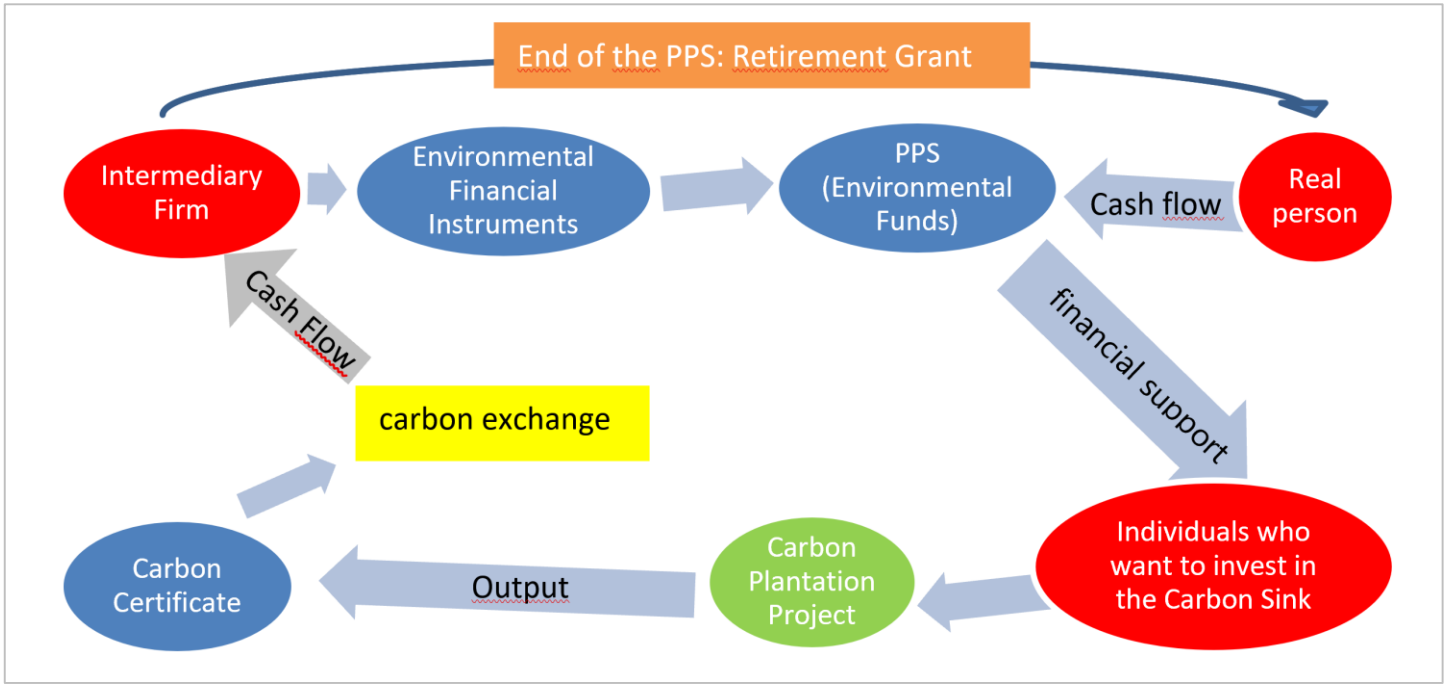

Figure 8. New financing method based on PPS for carbon sink projects

In this respect, the Real or Legal Person X considers the Private Pension System as a financer tool regarding the expansion of the Forests and in this regard, it deems an ER loan for the wooded areas, which are subject to emission, he will create or previously-created which is considered as a corporate bond.

For this purpose, X applies at the intermediary firm for evaluating the ERPAs. The intermediary firm calculates the costing for the real/legal X. There are basically two types of costs. First, the costs of afforestation, and second, the costs of carbon credit. A project for the area to be created for carbon emission is required and this project can be provided by the intermediaries to X. By independent institutions where intermediary institutions and ERPAs will be considered, Real /legal X person has been issued an investment vehicle, and undertakes to open new sink areas with a certain percentage of the revenues to be obtained by the agreement.

X, whose project has been accepted, ERPAs are audited and became the actor of the forestry, is supported by the portfolio which has investment tools, and is being included in the Private Pension System. Additional tax incentives may be offered if individuals are included in the system and invest in portfolios of financial instruments created from afforestation projects and they may have the possibility of granting more state contributions to citizens' sensibility.

It may be one of the advantages of this system to further embody an abstract concept financing it, such as the creation of state credit through a special afforestation law (which may also be in the form of a bond / treasury bill), drawing the attention of individuals, the creation of public opinion and a more liveable environment.

In this context, if the issue is broadened, the main objectives for forest villagers should be increased forestation and reforestation, preventing deforestation; and more restoration of the deteriorated agricultural lands and meadows/pastures.

Discussion

The efforts to prevent climate change have reached the present day following the Rio Earth Summit in 1992, based on the emerging sustainable resource management. In this period, many different methods and projects have been conducted. The most important point in these studies is the determination of the financing sources to be used for the projects. No matter how much it is a beneficial project because it cannot be conducted unless it is provided with sufficient financing.

Many different financing methods have been used for afforestation efforts. These afforestation works, although not directly for the purpose of carbon accumulation, are similar in terms of being a forestation project. As reported by Koçar (1999), afforestation studies have been granted credit by the Farmers' Chamber for 40 years with 5% interest since 1936 in the United States. Tax incentives are also provided. In Spain, up to fifty percent of the afforestation investments are donated according to the law, and up to 90 percent of the total cost can be granted credit. In Portugal, up to 70% of financial aid can be provided from the budget and funding created for the development of forestry in the private sector. The Forestry Authority Forestry Commision, an official institution for afforestation efforts in the UK, awards a grant up to 70% during planting and in 5 years awards a grant for the rest. In Australia, 40-50% of the cost of afforestation investments for different types of trees can be subsidized by the forest organization, but these supports are not sufficient. In Australia, the private sector covers costs such as the guarantee of purchase for forestation, silvicultural support, rejuvenation costs. Forestry efforts in the Netherlands are provided by the Ministry of

Agriculture and Fisheries as tax reductions and direct grants. The National Forestry Fund established in Argentina supports forestry projects and in Chile, it is stated that 50% income tax reduction is provided by law in forestry efforts (Koçar, 1999). As afforestation efforts are covered by the forestry organization in Turkey, financing is provided by institutions and treasury. On the other hand, forestry and treasury lands are allocated to private afforestation investments and low-interest loans with government support are provided.

All of the aforementioned economic stimulus and supports are mostly state-funded financial resources. These resources may be interrupted by crises that may occur or changes in the main objectives and investment priorities of the state institutions. For this reason, it is essential to develop financial resources and integrate investments of real persons and the private sector into the forestry sector. By this way, it will be possible for the savings of institutions and individuals to turn them into an investment and to contribute to the environmental protection efforts together with reducing the carbon emission rates of the country by reducing the carbon emissions as well. In this context, the integration of afforestation investments to PPS system through special bonds in order to increase carbon capture will ensure that investors and entrepreneurs are guaranteed by laws and regulations.

Bonds, stocks, etc. have been proposed for vehicles as a financing tool poplar plantations in Turkey (Koçar, 1999). However, it is stated that there is no possibility of financing with current bond transactions. For the afforestation investments, it is stated that it may be possible to use the coupon bond or stock exchange with exchangeable bonds and the necessity of legal regulations is mentioned. The proposed bond in the proposed system is proposed as a main framework. In the detailed system to be formed within this scope, a new structure should be prepared with various arrangements by considering the structure of the bond which is similar to the growth of a tree in general. In this context, it will be possible to use the carbon credits obtained from the afforestation investments of the intermediary institutions such as banks during the determined bond period. In addition, the increasing carbon ratios in the afforestation areas each year represent an increasing value in the carbon market. On the basis of the PPF, this will also prevent the issuers of the bond issuers on an annual basis, thus avoiding the pressure of cash outflows on an annual basis. At the same time, the intermediary institutions will be able to evaluate the revenues from the sale of carbon credits due to the long term.

It is obvious that carbon emission contracts included in private pension funds may have some macroeconomic effects. These can be expressed as:

- Contributing to society with a more livable environment with the increase in wealth levels of individuals with additional income in retirement,
- Use of long-term fund accumulation as a financial tool to reduce greenhouse gas emission in increasing carbon sink areas,
- With the increasing ability of public and private sector to borrow, allocating more resources by the public sector to the efforts that will create environmental awareness,
- When allocating resources for employment-increasing investments, more resources can be allocated to sectors related to afforestation and forest efforts,
- Paving the way for the investors financially, who wish to carry out efforts to reduce the carbon emission, to carry out afforestation efforts through the extension of the term in the financial market,
- As environmental factors begin to improve, real and legal persons want to allocate more resources to this subject and deepening of money and capital markets,
- To contribute to sustainable growth and fight against inflation together with forestry efforts.

It is also considered that there are possible financial opportunities and expectations for the creation of carbon contracts in private pension funds. These can be expressed as:

- The return of funds that provide hedging against the aging of the population as environmental protection to the young population,
- Monitoring contributions to forestry on the basis of individual accounts in the use of long-term savings,
- Establishment of the severance pay fund for the employees in the forest sector by utilizing the infrastructure of the private pension system,
- In particular, laying out a little more risky products with less return softening the life insurances which offers risk-weighted products.
- A more competitive and dynamic market structure developing along with the rapid access of forest products to the end user with the widespread use of fast-growing species in forestry,
- Increasing the tax incentives to real or legal persons who consider carbon emissions as a threat and who spend funds to reduce impacts in this way,
- Strengthening the legislation and institutional structure of private pension in a way more sensitive to the environment

Conclusion

With the reduction of a number of scientific risks in climate change, the creation of forest sinks has recently become the focus of world science, priorities that require harmonious forestry efforts have been identified as an environmental area. When we look at the international policies on climate change, it is known that carbon sink areas, in other words, the forest carbon captures, are the easiest and cheapest way to reduce carbon emissions.

First of all, forests play an invaluable and critical role in climate change as well as in reducing carbon emissions as the largest carbon sink and the most important source of emissions. Trees are considered to be carbon concentrators.

Especially deforestation affects the total global greenhouse gas emission rates per year significantly. It is important to control and prevent deforestation. The use of forests as a carbon sink is often pushed aside when creating an emission pool. With regard to policies on the side of forestry, it is an irony. In order to prevent the global temperatures from rising rapidly on the average, it is being tried to give war on other fronts instead of the "Forestry Front." The benefits of global carbon markets as a means of combating climate change should be recognized in the forestry sector. As forestry and forest areas are becoming an international policy, forestation and the creation of carbon sink areas will become more supported.

Institutions or individuals need to take urgent action to raise awareness about carbon emissions. To this end, the impact on the international financial sector should also be reviewed when making emission reduction agreements. In order to reduce carbon emissions, it is natural for decision makers to want to see their returns in advance. Therefore, this type of market needs to be formed urgently. As mentioned briefly in this study, the evaluation of carbon certificates in a separate market by private pension firms would be a very appropriate decision. The diversity in financial markets will increase with the evaluation of hybrid financial instruments produced with carbon certicates in the capital markets. Individuals who want to be environmentally conscious should be directed to these types of financial instruments when creating their individual retirement portfolios.

As Turkey does not take part in the mandatory carbon market, to impose any sanctions on carbon emission reduction is beside the point. The carbon emission reduction projects based on volunteerism and the creation of the targeted carbon exchange are considered to be somewhat impossible. However, this reduction is more realistic and easy to implement within the framework of yielding economic profit investments. In particular, the existence of some features to prevent the exit from the system for at least 10 years increases the acceptability of the lower income at the beginning. Moreover, it is thought that the idea of investing in the future will be more accepted by people by helping people to reduce climate change.

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RESEARCH ARTICLE

Cross-stress Tolerance (Cold and Salt) in Plants Have Different Seed Nutrient Content (Maize, Bean and Wheat)

Yavuz Demir*

Atatürk University, Faculty of Education, Department of Biology, Erzurum/Turkey

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Salt stress

ABSTRACT

The aim of this study was to determine cross-stress tolerance in plants have different seed nutrient content (maize, bean and wheat). For this purpose, salt (50 and 100 mM NaCl) and cold stress (12/7ºC) separately or in combinations (cross stress) were applied and studied the alterations of root and stem growth, total soluble protein content and antioxidant enzyme activities (superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX)) associated with induction of cold hardiness by salt stress. Salt and cold stress and its combinations caused inhibition of root and stem growth, and antioxidant enzyme activities (SOD, CAT, POD and APX) were significantly increased or decreased due to both salt, cold stress and its combinations. The soluble protein content increased in maize and wheat while decreased in bean in all applications. Cross-stress, on the other hand, decreased the soluble protein content according to alone salt or cold stress in all plants. As a result, there is not determined any relationship among cross-stress tolerance and growth, soluble protein content, antioxidant enzyme activities or plants have different energy sources. For example; while the highest increase in SOD, CAT, POD and APX activities were observed in maize, root-stem growth was most decreased in maize.

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Introduction

Plants are immobile and is faced with abiotic environmental stresses such as cold, temperature, soil salinity, drought and nutrient deficiency or excess throughout their life. Abiotic stress factors limit 50% or more productions of world agriculture (in point of yield- plant growth and development) (Yasuda, 2017). Plants have cellular and/or molecular responses that increase tolerance to stress in response to abiotic stresses. Although salt, water and cold stresses are clearly different from each other and each of them certain plant responses arise, also activates some common reactions. Low temperature, drought and salinity represent stress factors associated with plant cell dehydration.

Salinity is one of the most important stress factors that reduce the growth and efficiency of plants in various climates. It is an important problem especially in arid and semi-arid regions. Approximately 22% of the agricultural land in the world is salted (F.A.O. 2010). Biochemical and physiological responses to salt stress in plants vary and affect almost all plant processes. High salinity includes both ionic (chemical) and osmotic (physical) component. Salt stress usually cause water stress, ionic toxicity, nutritional imbalance (presence of nutrients in the soil, receive and transport in plant), oxidative stress, changes in metabolic processes (photosynthesis, lipid metabolism and protein synthesis), membrane disturbance, slow cell division and growth and genotoxicity, thus affect plant growth. These effects depend on the stage of the plant

* Corresponding author
E-mail address: ydemir@atauni.edu.tr

growth, the duration and density of stress. The most important effect of salt stress is the prevention of plant growth by reducing enzyme activities and biochemical components (Akladious and Mohamed, 2018; Kaleem et al., 2018).

Low temperatures cause typical symptoms of chilling such as senescence, inhibition of growth, water imbalance, mineral nutrition, respiratory and photosynthesis (at least decrease in chlorophyll and damage to the photosynthetic apparatus) (Erdal et al., 2015). It can lead to a higher cold tolerance that lead to molecular, biochemical and physiological changes when a plant exposure to a cold temperature and this called as acclimation (Mutlu et. al., 2016). Low temperature can lead to immediate mechanical stresses, changes in the activity of macromolecules and reduced osmotic potential in the cellular environment. Cold stress responses in plants are extremely complex events that alter the biochemical composition of cells to prevent damage caused by low temperatures. In addition, cold stress has a significant effect on plant morphology, which leads to decrease in growth and low productivity. One of the cold resistance factors is the ability of the antioxidant enzymes to maintain their activity during cold stress and to recover relatively quickly after the plants are transferred to hot conditions. (Eremina et al., 2016; Zhang et al., 2014). Therefore, it is vital to develop products that are resistant to salt and cold stress in order to feed the growing population in the world

Plants are usually not only subject to a continuous stress factor that potentially damages the plant at the same density, but at same or different times of their lives are exposed to various stress factors (Foyer et al., 2016). It may vary from time to time in the variety and intensity of the stress throughout the life of the plant. Plants are often exposed to multiple stresses such as drought and heat, or drought and cold. The combination of several stresses can cause more damage than alone stress. While answers to a single stress such as cold, drought and heat have been extensively researched, little is known about plant response to multiple simultaneous stresses (Lee and Back, 2016). Cross-stress tolerance can be achieved when the response to stress by the plants increases the performance of these individuals in another type of stress. This tolerance, therefore, means that plants are exposed to different stress factors at different times, which is usually seen in plants in nature. Cross-tolerance to environmental stress is a common phenomenon in plants, and exposure to some form of stress provides an overall increase in resistance to a range of different stresses (Yasuda, 2017). Cross stress tolerance is important in the daily life of plants in nature. Therefore, plant plasticity is necessary to overcome such stresses that occur repeatedly in the life of plants; this creates acclimation mechanisms that allow them to react better to single or multiple stresses of the same or different nature at all times. Cross-tolerance occurs due to synergistic co-activation of non-specific stress-sensitive pathways that cross biotic-abiotic stress limits (Yasuda, 2017). Cross stress tolerance is a sophisticated molecular process involving morphological, anatomical, physiological and biochemical changes in cell, organ and all plant levels. Cross-tolerance events are often associated with the regulation of gene expression and increased ROS (reactive oxygen species) production by H_2O_2, oxidative signal and redox signaling (Munne-Bosch and Alegre, 2013).

Although some mechanisms associated with stress memory in plants are known in addition to epigenetic modifications and morphological changes, such as accumulation of specific transcription factors or protective metabolites, cooperation among ecologists, physiologists, biochemists and molecular biologists in the present and near future is very important to understand cross-stress tolerance and stress “memory”.

In this study, it is aimed to investigate whether exposure to salt stress (NaCl) will increase the cold stress resistance of plants and are there any relationship between different seed nutrient content (maize, bean and wheat) and stress tolerance. Cross-stress tolerance has evaluated by determining changes in the root-shoot growth, total soluble protein content and antioxidant enzyme (SOD, CAT, POD and APX) activities.

Materials and Methods

Plant Material and Growth Conditions

Before germination, seeds of maize (*Zea mays* L. cv. Hido), bean (*Phaseolus vulgaris* cv. Kızılhaç) and wheat (*Triticum aestivum* L. cv. Bezostoja-1) were surface sterilized by using (0.01% w/v) sodium hypochlorite solution, and washed with sterile dH_2O. Seeds have different nutrient content were grown in control (25/20°C), salt stress (50 and 100 mM NaCl, 25/20°C, they were selected with preliminary studies), cold stress (12/7 °C), cold (12/7 °C)+salt stress (50 mM NaCl) and cold (12/7 °C)+salt stress (100 mM NaCl) conditions for 7 days in petri dishes. On the 7th day, all the plants were harvested (the tissues were rinsed three times in distilled water after harvested) and analyzed.

Measurement of Root and Stem Length of Plants

The root and stems of the plants were cut with the help of the bust of the joints and the lengths were measured with the help of the millimetric ruler. Roots measured based on the length of the main root (Bozcuk, 1978). The lengths of the root and stem of the applications were separately measured and divided by the number of plants, and mean root and stem length were calculated as cm / plant.

Determination of Total Soluble Protein Content

Total soluble protein contents in the root and stem were measured according to the method of Smith et al. (1985). Total soluble protein content was determined as µg protein/gr tissue.

Determination of Antioxidant Enzyme Activities

For the enzyme assays (SOD, CAT, POD and APX), leaf tissues (0.5 g) were homogenized in liquid nitrogen, and 5 ml 10 mmol l^{-1} K–P buffer (pH 7.0) containing 4% (w/v) polyvinylpyrrolidone (PVPP) and 1 mmol l^{-1} disodium ethylenediamine tetraacetic acid (EDTA) was added. The homogenates were centrifuged at 12,000xg and 4 °C for 15 min, and the supernatant was used to determine enzymes activities. SOD activity was measured according to Elstner and

Heupel (1976). CAT activity was measured according to Gong et al. (2001). POD activity was determined according to Yee et al. (2002). APX activity was determined according to Nakano and Asada (1981).

Results and Discussion

In the study, the change in growth, which is an important indicator of the plant's vital functions and the evaluation of plant conditions, has been chosen as a criterion. All stress conditions (50 and 100 mM NaCl and cold) were determined to inhibit root and stem elongation of maize, bean and wheat (Table 1). These results are consistent with studies reporting that salt and cold stress reduce plant growth (Mohamed and Latif, 2016; Zhou et. al., 2018). Suppression of plant growth under environmental stress may be due to decreased cell division, elongation and apical meristem activity. Decrease in shoot and root length in salt stress may be due to either the inhibitory effect of water shortage on growth-promoting hormones or the reduction in water absorption and activity of metabolic events (Akladious and Mohamed, 2018; Akladious and Hanafy, 2018; Mutlu et. al., 2016). This inhibitory effect may possibly be due to the decrease in intracellular CO_2 concentration and the effect of salt on the stoma and photosynthesis due to the deterioration of photosynthetic enzymes, chlorophyll and carotenoids (Zhou et. al., 2018). At the same time, in the case of cross stress (Cold + NaCl (50 and 100 mM), these inhibitions were determined to be more (Table 1). It was observed that root-stem growth more decreased as salt concentration increased in cold stress. Exposure of a plant to a single stress can lead to a broad spectrum of abiotic stresses and, in some cases, to tolerance to biotic stresses, and this is known as cross-stress tolerance. Gaining abiotic stress tolerance or cross stress tolerance requires activation of mechanisms that minimize cellular damage or deterioration. Antioxidant systems play a vital role in triggering cross-tolerance through ROS scavenge and / or signal functions (Hossain et. al., 2018). However, in this study, although the ROS scavenge capacity maximum increased in the maize among seeds have different seed nutrient content in stresses (salt and cold) and cross stress conditions, the root-stem growth was the most negatively affected in maize plant. There was no significant improvement observed in cross stress applications according to stress alone. Even the severity of stress increased.

Table 1. Effects of salt (50 and 100 mM NaCl), cold stress (12/7°C) and cross stress (Cold + NaCl (50 and 100 mM)) on plant root and stem lengths (cm) with different seed nutrient content

	MAIZE				BEAN				WHEAT			
Applications	**Root**	**Change %**	**Stem**	**Change %**	**Root**	**Change %**	**Stem**	**Change %**	**Root**	**Change %**	**Stem**	**Change %**
Control	12.6		6.8		9.5		2.4		12.8		11.4	
50 mM NaCl	9.8	-22.2	5.2	-23.5	9.2	-3.2	1.9	-20.8	10.4	-18.8	9.8	-14
100 mM NaCl	6.4	-49.2	3.7	-45.6	6.3	-33.7	1.4	-41.7	8.0	-37.5	6.3	-44.7
Cold (12/7°C)	7.8	-38.1	5.2	-23.5	6.9	-27.4	1.9	-20.8	9.7	-24.2	8.3	-27.2
		-53.2		-53		-40		-33.3		-37.5		-38.6
Cold+50 mM NaCl	5.9	-39.8	3.2	-38.5	5.7	-38	1.6	-15.8	8.0	-23.1	7	-28.6
		-24.4		-38.5		-17.4		-15.8		-17.5		-15.7
		-69		-63.2		-51.6		-41.7		-51.6		-56
Cold+100 mM NaCl	3.9	-39	2.5	-32.4	4.6	-27	1.4	0	6.2	-22.5	5.0	-20.6
		-50		-52		-33.3		-26.3		-36.1		-39.8

The soluble protein amounts in maize and wheat increased in all applications (salt, cold and cross stress (salt + cold)) (Table 2). It has been reported that the soluble protein amounts in maize and potatoes increases with increasing salt concentration (Abd El-Samed et. al., 2004; Ryu et. al., 1995). In previous studies it was determined that the soluble protein amount in wheat leaves increased significantly under cold stress (Turk et al., 2014). Protein changes have proven to be a key feature of cold resistance. The soluble protein amount in bean plant reduced in all applications (salt, cold and cross stress (salt + cold)) (Table 2). Öztürk et al. (2012) reported that salt stress reduces the soluble protein amount in the pea. Reduction in protein content is a common response to salinity stress. The reason for this is that amino acids in proteins react with active radical and degrade. High concentrations of NaCl (100 and 150 mM) caused a significant reduction in the total soluble protein content of cowpea leaves according to their controls. Protein degradation in a saline environment may originate from reduction in protein synthesis, increased proteolysis, and reduction in amino acid availability, and denaturation of enzymes involved in protein synthesis, or the reaction of amino acid of proteins with active radical and degradation. Although the highest increase in the soluble protein amount was seen in maize plants in stress conditions, the highest growth inhibition was also observed in maize. As a result, there was no correlation between the degree of maize susceptibility and the soluble protein amount in the root and stems in all stress conditions.

Table 2. Effects of salt (50 and 100 mM NaCl), cold stress (12/7ºC) and cross stress (Cold + NaCl (50 and 100 mM)) on the soluble protein content (mg g^{-1} FW) of plants with different seed nutrient content

	MAIZE		BEAN		WHEAT	
Applications	**Protein**	**Change %**	**Protein**	**Change %**	**Protein**	**Change %**
Control	1.146		1.996		1.270	
50 mM NaCl	1.576	37.5	2.000	0.2	1.463	15.2
100 mM NaCl	1.690	47.5	1.987	-0.5	1.499	18
Cold (12/7ºC)	1.562	36.3	1.990	-0.3	1.527	20.2
Cold+50 mM NaCl	1.490	30 -5.5 -4.6	1.975	-1.1 -1.3 -0.8	1.431	12.7 -2.2 -6.3
Cold+100 mM NaCl	1.550	35.3 -8.3 -0.8	1.982	-0.7 -0.3 -0.4	1.435	13 -4.3 -6

Under normal conditions, ROS is effectively scavenged by antioxidant systems. Nevertheless, very low concentrations of ROS assume a positive function as signaling molecules that cause the formation of defense responses in plants. However, when plants are exposed to environmental stresses such as salinity and cold, the antioxidant system cannot prevent the formation of excess ROS (such as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-)). Excess ROS produced in cells triggers phytotoxic reactions such as lipid peroxidation, protein degradation and DNA mutation causing cellular damage (Bezirganoglu et. al., 2018). The capacity of plants to withstand stress conditions often depends on their ability to activate an adequate antioxidant response. Preservation of the redox balance is a prerequisite for the development of tolerance against both biotic and abiotic stresses (Jiang *et al.*, 2012). Some plant species have the potential to protect cellular systems from the effects of this ROS by increasing the activity of enzymatic (SOD, CAT, POD) enzymes and non-enzymatic (ascorbate and glutathione) substances (Agarwal and Pandey, 2004).

Superoxide dismutase (SOD, EC 1.15.1.1) is a metallic enzyme that catalyzes the conversion to the less toxic H_2O_2 by protecting the plant against the harmful effects of superoxide radical ($O_2^{\bullet-}$) in chloroplast ($2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$). Compared to plants in terms of SOD activity in 50 mM NaCl stress; SOD activity increased in maize and wheat, while decreased in bean. SOD activity increased in maize while decreased in bean and wheat in 100 mM NaCl stress (Table 3). These results are consistent with studies showing that SOD activity increases in tomato, wheat and pea plants under salt stress (Doğan et. al., 2010; Jahantigh et. al., 2016; Öztürk et. al., 2012). The increase in SOD activity in the leaves exposed to salt stress may be due to activation of pre-existing SOD or synthesis of new SOD under salt conditions. SOD activity in cold stress increased in maize while decreased in bean and wheat. Previous studies have reported increased SOD activity in cold stress (Erdal et. al., 2015; Bezirganoglu et. al., 2018). It is reported that SOD activity decreases in cold tolerant barley and increases in cold sensitive barley (Mutlu et. al., 2016). The decrease in SOD activity and excessive $O_2^{\bullet-}$ formation may be one of the main factors which causes metabolic deterioration in cold stress. In the case of cross stress (Cold + NaCl (50 and 100 mM)), SOD activity increased in maize compared to control while decreased in bean and wheat. In the case of cross stress, SOD activity was reduced in maize, beans and wheat according to alone 50 mM NaCl stress. It increased in bean but decreased in maize and wheat according to alone 100 mM NaCl stress. In cold+50 mM NaCl cross stress condition, SOD activity was decreased bean, maize and wheat according to cold stress alone (12/7 ºC). In cold+100 mM NaCl cross stress condition, SOD activity increased in bean while decreased in maize and wheat according to cold stress alone (12/7ºC).

Table 3. Effects of salt (50 and 100 mM NaCl), cold stress (12/7ºC) and cross stress (Cold + NaCl (50 and 100 mM)) on the SOD activities (U mg−1 protein) in plants with different seed nutrient content

	MAIZE		BEAN		WHEAT	
Applications	**SOD Activity**	**Change %**	**SOD Activity**	**Change %**	**SOD Activity**	**Change %**
Control	195		340		208	
50 mM NaCl	262	34.6	315	-7.4	213	2.4
100 mM NaCl	268	37.4	296	-13	182	-12.5
Cold (12/7ºC)	267	37	305	-10.3	168	-19.2
Cold+50 mM NaCl	251	28.7 -4.2 -6	290	-14.7 -8 -5	145	-30.3 -32 -13.7
Cold+100 mM NaCl	213	9.2 -20.5 -20.2	308	-9.4 4.1 1	109	-47.6 -40.1 -35.1

Catalase (CAT, EC 1. 11.1.6) is the most effective enzyme to prevent oxidative damage by breaking down H_2O_2 in peroxisomes and glioxisomes in all living things. CAT converts H_2O_2, H_2O and molecular O_2 in peroxisomes. Since CAT is an unstable enzyme, it is determined that it can be inhibited by H_2O_2 when exposed to high light intensity and stress. All stress conditions (Cold, 50 and 100 mM NaCl and Cold + NaCl (50 and 100 mM)) were determined to increase CAT activity in maize leaves. It was determined that CAT activity increased as salt concentration increased in cold stress (Table 4). Previous studies have reported that salt and cold stress increase CAT activity (Demir and Öztürk, 2004; Esim et. al., 2014; Bezirganoglu et. al., 2018). All stress conditions (Cold, 50 and 100 mM NaCl and Cold + NaCl (50 and 100 mM)) were generally determined to inhibit CAT activity in bean leaves (except for cold + 50 mM NaCl). It was reported that salt and cold stress inhibits CAT activity (Mutlu et. al., 2013; Öztürk et al., 2012; Keles and Oncel, 2002). It was determined that CAT activity decreased as salt concentration increased in cold stress. Stress conditions (Cold, 50 and 100 mM NaCl) increased CAT activity in wheat leaves. But CAT activity in cases of cross stress (Cold + NaCl (50 and 100 mM)) decreased as salt concentration increased in cold stress.

Table 4. Effects of salt (50 and 100 mM NaCl), cold stress (12/7°C) and cross stress (Cold + NaCl (50 and 100 mM)) on the CAT activities (U mg−1 protein) in plants with different seed nutrient content

	MAIZE		BEAN		WHEAT	
Applications	**CAT Activity**	**Change %**	**CAT Activity**	**Change %**	**CAT Activity**	**Change %**
Control	0.020		0.344		0.100	
50 mM NaCl	0.045	125	0.326	-5.2	0.125	25
100 mM NaCl	0.084	320	0.290	-15.7	0.120	20
Cold (12/7°C)	0.037	85	0.287	-16.6	0.110	10
Cold+50 mM NaCl	0.051	155 13.3 37.8	0.302	-12.2 -7.4 5.2	0.103	3 -17.6 -6.4
Cold+100 mM NaCl	0.070	250 -16.7 89.2	0.255	-26 -12.1 -11.2	0.105	5 -12.5 -4.6

Peroxidase (POD, EC 1.11.1.7) is an enzyme involved in various defense mechanisms, including lignification, auxin metabolism, salt tolerance and heavy metal stress, and protects the cell against oxidative damage. Therefore, POD is often used as a parameter to determine changes in growth and metabolism under environmental stress conditions. It has been determined that the activity of peroxidases, which are related to physiological events and played an active role in metabolism, have increased its activity under very different stresses. Stress conditions (Cold, 50 and 100 mM NaCl) increased POD activity in maize leaves but significantly reduced activity in cross stress conditions (Cold + NaCl (50 and 100 mM)) according to controls. It was determined that POD activity decreased as salt concentration increased in cold stress. It was determined that 50 and 100 mM NaCl stress inhibited POD activity in bean leaves, and cold stress alone caused an increase whereas cross stress (Cold + NaCl (50 and 100 mM)) caused generally a significant decrease in the activity according to controls. It was determined that POD activity decreased as salt concentration increased in cold stress. Stress conditions (Cold, 50 and 100 mM NaCl) inhibited POD activity in wheat leaves, and cross stress conditions (Cold + NaCl (50 and 100 mM)) showed statistically significant more decrease in the activity according to controls. It was determined POD activity decreased as salt concentration increased in cold stress (Table 5). Previous studies have reported that salt and cold stress increase POD activity (Demir and Öztürk, 2004; Öztürk et. al., 2012; Turk et. al., 2014). It was suggested that POD activity reduced in cold stress (Erdal et. al., 2015).

Table 5. Effects of salt (50 and 100 mM NaCl), cold stress (12/7°C) and cross stress (Cold + NaCl (50 and 100 mM)) on the POD activities (U mg−1 protein) in plants with different seed nutrient content

	MAIZE		BEAN		WHEAT	
Applications	**POD Activity**	**Change %**	**POD Activity**	**Change %**	**POD Activity**	**Change %**
Control	1460		57.500		1424	
50 mM NaCl	2394	64	46.000	-20	1280	-10.1
100 mM NaCl	3052	109	32.700	-43.1	928	-34.8
Cold (12/7°C)	1940	33	58.600	2	703	-50.6
Cold+50 mM NaCl	2130	46 -11 9.8	34.900	-39.3 -24.1 -40.4	700	-50.8 -45.3 -0.43
Cold+100 mM NaCl	1880	28.8 -38.4 -3.1	32.800	-43 0.3 -44	776	-45.5 -16.4 10.4

Ascorbate peroxidase (APX) (EC 1.11.1.11) has an important role in defense against ROS in many organisms (such as plants, algae, whips). APX family has a higher affinity to H_2O_2 than CAT. APX protects cells against H_2O_2 not only under normal conditions, but also under stress conditions (Öztürk et al., 2012). It was determined that all stress conditions (Cold, 50 and 100 mM NaCl and Cold + NaCl (50 and 100 mM)) increased APX activity in maize leaves. In the cold stress, APX activity increased as salt concentration increased. In general, stress conditions (50 and 100 mM NaCl and Cold + NaCl (50 and 100 mM)) inhibited APX activity in bean leaves (except for cold stress) according to controls (Table 6). It was also found out that APX activity decreased as salt concentration increased in cold stress. It was determined that salt stress (50 and 100 mM NaCl) increased APX activity in wheat leaves, and cold stress inhibited the activity. Cross stress conditions (Cold + NaCl (50 and 100 mM)) resulted in a significant decrease in activity relative to salt stress and an increase in cold stress. APX activity was inhibited as salt concentration increased in cold stress. Previous studies have explained increased APX activity in salt and cold stress conditions (Doğan et. al., 2010; Turk et. al., 2014; Erdal et. al., 2015; Bezirganoglu et. al., 2018). Reduced APX activity in salt and cold stress conditions has also been reported in previous studies (Öztürk et. al., 2012; Lukatkin, 2002).

Table 6. Effects of salt (50 and 100 mM NaCl) and cold stress (12/7ºC) on the APX activities (U mg−1 protein) in plants with different seed nutrient content

	MAIZE		BEAN		WHEAT	
Applications	**APX Activity**	**Change %**	**APX Activity**	**Change %**	**APX Activity**	**Change %**
Control	0.179		0.257		0.250	
50 mM NaCl	0.236	31.8	0.196	-23.7	0.306	22.4
100 mM NaCl	0.283	58.1	0.190	-26.1	0.326	30.4
Cold (12/7ºC)	0.242	35.2	0.283	10.1	0.211	-15.6
Cold+50 mM NaCl	0.245	37 3.8 1.2	0.185	-28 -5.6 -34.6	0.270	8 -11.8 28
Cold+100 mM NaCl	0.292	63.1 3.2 20.7	0.175	-32 -8 -38.2	0.255	2 -21.8 21

Conclusion

As a result; salt, cold and cross stress inhibited root-stem growth of maize, bean and wheat plants. Cross stress has more inhibitory effect than alone salt or cold stress. On the basis of the plant variety, the most inhibition was seen in maize, wheat and bean, respectively. The total soluble protein content increased in maize and wheat while decreased in bean in all applications. Cross-stress, on the other hand, decreased the soluble protein content according to alone salt or cold stress in all plants. No cross-tolerance was observed between the amount of total soluble protein and root-stem growth. Antioxidant enzyme activities (SOD, CAT, POD and APX) increased in maize while decreased in bean. In wheat, CAT and APX activities increased while SOD and POD decreased. In the cases of cross stress, it has shown increases and decreases compared to plant types. No cross-tolerance was observed among root-stem growth, antioxidant enzyme activities and different seed nutrient content in all plants. For example, the highest increase in SOD, CAT, POD and APX activities was observed in maize, while root-stem growth decreased most in maize.

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RESEARCH ARTICLE

Effects of Lovastatin Suplemented Diet on Laying Performance, Egg Quality, Yolk Lipid Profile and Some Serum Parameters in Laying Hens

Özlem Ekinci[1] • Adem Kaya[2] • Hatice Kaya[2] • Nurinisa Esenbuğa[2*] • Muhlis Macit[2]

[1]*Ministry of Agriculture and Forestry, General Directorate of Livestock, Department of Poultry, Lagomorphs and Apiculture, Ankara/Turkey*
[2]*Atatürk University, Faculty of Agriculture, Department of Animal Breeding & Husbandry, Erzurum/Turkey*

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ABSTRACT

This study was carried out to determine the effects of lovastatin supplementation on laying performance, egg quality, yolk lipid profile and some serum parameters in Lohmann LS white commercial laying hens reared in poultry houses of Food and Livestock Application and Research Center of Ataturk University. In this experiment, Lohmann layers (n=48, 46 wks of age) were randomly divided into two groups such as control (C) fed with basal diet and treatment (L) group fed with diet including 0,0059 % of lovastatin. After one week of the adaptation period, experiment lasted for five weeks. During the experimental period, hens were fed as ad-libitum and water through nipples was available for all the times. Lovastatin supplementation increased feed consumption (FC) and feed conversion ratio (FCR). Except for yolk color, other egg quality traits were not affected by diet including 0,0059 % of lovastatin. Hens fed with treatment diet had greater triglyceride and phosphatidyl serine values than hens fed with basal diet. Differences between the groups in terms of the levels of egg yolk and serum cholesterol were not significant in present study. These differences could be attributed to short experimental period and low lovastatin added to basal diet of hens. In conclusion, further studies should be conducted to clarify the effects of lovastatin supplementation on laying performance, egg quality, yolk lipid profile and some serum parameters in laying hens fed with diets including lovastatin at different levels during long feeding period.

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Introduction

Egg is one of the most important foods with its high protein value, rich in vitamins and minerals and low in calorie. Scientific and technological developments in poultry have enabled the egg to be produced in abundant and economical ways in recent years, but egg consumption has not been reached of the desired level because of cholesterol content. Egg contain about 200 mg of cholesterol and is considered a major source of dietary cholesterol (Çakır and Yalçın 2004; Elkin et al., 1999; Mori et al., 2000; Kim et al., 2004).

Egg cholesterol level is influenced by genetic, age and nutritional factors. Nutritional factors, such as type of fat, dietary fiber, the amount of vitamin C, can affect egg cholesterol level (Naber, 1976; Çakır and Yalçın 2004). There

* Corresponding author
E-mail address: esenbuga@atauni.edu.tr

are many studies dealt with the effect of genetic selection and various dietary factors on egg cholesterol levels. However, it is extremely difficult to reduce egg cholesterol levels by dietary manipulations. Genetic selection programs have resulted in only modest changes in egg cholesterol levels (Elkin and Rogler 1990; Mori et al., 2000).

Therefore, much attention has been focused on the use of several pharmacological agents to reduce the cholesterol content of eggs. Lovastatin, simvastatin, and atorvastatin have been shown to reduce egg cholesterol content as well as liver and plasma cholesterol concentration (Luhman et al. 1990; Elkin et al., 1999; Mori et al., 1999; Kim et al., 2004).

Lovastatin is a statin drug, used for lowering cholesterol in those with hypercholesterolemia to reduce risk of cardiovascular diseases. It acts as competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) and it inhibits cholesterol synthesis by making an inhibitory effect (Elkin and Rogler 1990; Mori et al., 1999; Kim et al., 2004) .

This study was carried out to determine the effects of lovastatin supplementation on laying performance, egg quality, yolk lipid profile and some serum parameters in Lohmann LS white commercial laying hens.

Materials and Methods

This research was carried out at Atatürk University Food and Livestock Application and Research Center in accordance with permit of Local Ethic Committee on Animal Experiment considering the project (BAP 2002/12) supported by Ataturk University in Erzurum, Turkey (39°55'N, 41°16'W).

In this experiment, Lohmann layers (n=48, 46 wks of age) were randomly divided into two groups such as control and treatment, and placed into cages (50x46x46 cm, widthxdepthxheight). Each group was replicated in 6 cages, 4 hens per cage. The control (C) and treatment (L) were fed with basal diet in mash form and diet including 0,0059 % of powdery lovastatin, respectively. Firstly, a premixture including basal feed and lovastatin at recommended proportion on prospectus was prepared in a mixer and then this homogenized premixture was added into basal feed in feed unit. After one week of the adaptation period, experiment lasted for five weeks. During the experimental period, hens were fed as ad-libitum and water through nipples was available for all the times. Hen house was lit for 17h. The experimental diet in mash form (16.4% CP, 2670 Kcal ME/kg) was obtained from a commercial feed mill in Erzurum.

Egg production and feed consumption were measured daily, egg weight was measured biweekly and body weight was measured at the beginning and the end of the experiment. Eighteen eggs from each group were taken and stored for 24 h at room temperature at the beginning and end of the experiment to determine egg quality parameters such as shape index, shell strength, shell thickness, yolk index, albumen index and Haugh unit. Yolk color was estimated according to the CIE standard colorimetric system (Yolk Colour Fan, the CIE standard colorimetric system, F. Hoffman-La Roche Ltd., Basel, Switzerland). After 4wk of lovastatin administration, the blood samples were taken from the wing vena (10 hens) using heparinized tubes for blood parameters. In addition, ten eggs from each group were collected at the end of the experimental period to determine yolk lipid profiles by the HPTLC methods (Hara and Radin 1978; Macala et al. 1983). The yolk lipids were seperated into following classes: cholesteryl ester (CE), triglyceride (TG), free fat acid (FFA), cholesterol (COL), phosphatidylserine (PS) ve phosphatidylcholine (PC).

Performance and egg quality characteristics were tested with One-way ANOVA. t-test was used for egg lipid profile and blood parameters. All statistical analyses have been made with the SPSS 10.01 (SPSS 1996) package software.

Results and Discussion

The effects of lovastatin on laying performance are presented in Table 1. As seen in Table 1, the highest feed consumption and FCR were observed in the lovastatin group. Differences between the groups were significant ($p<0.05$). Although the effect of time on feed consumption was found significant ($p<0.01$), the effect of the group x time interaction was insignificant. However, Elkin and Rogler (1990) and Kim et al (2004) did not observe significant change in feed consumption and FCR among groups.

Table 1. The effect of lovastatin on laying performance

	C	L				
	Mean	Mean	SEM	Group	Time	CxL
Feed consumption (g/d)	95.16	103.12	2.76	*	**	ns
Egg production (%)	72.37	69.43	2.74	ns	**	ns
Egg weight (g)	66.01	64.61	0.84	ns	ns	ns
FCR (kg feed/kg egg)	1.86	2.25	0.20	*	ns	*
Cracked egg yield (%)	4.41	10.80	1.96	ns	ns	ns

*(P<0.05), **(P<0.01), ns: non significant

The differences in egg production, egg weight and cracked egg yield between groups were insignificant. The lowest egg production was observed in the lovastatin group compared to the control group. Elkin and Rogler (1990) reported that lovastatin had no effect on egg production, egg weight and cracked egg yield. The findings of Luhman et al (1990) and Kim et al (2004) also supported the findings obtained from present study.

Shape index, shell strength, shell thickness, shell weight, yolk color, yolk index, albumin index and haugh unit were determined as egg quality traits of laying hens (Table 2).

It was determined that except for yolk color there was no effect on the shape index, shell strength, shell thickness, shell weight, yolk index, albumin index and Haugh unit. The laying hens fed with diet including lovastatin produced eggs with yolk color significantly higher than the control. The effect of time on yolk color was significant ($p<0.05$). Results related to yolk color were similar with the findings of Mori et al (2000), they reported that drug addition did not affect the shell weight, shell thickness, albumin and shell quality.

Table 2. The effect of lovastatin on egg quality traits of laying hens

	C	L				
	Mean	Mean	SEM	Group	Time	CxL
Shape index (%)	73.72	74.73	1.07	ns	ns	ns
Shell strenght (kg/cm^2)	0.57	0.474	0.096	ns	ns	ns
Shell thickness (mm×10^{-2})	0.34	0.337	0.014	ns	*	ns
Shell weight (g)	6.97	7.28	0.25	ns	ns	ns
Yolk color	7.21	8.05	0.18	**	*	ns
Yolk index (%)	38.90	39.79	0.88	ns	ns	ns
Albumen index (%)	7.45	8.77	0.64	ns	ns	ns
Haugh unit	77.70	83.03	2.64	ns	ns	ns

*(P<0.05), **(P<0.01), ns: non significant

The egg yolk lipid profile of the eggs collected at the end of the experiment are given in Table 3. There was no difference among groups except for TG and PS. The differences between the groups in terms of TG and PS were significant ($p<0.05$). Elkin and Rogler (1990) reported that by adding lovastatin in the amount of 0.059-0.0265%, egg cholesterol could be reduced by 15.5%. Luhman et al (1990) observed that relatively low doses of lovastatin or colestipol did not reduce the egg yolk cholesterol. Kim et al (2004) found that oral intake of 0.06% provastatin reduced egg cholesterol by 20% when compared to control group. Mori et al (2000) reported that lovastatin had no significant effect on egg yolk cholesterol. The lack of significant differences in present study may be due to the low proportion of lovastatin.

Table 3. The effect of lovastatin on egg yolk lipid profiles of laying hens

	C	L	P
	Mean±SEM	Mean±SEM	
CE (%)	4.87±0.80	4.32±0.60	ns
TG (%)	56.22±0.62	60.38±1.58	*
FFA (%)	0.39±0.11	0.32±0.03	ns
COL (%)	20.41±0.51	20.23±0.41	ns
PS (%)	0.28±0.01	0.56±0.08	*
PC (%)	6.34±0.36	5.31±0.46	ns

CE, cholesteryl ester; TG, Triglyceride; FFA, Free fat acid; COL, cholesterol; PS, Phosphatidylserine; PC, Phosphatidylcholine
*(P<0.05), **(P<0.01), ns: non significant

Table 4. The effect of lovastatin on serum parameters of laying hens

	C	L	P
	Mean±SEM	Mean±SEM	
Uric acit (µmol/L)	7.43±0.48	4.40±0.37	**
Total protein (g/L)	4.71±0.29	4.95±0.21	ns
Albumin (g/L)	1.40±0.08	1.56±0.10	ns
Globulin (g/L)	3.31±0.24	3.39±0.12	ns
Alkalin phosphatase (U/L)	11164.00±749.83	6114.00±1589.62	*
Triglycerides (mg/L)	874.00±148.94	1230.50±236.86	ns
Cholesterol (mmol/L)	97.00±13.069	124.00±10.78	ns
HDL (g/L)	16.50±3.26	17.50±2.84	ns
VLDL (g/L)	174.83±29.79	246.00±47.40	ns
LDL (g/L)	89.00±28.55	46.50±3.85	ns

*(P<0.05), **(P<0.01), ns: non significant

There were no statistically differences total protein, albumin, globulin, triglycerides, cholesterol, HDL, VLDL and LDL values between the groups ($p>0.05$) (Table 4). The mean values of uric acid and alkalin phosphatase in lovastatin group were lower than in the control group ($p<0.05$ and $p<0.01$, respectively). Uric acid, a nitrogenous end-product of amino acid and purine catabolism. In brief, it is the main end-product of N metabolism in birds. Alkalin phosphatase (ALP) is an enzyme found in intestinal contents and tissues such as liver, bone, and kidney which are sources of plasma alkalin phosphatase, and helps to breakdown of proteins in animal body. The low ALP value in the blood serum is not a disease or a case. Lovastatin from statins group used to decrease the levels of cholesterol induces a decline in the serum uric acide and ALP values (Alberts, 1998). But, the mechanism by which lovastatin effects on low uric acid and ALP values in the blood serum of laying hens is not completely known. Elkin et al. (1999) reported that VLDL did not affect cholesterol levels in

lovastatin or simvastatin, these researchers' findings were similar with results obtained from present study. Although Mori et al. (2000) found that lovastatin with 0.005% tended to decrease the mean of triglyceride (14.9%) and total cholesterol (10.1%), Mori (2000) observed that 0.001% lovastatin caused a significant reduction of triglyceride (38.5%) and cholesterol (36.5%) levels. Kim et al. (2004) reported that 0.08% of lovastatin decreased the plasma total cholesterol concentration by 28% compared to the control group. Similar to the present study, Elkin and Rogler (1990) and Hugget et al (1993) found that plasma cholesterol and triglyceride concentrations were not affected of lovastatin.

In this study, laying performance, egg quality traits, egg yolk lipid profile and some blood parameters were examined. It has been determined that the results related to egg yolk and serum cholesterol parameters, performance and egg quality traits from present study are different when compared to the findings of other studies. Lovastatin did not affect egg quality traits except for yolk color. Feeding with relatively low doses of lovastatin did not affect serum and egg yolk cholesterol parameters.

Conclusion

In conclusion, further studies should be conducted to clarify the effects of lovastatin supplementation on laying performance, egg quality, yolk lipid profile and some serum parameters in laying hens fed with diets including lovastatin at different levels during long feeding period.

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RESEARCH ARTICLE

Effect of Garlic (*Allium sativum* L.) on the Microbiological, Chemical and Sensorial Quality of Smoked Atlantic Mackerel (*Scomber scombrus* Linnaeus, 1758) Stored in Vacuumed Packets at Refrigerator Temperature (+4ºC)

Hünkar Avni Duyar* • Zafer Hakan Kalaycı • Sabri Bilgin

Sinop University, Faculty of Fisheries, Department of Fisheries and Fish Processing, Sinop/Turkey

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ABSTRACT

In this study, the effect of garlic (*Allium sativum* L.) on the microbiological, chemical and sensorial quality of smoked Atlantic Mackerel (*Scomber scombrus* Linnaeus, 1758) stored in vacuumed packets at refrigerator temperature (+4ºC) were investigated. The results of total volatile basic nitrogen (TVB - N) in control group (without garlic contains 10% salt) showed that the product reached the limit of consumable value on the 43rd day (TVB - N = 36.45±1.133 mg / 100 g) and on the 67th day in the garlic group (with 2% garlic contains 10% salt) (TVB - N = 35.69±1.026 mg / 100 g) ($P < 0.05$). It was observed that garlic group values were higher than control group in terms of sensory analysis (texture, appearance, odour and taste). However, it was determined that the value of taste did not fall below the consumable value in both groups. These results showed that the shelf life of smoked Atlantic Mackerel was 24 days longer with garlic application and the garlic supplement increased both the shelf life of the product and gave sensory appreciation to the product.

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Introduction

Atlantic Mackerel (*Scomber scombrus* Linnaeus, 1758) abundant in cold and temperate shelf areas, forms large schools near the surface. They overwinter in deeper waters but move closer to shore in spring when water temperatures range between 11°C and 14°C. Mainly diurnal, it feeds on zooplankton and small fish. The species is traded fresh, frozen, smoked and canned (Froese & Pauly, 2018).

Smoking is a traditional preserving method used for both fish and meat products around the world and also smoked fish products have wide acceptance today due to their accustomed taste and aroma as well as longer shelf life as a result of the combined effects of dehydration, antimicrobial and antioxidant activities of several smoke constituents mainly: formaldehyde, carboxylic acids and phenols (Doe, 1998). Smoking gives the special color and flavor to the food and extends its shelf life via the effects of dehydration, antimicrobial and antioxidant of the smoke compounds. Smoking also changes the texture of product. Several factors contribute to the quality and safety of such products. These factors are the quality of raw material, salt concentration used water activity of the final product, heat through the smoking process, the quantity of smoke, packaging methods, hygienic circumstances and storage conditions (Koral et al., 2010).

* Corresponding author
E-mail address: had052@gmail.com

Atlantic Mackerel used in this study is an important pelagic fish caught in the North Atlantic including the Mediterranean with high economic value (Froese & Pauly, 2018). The fat content is about 6-23%, water content is 56-74% and protein content is 18-20 % throughout the year (NOAA, 2014). It is considered one of the more healthy fish because it is rich in omega-3 fatty acids and an excellent source of selenium, niacin, and vitamins B6 and B12 (NOAA, 2014). Generally, it is sold freshly and frozen, but it is exported from Norway to Turkey as frozen and consumed by Turkish people as frozen.

The use of synthetic antioxidant has been very effective in controlling food rancidity. However, synthetic antioxidants have frequently been associated with certain health problems (Pakawatchai et al., 2009). This has necessitated the use of natural antioxidants, such as ginger (Iheagwara, 2013; Frank et al., 2014), garlic (Duyar et al., 2016), in the prevention of rancidity in smoked fish. It is also important to apply herbal preservatives and sweeteners to extend the fish flavor and shelf life (Balıkçi, 2009; Frank et al., 2014; Duyar et al., 2016).

Garlic is the edible bulb from a plant of the Allium (onion) genus, commonly used for flavoring in cooking and for its beneficial effects for human health. Garlic is among the oldest of all cultivated plants. It has been used as a medicinal agent for thousands years. This remarkable plant has multiple beneficial effects such as antimicrobial, antithrombotic, hypolipidemic, antiarthritic, hypoglycemic, antitumor and antioxidant activities. A large number of studies have demonstrated the antioxidant activity of garlic by using. It grows in many parts of the world and is a popular ingredient in cooking due to its strong smell and delicious taste. It is high in a sulfur compound called allicin, which is believed to bring most of the health benefits. However, throughout ancient history, the main use of garlic was for its health and medicinal properties. Garlic not only possesses antibacterial, antifungal and antimicrobial properties, but also its consumption positively influences human circulatory and immune system and hence has a wide range of health benefits (Banerjee & Maulik, 2002, Păcurar & Krejci, 2010). It has been proven effective as a hypolipidemic, antimicrobial, antihypertensive, hepatoprotective, and insecticidal agent in various human and animal therapies (Shakya & Labh, 2014). Nowadays people are very conscious of consuming natural products that no longer contain chemical preservatives. This has led researchers to work on the use of natural materials such as garlic instead of chemicals to produce new flavors and to ensure shelf life for products that are produced.

The aim of this study was to determine the effect of garlic (*Allium sativum* L.) on the microbiological, chemical and sensorial quality of smoked Atlantic Mackerel (*Scomber scombrus* Linnaeus, 1758) stored in vacuumed packets at refrigerator temperature (+4ºC).

Materials and Methods

Fish Samples

Atlantic Mackerel (*Scober scombrus*) exported from Norway to Turkey, were bought from Sinop fishermen in boxes as frozen blocks. The mean length of the *S. scombrus* used in the research is 35 ± 3 cm and the mean weight is 350±20 g (n = 200).

Preparation of fish samples

Gutting was carried out manually after beheading and washing in running tap water. Fish were divided into 2 groups as control group (without garlic) and garlic group (with garlic) to be transferred to salt solution. Two salted brines were prepared for salting before smoked. For the control group, a salt solution of 10% (10 g salt to 100 ml fresh water) was prepared with 1/3 (fish / salt). For garlic supplement group; 10% salt solution was prepared and crushed garlic pieces were added so that the brine ratio would be 2% to prepare the fish to stand.

Salting before hot smoke

The first group (control group) prepared for smoked; it was left for one hour on salt water containing 10% salt. The second group of smoked samples (garlic group) was stored for one hour in salt water containing 10% salt and 2% garlic. At the end of the one hour, the fish were removed from the salt water and washed to remove excess salt under the tap water. Then, the fish were hung with hooks, lined up at appropriate intervals in the smoke oven, kept at room temperature for 20 minutes to drain excess water. After this process, smoking process was started

Smoking

The smoking process was carried out in a semi-mechanical smoke oven by hot smoke method. In the production of smoke, sawdust obtained from beech wood is used. The smoking process was carried out in three stages: In the first step, at 30°C for 40 minutes it was expected hardened skin. In the second step, lightly soaked sawdust was placed in a metal tray and the fishes were allowed to cook for 60 minutes at 50°C in sawdust smoke. In the third and last stage, the temperature was raised to 80°C and smoke was made for 45 minutes. Smoked fishes were thawed 30 minutes at room temperature. Later, remove the filets of fishes for packing and stocking.

Packing and stocking

From each sample prepared for packaging, each fillet was packed with a vacuum packed device (Abant brand, MG 42 model table top vacuum machine) by placing a polyethylene vacuum bag. The vacuum packed filet is stored in the refrigerator (4 ± 1°C) during the study.

pH Determination

10 grams of smoked fish samples were weighed and 20 ml of purified water was added. These samples were homogenized for 1 minute and then pH measurements were made by immersing the pH-meter probe in the homogenized fish (Vural & Oztan, 1996). Smoked fish are food items that are quickly defeated. The pH values of smoked fish range from 5.4 to 6.9 (Varlık, 2004).

Water Activity (aw)

Nova AG, LabSwift-aw that Automatic water activity machine was used for determination of water activity.

Determination of Total Volatile Basic Nitrogen (TVB-N)

The TVB-N assay was performed according to the Lücke-Geidel method modified by Antonacopoulus and the results were given as mg / 100g (Varlik et al., 1993). A 10 g fragmented sample was placed in a balloon. Then add 1 g of magnesium oxide (MgO) and a few drops of silicone oil and some purified water to prevent foaming and also 10 ml of 3% boric acid (H_3BO_3), 8 drops of tashiro indicator mixture and about 100 ml of purified water were added to the 500 ml Erlenmeyer used as a titration vessel. The sample was placed in a balloon, dish and other balloon heater with pure water, and then subjected to distillation for 15-20 minutes by connecting to the coolant tap. The resulting distillate was titrated with 0.1 N hydrochloric acid (HCl) and the total volatile basic nitrogen content was calculated according to the following formula (Inal, 1992, Varlik et al., 1993):

TVB-N (mg/100 g) = Spent (HCl) x (0.0014008) x (100) x (1000) / Sample amount (g)

There are differences according to TVB-N value in the classification of the quality of fishery products according to various researchers. The TVB-N values obtained in our study were evaluated based on the quality values of Varlık et al. (1993).

Determination of Thiobarbituric Acid (TBA)

The number of thiobarbituric acid was determined according to Erkan & Özden (2008). The homogenized sample was weighed 1.90-2.00 g (± 0.01) into the tube and 100 µl (0.10% = 1 g / l of ethanol prepared BHT) was added. 16-25 ml of TCA (trichloracetic acid) (5%) was added to it and ultra-thoracic was removed and filtered through filter paper. After placing 5 ml of the filtrate on the tube, the same amount of TBA reagent (0.02 mol / l of 10% glacial acetic acid prepared, weighing 0.2883 g for 100 cc) was added. The tubes were held in a water bath at 70-80°C for 30 minutes and after the tubes had been cooled, they were read against the corpuscles in a spectrophotometer set to a wavelength of 532 nm.

Microbiological Analyzes

For microbiological analyzes, samples were taken from aseptic conditions from two packets of each group. 10 g of fish meat samples were weighed and added with 90 ml of 0.85% sterile saline (8.5 g NaCl, 1000 ml of purified water). The pre-sterilized homogenizer was homogenized and diluted 10^{-1}. Dilutions in the form of dilution solution consisting of 1 ml dilution + 9 ml saline were formed from the homogenized sample. Diluted dilutions of up to 10 were then made, and two parallel runs from each dilution and microbiological analyzes were made using the bulk plate method (Baumgart, 1986, Varlik et al., 1993). Following the incubation, petri dishes were counted and the results were given as log cfu / g.

Total mesophilic aerobic bacteria (TMAB) and total psychrophilic aerobic bacteria (TPAB) count

Plate Count Agar (PCA) medium was used for total mesophilic aerobic bacteria and total psychrophilic aerobic bacteria counts. Samples from smoked fish samples were taken under aseptic conditions and weighed 10 g. The weighed sample was disintegrated and homogenized in sterile disruptor with 90 ml of 0.85% physiological salt water (8.5 g NaCl, 1000 ml distilled water). The first dilution (10^{-1}) was performed with this procedure (Gurgun and Halkman, 1990). Dilutions of 10^{-2}, 10^{-3} and 10^{-4} were then prepared using 0.85% physiological salt water as diluent fluid. Sowing was done by transferring 1 ml of sterilized-end automatic pipettes (eppendorf, 1000 µL) from each of the dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} ratios. Plate Count Agar (PCA) broth which has been sterilized and cooled to 44-46 ° C was added 10-15 ml by pouring method to petri dish boxes and mixed (Roger et al., 1987, Göktan, 1990). The petri dishes were then incubated for 3 days at 28°C in an incubator for total mesophilic aerobic bacteria counts and 10 days at 7°C for total psychrophilic aerobic bacteria counts (Roger et al., 1987, Göktan, 1990). Then, total mesophilic and psychrophilic aerobic bacteria counts were calculated (Gurgun & Halkman, 1990).

Total yeast and mold count (TYM)

Potato Dextrose Agar (PDA) is used for the cultivation of fungi. Potato Dextrose Agar (PDA) is a general purpose medium for yeasts and molds that can be supplemented with acid or antibiotics to inhibit bacterial growth. Potato Dextrose Agar medium was used for yeast and mold count. Dilutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} ratios were prepared from homogenized smoked fish samples. Sowing was performed by transferring 1 ml of eppendorf (1000 µL) into 2 sterile empty petri dishes from each of dilutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} ratios. Sterilized petri dishes were placed at a temperature of 44-46 ° C and poured into the PDA medium by pouring 10-15 ml. After the fermentation, the petri dishes were inverted and the number of yeast and mold was counted after incubation for 3 days at 28°C (Roger et al., 1987; Göktan, 1990; Varlık et al., 1993).

Total coliform bacteria count

Dilutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} ratios were prepared from homogenized smoked fish samples. Sowing was performed by transferring 1 ml of eppendorf (1000 µL) into 2 sterile empty petri dishes from each of dilutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} ratios. Sowing petri dishes were placed at a temperature of 44-46°C with 10-15 ml of Violet Red Agar (VRBA) medium and than the petri dishes were inverted after the supernatant and incubated for 1 day at 35°C (Roger et al., 1987; Gökalp et al., 1999).

Sensory Analysis

For sensory analysis, smoked fish were placed on labeled plates and presented to panelists. The panelist group of six people evaluated the appearance, smell, taste and texture of the fish. A score of 5 was considered the limit value and below this value was considered not to be consumed (Koral, 2006).

Panelists were asked to make a good assessment from 1 to 10 points in terms of point criteria by the modified method of Koral (2006). Panelists evaluated the sensory analysis using a 0-10 hedonic scale (10-8: very good; 8-6: good; 6-5: consumable and <5: spoilage)

Statistical Analysis

Significant differences between two groups were determined using the t test or/and Mann-Whitney test of PAST software with a significance level of P < 0.05.

Results

Chemical Contents

Total volatile basic nitrogen and thiobarbituric acid results during the storage days are shown in Table 1 and Figure 1. The TBA values increased both control and garlic groups depending on storage days and the TBA values did not exceed the consumable limit in both groups during the storage times (Table 1, Figure 1). The TBA values between two groups were not significantly different until the 7th days (P > 0.05). From day 7 to day 43, the difference between the two groups was statistically significant (P < 0.05).

The TVB – N values was higher in control group than garlic group during the storage times and the TVB – N values increased regularly both groups depending on storage days. The TVB – N values between two groups were not significantly different on days 0th, 16th, 19th and 25th days (*P* > 0.05). On the other days of storage, the difference between the TVB – N values of two groups was found to be statistically significant (*P* < 0.05). The results of the TVB – N in control group showed that the product reached the limit of consumable value on the 43rd day (TVB – N = 36.45±1.133 mg / 100 g) and on the 67th day in the garlic group (TVB – N = 35.69±1.026 mg / 100 g).

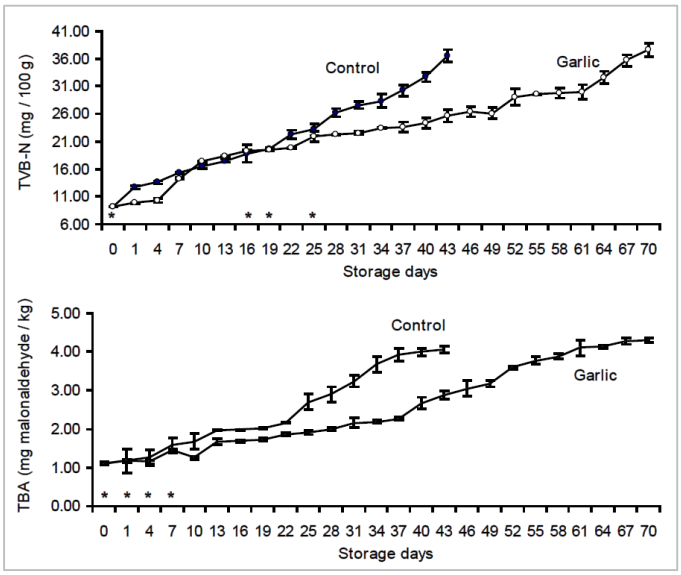

Figure 1. Total volatile basic nitrogen (TVB-N) and thiobarbituric acid (TBA) results during the storage days. The days marked with stars are statistically insignificant between two groups (P > 0.05)

Table 1. Total volatile basic nitrogen (TVB – N) and thiobarbituric acid (TBA) results during the storage days

Storage Days	Total volatile basic nitrogen (TVB – N) (mg / 100 g)		Thiobarbituric acid (TBA) (mg malonaldehyde / kg	
	Control	Garlic	Control	Garlic
0	9.20±0.026[a]	9.20±0.072[a]	1.11±0.031[a]	1.11±0.040[a]
1	12.65±0.265[a]	9.89±0.118[b]	1.17±0.306[a]	1.18±0.038[a]
4	13.59±0.361[a]	10.32±0.427[b]	1.26±0.200[a]	1.15±0.041[a]
7	15.29±0.087[a]	14.27±0.115[b]	1.58±0.210[a]	1.44±0.035[a]
10	16.47±0.347[a]	17.34±0.066[b]	1.68±0.215[a]	1.26±0.039[b]
13	17.49±0.262[a]	18.27±0.111[b]	1.97±0.020[a]	1.66±0.083[b]
16	18.80±1.600[a]	19.32±0.100[a]	1.98±0.010[a]	1.69±0.042[b]
19	19.64±0.120[a]	19.55±0.183[a]	2.01±0.031[a]	1.73±0.040[b]
22	22.32±0.763[a]	19.81±0.092[b]	2.15±0.012[a]	1.86±0.046[b]
25	23.16±1.037[a]	21.96±0.900[a]	2.69±0.212[a]	1.91±0.061[b]
28	26.20±0.780[a]	22.23±0.108[b]	2.90±0.200[a]	1.99±0.038[b]
31	27.57±0.626[a]	22.48±0.274[b]	3.23±0.153[a]	2.15±0.122[b]
34	28.31±1.222[a]	23.46±0.089[b]	3.67±0.218[a]	2.19±0.040[b]
37	30.28±1.041[a]	23.58±0.976[b]	3.93±0.164[a]	2.26±0.036[b]
40	32.70±0.872[a]	24.32±0.930[b]	4.00±0.092[a]	2.67±0.142[b]
43	36.45±1.133[a]	25.63±1.110[b]	4.06±0.076[a]	2.88±0.101[b]
46		26.40±0.917		3.05±0.200
49		26.06±1.005		3.17±0.083
52		28.97±1.487		3.60±0.040
55		29.55±0.092		3.78±0.101
58		29.82±0.890		3.88±0.061
61		29.98±1.271		4.10±0.200
64		32.58±1.037		4.13±0.040
67		35.69±1.026		4.27±0.092
70		37.59±1.254		4.31±0.061

The different superscript on the same line represent statistical differences between the two groups (P < 0.05).
Analyzes were performed in triplicate (n = 3).

aW and pH Values

Water activity (aw) and pH fluctuations during the storage days are shown in Table 2 and Figure 2. Although small statistically significant increases and decreases were detected pH values between two groups during storage ($P < 0.05$), the pH value was found to be within the range of pH = 6 and 6.5 values recommended for freshness (Table 2, Figure 2). The average water activity (aW) values was found between 0.97±0.003 and 0.99±0.003 in control and garlic groups during the storage days (Table 2, Figure 2).

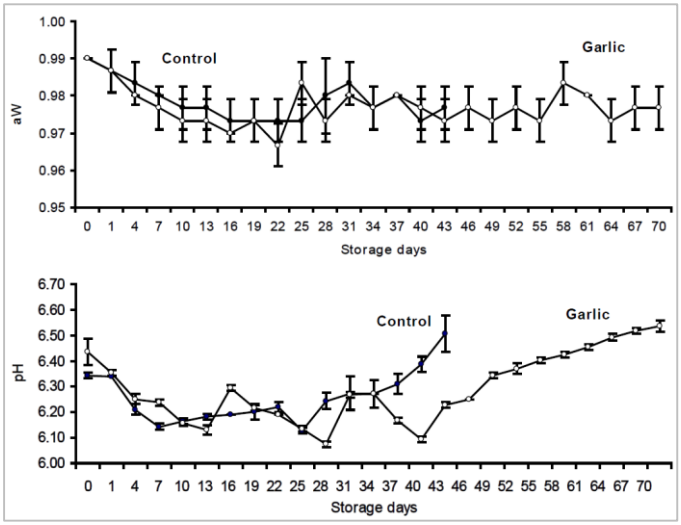

Figure 2. Water activity (aW) and pH fluctuations during the storage days

Table 2. Water activity (aw) and pH results during the storage days

Storage Days	aw		pH	
	Control	Garlic	Control	Garlic
0	0.99±0.000[a]	0.99±0.000[a]	6.34±0.003[a]	6.44±0.102[a]
1	0.99±0.003[a]	0.99±0.003[a]	6.34±0.000[a]	6.35±0.003[a]
4	0.98±0.003[a]	0.98±0.000[a]	6.21±0.006[a]	6.25±0.006[b]
7	0.98±0.000[a]	0.98±0.003[a]	6.14±0.003[a]	6.24±0.003[b]
10	0.98±0.003[a]	0.97±0.003[a]	6.16±0.003[a]	6.16±0.003[a]
13	0.98±0.003[a]	0.97±0.003[a]	6.18±0.003[a]	6.13±0.006[b]
16	0.97±0.003[a]	0.97±0.000[a]	6.19±0.000[a]	6.29±0.003[b]
19	0.97±0.003[a]	0.97±0.003[a]	6.20±0.002[a]	6.22±0.003[a]
22	0.97±0.003[a]	0.97±0.003[a]	6.22±0.006[a]	6.19±0.000[b]
25	0.97±0.003[a]	0.98±0.003[a]	6.13±0.032[a]	6.13±0.003[a]
28	0.98±0.006[a]	0.97±0.003[a]	6.24±0.009[a]	6.07±0.003[b]
31	0.98±0.003[a]	0.98±0.000[a]	6.27±0.019[a]	6.27±0.003[a]
34	0.98±0.003[a]	0.98±0.003[a]	6.27±0.015[a]	6.27±0.000[a]
37	0.98±0.000[a]	0.98±0.000[a]	6.31±0.012[a]	6.17±0.003[b]
40	0.97±0.003[a]	0.98±0.003[a]	6.39±0.009[a]	6.09±0.003[b]
43	0.98±0.003[a]	0.97±0.003[a]	6.51±0.020[a]	6.23±0.003[b]
46		0.98±0.003		6.25±0.000
49		0.97±0.003		6.34±0.003
52		0.98±0.003		6.37±0.006
55		0.97±0.003		6.40±0.003
58		0.98±0.003		6.42±0.003
61		0.98±0.000		6.45±0.003
64		0.97±0.003		6.49±0.003
67		0.98±0.003		6.52±0.003
70		0.98±0.003		6.54±0.007

The different superscript on the same line represent statistical differences between the two groups ($P < 0.05$).
Analyzes were performed in triplicate (n = 3).

Microbiological Contents

Total psychrophilic aerobic bacteria (TPAB), total mesophilic aerobic bacteria (TMAB), total coliform bacteria and total yeasts and molds (TYM) results are shown in Table 3 and Figure 3. Total psychrophile aerobics bacteria (TPAB) spillage was not detected in the first 4 days of storage in both groups. No statistical difference could be detected between the two groups on the 7th, 10th, 19th, 22nd, 28th and 31st days of the storage period ($P > 0.05$). However, on the 13th, 16th, 31st, 34th, 37th and 40th days of storage, the total psychrophilic aerobics bacteria count of the control group was found to be statistically higher than the garlic group ($P < 0.05$).

The total number of aerobic mesophilic bacteria (TMAB) showed a steady increase in the storage process in both groups. The TMAB values was found to be 6.63±0.090 log cfu / g in the control group and 4.40±0.062 log cfu / g in the garlic group on the 43rd day of the storage period. This value was found as 6.26±0.029 log cfu / g in the garlic group on the 70th day. The difference in total mesophilic aerobic bacteria count between control and garlic groups during storage was found to be statistically insignificant ($P > 0.05$).

Total yeast and mold (TYM) values were found to be 4.55±0.030 log cfu / g in the control group and 4.09±0.079 log cfu / g in the garlic group on the 43rd day of storage. This value was determined as 5.34±0.075 log cfu / g in the garlic group on the 70th day. The TYM value of the garlic group was statistically higher than that of the control group until the 13th day of the storage period ($P < 0.05$), whereas the values of the control group after the 13th day were statistically higher than the values of the garlic group ($P < 0.05$).

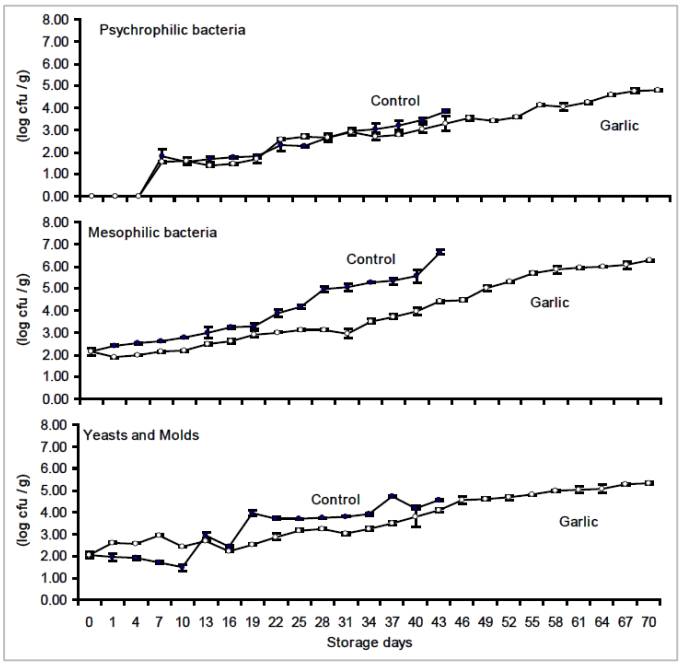

Figure 3. Microbiological fluctuations during the storage days. Vertical lines refer to standard erros (S.E.)

Table 3. Total psychrophilic aerobic bacteria (TPAB), total mesophilic aerobic bacteria (TMAB), total coliform bacteria and total yeasts and molds (TYM) results

Storage Days	Total psychrophilic aerobic bacteria (log cfu/g)		Total mesophilic aerobic bacteria (log cfu/g)		Total coliform bacteria (log cfu/g)		Yeasts and Molds (log cfu/g)	
	Control	Garlic	Control	Garlic	Control	Garlic	Control	Garlic
0	<1.47	<1.47	2.13±0.173^{a}	2.13±0.153^{a}	<1.47	<1.47	2.05±0.050^{a}	2.05±0.150^{a}
1	<1.47	<1.47	2.41±0.029^{a}	1.88±0.035^{b}	<1.47	<1.47	1.95±0.182^{a}	2.59±0.030^{b}
4	<1.47	<1.47	2.51±0.031^{a}	1.99±0.031^{b}	<1.47	<1.47	1.92±0.075^{a}	2.57±0.033^{b}
7	1.83±0.297^{a}	1.57±0.020^{a}	2.60±0.030^{a}	2.15±0.046^{b}	<1.47	<1.47	1.72±0.059^{a}	2.94±0.060^{b}
10	1.54±0.045^{a}	1.60±0.175^{a}	2.76±0.028^{a}	2.19±0.052^{b}	<1.47	<1.47	1.47±0.141^{a}	2.42±0.017^{b}
13	1.68±0.119^{a}	1.39±0.036^{b}	2.99±0.242^{a}	2.48±0.030^{b}	<1.47	<1.47	2.94±0.121^{a}	2.67±0.035^{b}
16	1.78±0.045^{a}	1.48±0.035^{b}	3.22±0.075^{a}	2.61±0.079^{b}	<1.47	<1.47	2.42±0.060^{a}	2.23±0.036^{b}
19	1.80±0.069^{a}	1.70±0.176^{a}	3.28±0.114^{a}	2.92±0.142^{b}	<1.47	<1.47	3.95±0.135^{a}	2.50±0.046^{b}
22	2.30±0.225^{a}	2.57±0.035^{b}	3.87±0.173^{a}	3.00±0.017^{b}	<1.47	<1.47	3.72±0.062^{a}	2.87±0.148^{b}
25	2.28±0.045^{a}	2.69±0.076^{b}	4.17±0.079^{a}	3.11±0.030^{b}	<1.47	<1.47	3.69±0.032^{a}	3.16±0.063^{b}
28	2.67±0.113^{a}	2.65±0.173^{a}	4.97±0.131^{a}	3.13±0.046^{b}	<1.47	<1.47	3.73±0.029^{a}	3.23±0.067^{b}
31	2.95±0.045^{a}	2.90±0.178^{a}	5.06±0.159^{a}	2.95±0.198^{b}	<1.47	<1.47	3.80±0.030^{a}	3.02±0.062^{b}
34	3.05±0.224^{a}	2.70±0.175^{b}	5.28±0.030^{a}	3.51±0.096^{b}	<1.47	<1.47	3.90±0.058^{a}	3.23±0.078^{b}
37	3.20±0.226^{a}	2.80±0.070^{b}	5.33±0.131^{a}	3.71±0.105^{b}	<1.47	<1.47	4.72±0.063^{a}	3.48±0.079^{b}
40	3.45±0.090^{a}	3.04±0.185^{b}	5.55±0.285^{a}	3.94±0.171^{b}	<1.47	<1.47	4.18±0.061^{a}	3.80±0.477^{b}
43	3.85±0.045^{a}	3.28±0.350^{b}	6.63±0.090^{a}	4.40±0.062^{b}	<1.47	<1.47	4.55±0.030^{a}	4.09±0.079^{b}
46		3.53±0.105		4.48±0.060		<1.47		4.56±0.159
49		3.41±0.036		5.02±0.131		<1.47		4.62±0.062
52		3.59±0.035		5.30±0.030		<1.47		4.67±0.096
55		4.13±0.038		5.69±0.063		<1.47		4.80±0.017
58		4.05±0.175		5.85±0.171		<1.47		4.98±0.046
61		4.24±0.070		5.94±0.033		<1.47		5.03±0.156
64		4.59±0.034		5.99±0.030		<1.47		5.07±0.193
67		4.77±0.107		6.04±0.165		<1.47		5.28±0.045
70		4.79±0.035		6.26±0.029		<1.47		5.34±0.075

The different superscript on the same line represent statistical differences between the two groups ($P < 0.05$).
Analyzes were performed in triplicate (n = 3).

Sensory Analysis

Changes in sensory analysis (texture, appearance, odour and taste) scores during the storage time are shown in Table 4 and Figure 4. It was determined that garlic group values were higher than control group in terms of sensory analysis (texture, appearance, odour and taste) during storage. Texture, appearance and odour values were determined as 10 in the first 4 days in both groups. On the assessment of texture, the control group decreased below the consumable value on the 40th day ($P < 0.05$) and the garlic group on the 61st day. These results were found to be lower than the consumable limit value of the garlic group on the 55th day and the control group on the 43rd day ($P < 0.05$) after evaluation on the odour. However, it was determined that the value of taste did not fall below the consumable value in both groups. According to the results of this study, it was determined that the control group fell below the consumable limit value on the 40th day ($P < 0.05$). According to the results of the sensory evaluation mentioned above, it was determined that appearance values of the control group dropped below the consumption limit value (5) on the 40th day and the garlic group on the 70th day.

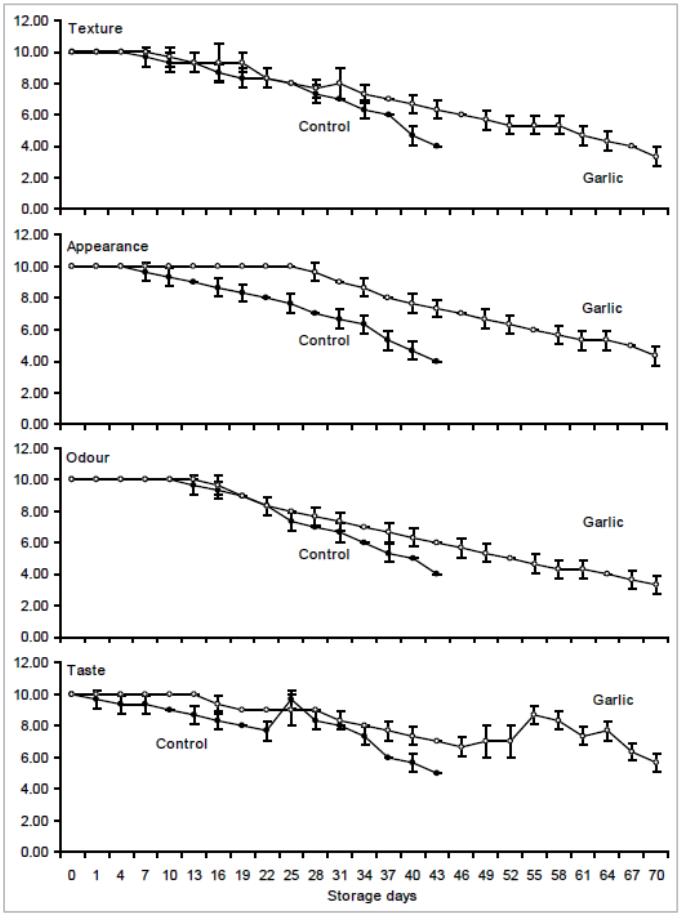

Figure 4. Changes in sensory analysis (texture, appearance, odour and taste) scores during the storage days. Vertical lines refer to standard error

Table 4. Changes in sensory analysis (texture, appearance, odour and taste) scores

Storage Days	Texture		Appearance		Odour		Taste	
	Control	Garlic	Control	Garlic	Control	Garlic	Control	Garlic
0	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a
1	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	9.67±0.333^a	10.00±0.000^a
4	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	9.33±0.333^a	10.00±0.000^a
7	9.67±0.333^a	10.00±0.000^a	9.67±0.333^a	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	9.33±0.333^a	10.00±0.000^a
10	9.33±0.333^a	9.67±0.333^a	9.33±0.333^a	10.00±0.000^a	10.00±0.000^a	10.00±0.000^a	9.00±0.000^a	10.00±0.000^a
13	9.33±0.333^a	9.33±0.333^a	9.00±0.000^a	10.00±0.000^a	9.67±0.333^a	10.00±0.000^a	8.67±0.333^a	10.00±0.000^a
16	8.67±0.333^a	9.33±0.667^a	8.67±0.333^a	10.00±0.000^a	9.33±0.333^a	9.67±0.333^a	8.33±0.333^a	9.33±0.333^a
19	8.33±0.333^a	9.33±0.333^a	8.33±0.333^a	10.00±0.000^a	9.00±0.000^a	9.00±0.000^a	8.00±0.000^a	9.00±0.000^a
22	8.33±0.333^a	8.33±0.333^a	8.00±0.000^a	10.00±0.000^a	8.33±0.333^a	8.33±0.333^a	7.67±0.333^a	9.00±0.000^a
25	8.00±0.000^a	8.00±0.000^a	7.67±0.333^a	10.00±0.000^a	7.33±0.333^a	8.00±0.000^a	9.67±0.333^a	9.00±0.577^a
28	7.33±0.333^a	7.67±0.333^a	7.00±0.000^a	9.67±0.333^a	7.00±0.000^a	7.67±0.333^a	8.33±0.333^a	9.00±0.000^a
31	7.00±0.000^a	8.00±0.577^a	6.67±0.333^a	9.00±0.000^a	6.67±0.333^a	7.33±0.333^a	8.00±0.000^a	8.33±0.333^a
34	6.33±0.333^a	7.33±0.333^a	6.33±0.333^a	8.67±0.333^b	6.00±0.000^a	7.00±0.000^b	7.33±0.333^a	8.00±0.000^a
37	6.00±0.000^a	7.00±0.000^a	5.33±0.333^a	8.00±0.000^b	5.33±0.333^a	6.67±0.333^b	6.00±0.000^a	7.67±0.333^a
40	4.67±0.333^a	6.67±0.333^b	4.67±0.333^a	7.67±0.333^b	5.00±0.000^a	6.33±0.333^b	5.67±0.333^a	7.33±0.333^a
43	4.00±0.000^a	6.33±0.333^a	4.00±0.000^a	7.33±0.333^b	4.00±0.000^a	6.00±0.000^b	5.00±0.000^a	7.00±0.000^b
46		6.00±0.000		7.00±0.000		5.67±0.333		6.67±0.333
49		5.67±0.333		6.67±0.333		5.33±0.333		7.00±0.577
52		5.33±0.333		6.33±0.333		5.00±0.000		7.00±0.577
55		5.33±0.333		6.00±0.000		4.67±0.333		8.67±0.333
58		5.33±0.333		5.67±0.333		4.33±0.333		8.33±0.333
61		4.67±0.333		5.33±0.333		4.33±0.333		7.33±0.333
64		4.33±0.333		5.33±0.333		4.00±0.000		7.67±0.333
67		4.00±0.000		5.00±0.000		3.67±0.333		6.33±0.333
70		3.33±0.333		4.33±0.333		3.33±0.333		5.67±0.333

The different superscript on the same line represent statistical differences between the two groups ($P < 0.05$).
Analyzes were performed in triplicate (n = 3).

Discussion

TVB – N analysis is a criterion of the products of microbial and enzymatic degradation of protein and non-protein nitrogen compounds in fish meat and it is one of the most important parameters used in determining the quality of fresh and processed fish meat. Quality classification for TVB – N values (Varlık et al., 1993): samples containing 25 mg / 100 g TVB – N are very good, samples containing 30 mg / 100 g TVB – N are good, samples containing 30 - 35 mg / 100 g TVB – N are marketable, more than 35 mg / 100 g TVB – N are considered spoilt. Studies on TVB - N content have reported that fish shelf life is different for different fish species stored by different processing methods. Namely, Rainbow trout (*Oncorhynchus mykiss*) was salted at different rates and smoked hot and stored under refrigerator conditions and the TVB-N value of the 6% salted product reached 35.0 mg / 100 g at the end of the 87th day (Unal, 1995). In a study investigating the shelf life of a hot smoked bonito stored at refrigerator temperature, the TVB-N value reached 41.82±1.20 mg / 100 g on the 13th day of storage (Koral et al., 2010). Dondero et al. (2004) investigated the quality parameters by smoked salmon by the method of cold smoke and packed in vacuum and stored at different temperatures, shelf life has been determined as 46 days at the lowest temperature (0ºC) and 19 days at the highest temperature. TVB-N value of garfish meat smoked and stored in refrigerator conditions was determined as 37.47 mg / 100 g on 25th day and also the TVB-N value of garfish meat smoked and stored in room conditions was 38.87 mg / 100 g on the 9th day (Koral et al., 2009). In the present study, the results of TVB – N in control group showed that the product reached the limit of consumable value on the 43rd day and on the 67th day in the garlic group. The TVB - N results obtained in this study showed that the garlic treatment extended the mackerel shelf life by 24 days. When the TVB-N values obtained in this study were compared with other research results, TVB-N values increased similarly to other studies due to storage time (Muratore & Licciardello 2005; Bilgin, 2003; Bilgin et al 2007; Ünal, 1995). Furthermore, the reasons for the differences in the studies may be due to differences in packaging and storage conditions, different fish species, quality of raw material, salt concentration and duration, application of drying process and smoke density etc.

The value of TBA in the meat is a result of oil oxidation and is considered as one of the most important criteria of quality parameters (Varlık et al., 1993). A large part of the changes related to the degradation of fish occurs with the oxidation and disintegration of oils, resulting in a loss of the painful organoleptic value (Varlık et al., 1993). Schormuller (1969) reported that the amount of TBA used in determining the degree of oxidation in aquaculture should not be less than 3 mgMDA / kg in a very good material, not more than 5 in a good material, and a limit value of 7-8 mgMDA / kg in a good material and the aquatic products above this value are evaluated as spoilt. The TBA values obtained in our study were evaluated based on the quality values of Schormuller (1969).

It has been reported that there is no significant difference between the groups in terms of the TBA values after storage of hot smoked and vacuum packed bonito (*Sarda sarda*) on the refrigerator temperature for 60 days (Duyar et al., 2008). Yanar et al. (2006) examined the TBA value in smoked tilapia (*Oreochromis niloticus*) by hot smoked method using different salt solutions and at the end of the study, it was reported that the TBA values in all groups increased regularly according to the storage period in refrigerator conditions and this increase was directly proportional to the increase in salt concentration. In a study examining the quality parameters of goldfish stored at 4°C with hot smoke, the TBA value was reported to increase to 6.32 mg malonaldehyde / kg on the 28th day of storage (Ünlüsayın et al., 2001). In the present study, the TBA values increased both control and garlic groups depending on storage days and the TBA values did not exceed the consumable limit in both groups. As regards the TBA values reported in previous studies, numerical differences were observed as well as similarities with various research results. The reported differences in TBA between studies may be due to differences in raw materials used, differences in different smoke and storage conditions. In addition, it can be deduced that the garlic used in this study is the positive effect of antioxidant.

It has been reported that the pH value should be between 6.0 and 6.5 for freshness and between 6.8 and 7.0 for consumption limit value (Varlık et al., 1993). In this study, increases and decreases in pH value were determined. However, it has been found at the appropriate intervals of 6.0-6.5 recommended for freshness.

Microbiological parameters are important in determining the shelf life and quality of smoked products (Hansen et al., 1995). Total mesophilic aerobic bacteria (TMAB) and total psychrophile aerobic bacteria (TPAB) are important indicators in determining the microbiological properties of cold stored smoked products. These bacteria play an effective role in the degradation of hot and cold smoked products. In smoked products, the reported microbiological limit of consumption for these bacteria is 6 log cfu / g (Olafsdottir et al., 2005). Generally, when the total bacterial load is being assessed, acceptable microbiological lower limit for fish and fish products is reported as 6 log cfu / g and upper limit 7 log cfu / g (ICMSF, 1986). When the microbiological analysis values obtained in our study were compared with the results of previous studies, coliform bacteria encounter and storage-related bacterial growth were parallel to the results of the previous study (Balıkçı, 2009). When considering the numbers of yeast and mold with TMAB, TPAB, it is generally thought that the differences between the studies may be due to smoke type, sawdust type and raw material.

In this study, sensory evaluation results were regularly decreased in control and garlic groups depending on the storage period. But, the taste values increased on the 25th day in the control group and on the 55th day in the garlic group. This may be due to increasing Inosine monophosphate (IMP). The IMP is responsible for sweetness and characteristics associated with fresh fish muscle (Howgate, 2006). The garlic group showed better results than the control group in all four parameters (texture, appearance, odour and taste). The reason why the value of garlic group in the present study is lower than the control group; it is thought to be caused by the

combination of vitamin C and Organosulfur compounds, which are antioxidant properties found in the content of garlic.

Conclusion

From the above results, it can be concluded that garlic provide antioxidant and antimicrobial benefits to smoked Atlantic Mackerel stored in vacuumed packets at refrigerator temperature (+4ºC). Therefore, it is suggested that garlic as a natural herb, could be used to extend the shelf life of meat products, providing the consumer with food containing natural additives, which might be seen more healthful than those of synthetic origin. Further research is required to focus on understanding the mechanisms of action, in particular concentrations of active ingredients of garlic in either powder or fresh form which applied to smoked fish products.

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RESEARCH ARTICLE

Determination of Amino Acids Composition in Different Tissues of Whiting, *Merlangus merlangus euxinus* (Nordmann, 1840) from the Black Sea, Turkey

Özlem Bilgin[1*] • Uğur Çarlı[1] • Selahattin Erdoğan[1] • Murat Emrah Maviş[2] • Gökçe Göksu-Gürsu[2] • Muhittin Yılmaz[3]

[1]*Sinop University, Scientific and Technological Researches Application and Research Center (SUBITAM), Sinop/Turkey*
[2]*Sem Laboratuvar Cihazları Pazarlama San. ve Tic. Inc. R&D Center, Istanbul/Turkey*
[3]*Sinop University, Faculty of Engineering and Architecture, Department of Bioengineering, Sinop/Turkey*

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ABSTRACT

LC-MS/MS was used to determine the amino acid composition in muscle, ovary and liver of whiting, *Merlangus merlangus euxinus*, caught off the coast of Sinop province in the Black Sea. A total of 19 amino acids (AA) were found in the different samples. The essential amino acids (EAA) in the different tissues of whiting were 55.9% in the meat, 54.8% in the ovary and 51.7% in the liver of the total amino acids. The AA contents except for Arg, Glu, Pro and Tau in meat and ovary of whiting were not significantly different ($P \geq 0.05$), but the AA contents except for Cys, Tyr, Asp, Orn and Tau of these two tissues were significantly ($P < 0.05$) higher than the AA contents of the liver. These results showed that whiting ovaries have approximately as much protein as the meat.

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Introduction

Whiting, *Merlangus merlangus euxinus* (Nordmann, 1840), is one of the most important commercial demersal fish species in the Black Sea off the coast of Turkey and mainly fished by bottom trawl during autumn and winter and by gillnets throughout the year (Bilgin *et al.*, 2012). In the Black Sea reproduction activity of this species continues during the year and intensive spawning occurs at last three times a year: at the end of summer, in mid-autumn and in early winter (Bilgin *et al.*, 2012). Mazlum and Bilgin (2014) reported that food consumption was intense during spring and summer. The meat of this fish species is consumed locally throughout the year usually by cooking in oil (personal observation).

Seafoods are valuable sources of protein, fatty acids, minerals and vitamins (Tilami and Sampels, 2017). The taste of fish meat is closely related to the biochemical composition especially the protein content (Tilami and Sampels, 2017; Doğan and Ertan, 2017) and the biochemical composition of fish meat can be affected by water temperature and nutrients in the environmental (Doğan and Ertan, 2017). It was reported that the chemical composition of different fish species depends on variables such as season, sexual maturity,

* Corresponding author
E-mail address: ozlmbilgin28@gmail.com

reproduction time and the nutrient cycle (Limin *et al*., 2006; Özden and Erkan, 2011; Doğan and Ertan, 2017).

Amino acids AA may also be responsible for some of the taste and flavor of fish (Doğan and Ertan, 2017). Fish meat is also considered to be a good source of essential amino acids (EAA), i.e., Arg, Cys, His, Iso, Leu, Lys, Met, Phe, Thr, Tyr and Val. They also contain measureable amounts of Orn and Tau which are not found in proteins (Kim and Lall, 2000; Limin *et al*., 2006; Adeyeye, 2009; Erkan *et al*., 2010ab; Özden and Erkan, 2011; Doğan and Ertan, 2017). It was reported that in fish, 50-80% of the non-protein nitrogenous compounds are AA and significant amounts of these AA are Glu, Arg, Lys, Pro and Tau (Ruiz-Capillas and Moral, 2001; Doğan and Ertan, 2017). Gln is a α-amino acid that is used in the biosynthesis of proteins and its side chain is similar to that of Glu, except the carboxylic acid group is replaced by an amide (Tapiero *et al*., 2002; Watford, 2015). It is classified as a charge-neutral, polar amino acid. Both glutamate and glutamine (Glx) are not considered essential amino acids but they play important roles in maintaining growth and health in both neonates and adults (Watford, 2015). Their chemical characteristics are very similar as well. Both contain nitrogen, belong to a carboxylic acid chemical group, and both glutamate and glutamine are alkaline. Glutamine and glutamate with Pro, His, Arg and Orn comprise 25% of the dietary amino acid intake and constitute the glutamate family of amino acids, which are disposed of through conversion to glutamate (Tapiero *et al*., 2002).

The AA contents different fish species' muscles have been studied using high pressure liquid chromatography (HPLC) (Antoine *et al*., 1999; Kinm and Lall, 2000; Limin *et al*., 2006; Adeyeye, 2009; Erkan *et al*., 2010a; Erkan *et al*., 2010b; Özden and Erkan, 2011; Doğan and Ertan, 2017. Although the fish liver and gonads (especially ovary) are important internal organs, they are not generally consumed. Note that: the ovaries when available of whiting are cooked in oil and consumed by people living in the Black Sea region (personal observation).

In previously studies, there is no investigation related to amino acids profile of different tissues of fish species in the Black Sea. Moreover, LC-MS/MS (Liquid Chromatography Mass Spectrometer) device is able to separate, identify and quantify the requested substance in a mixture at an advanced level (Anonymous, 2016) and in this study we used firstly the Using LC-MS/MS was used to determine the amino acid composition of the muscle, ovary and liver of whiting (*Merlangus merlangus euxinus*) caught along the Sinop coast of the Black Sea.

Materials and Methods

Samples

A total of 3 kg newly caught whiting in the Black Sea were obtained from local fishermen in February 2018 in the Sinop region. Newly caught whiting specimens were brought to the laboratory in ice. The total length of each whiting was measured with a sensitivity of 1 mm. Specimen, gonad and liver wet weights were obtained using a balance with a sensitivity of 0.001 g. The average total length and wet weight of whiting used were 14.0±0.2 cm and 18.7±0.7 g, respectively. The average Gonodosomatic index, GSI = [(gonad weight / total fish weight) x 100)] and the average Hepatosomatic index, HSI = [(liver weight / total fish weight) x 100)] were 4.9±0.4% for GSI (Note that the maturity stages of the ovary used included stage I, stage II and stage III ovaries with stage III being the most/least mature according to Bowers (1954) and 4.2±0.2% for the HSI. After the length and weight determination, the whole edible muscle and liver of both males and females and ovary of females was minced and homogenized using homogenizer and than stored in a freezer at -20ºC for 3 days.

Determination of Amino Acids

The amino acids analyzes of the samples were made in duplicate using the SUBITAM's (Sinop University Scientific and Technological Researches Application and Research Center) Agilent Infinity 1260 HPLC system consisting of a binary pump, a degasser and autosampler coupled with 6460 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA).

For the measurement of the amino acid concentrations, samples were prepared by using Jasem LC-MS/MS amino acid kit (Sem Laboratuvar Cihazları Pazarlama San. ve Tic. Inc. Istanbul/TURKEY) which included five standards for calibration, labelled stable isotope mixtures of each targeted amino acid as internal standard (IS) with exception of isoleucine and histidine (leucine IS and 3-Methylhistidine were assigned respectively), mobile phases, reagents, chromatographic and mass detection parameters of the method with modified sample preparation procedure comprising an acidic hydrolysis process. The concentrations of the targeted amino acids were determined using both electrospray ionization (ESI) and multiple reactions monitoring (MRM). In this section we specified general process, detailed settings information are in the related forthcoming paragraph with Table 1a and b. The samples were hydrolyzed as follows: 0.5 g of sample was hydrolyzed with 4 ml of acidic hydrolysis reagent in a screw capped glass tube for 24 h at 110ºC.

When the sample was cooled to room temperature (~24ºC), the hydrolyzed sample was centrifuged (Hettich Universal 320 desktop air cooled centrifuge) at 4000 rpm (g force: 3756.48) for 5 min. (No loss of sample solid phase remains at the bottom and necessary part is supernatant) After that, 10 µl of supernatant was transferred into a sample vial and completed to 1 ml with distilled water in order to obtain 800 fold diluted hydrolysates. Subsequent to the hydrolysis step, kit sample preparation procedures for calibration standards and samples were as follows: 50 µL of the standard or diluted hydrolysate was transferred into a sample vial. Next, 50 µL of the labeled stable isotopes mixture was added as an internal standard and 700 µL of reagent-1 were added to the sample vial before swirling for 5 sec.

HPLC system was operated to inject 3 µL of prepared sample into the Jasem analytical column specified for amino acid analysis (depending on the analysis kit) maintained at 30ºC. Chromatographic separation was carried out using Jasem's mobile phase A and B with gradient elution at a flow rate of 0.7 ml/min. The HPLC elution was as follows: the initial

LC gradient of 22% A was held for 1 min. Then, the gradient was ramped to 78% A in 3 min. and held for 0.5 min. Finally, the column was equilibrated at 22% A for 3 min. The total running time was 7.5 min. Mass spectrometric detection was performed on Agilent 6460 triple quadrupole MS equipped with an ESI source in the positive ion mode. The optimal MS detector settings were as follows: drying gas temperature 150°C, drying gas flow 10 L/min, nebulizer pressure 40 psi (Gauge-Nebulizing takes place in a chamber in which is under the atmospheric pressure not in the vacuum) and capillary voltage of 2000 V (+). The positive ESI mode was operated for the detection of amino acid and IS as protonated form ($m/z = [M+1]^+$). Collision-induced dissociation (CID) of this precursor ion produced one major product ion for each amino acid and IS. MRM transitions of the amino acid and corresponding IS (precursor ion to product ion) were monitored at optimum fragmentation voltages (FV) and optimum collision energies (CE) (Table 1a and 1b). The peak area ratio of the amino acid to the assigned IS was evaluated for quantification of targeted amino acid concentration.

Table 1a. MRM transitions of amino acids and conditions

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	FV (v)	CE (v)
Phenylalanine	166.1	120.1	80	6
Tyrosine	182.1	165	80	1
Methionine	150.1	104.1	80	4
Aspartic acid	134.1	74.1	90	10
Threonine	120.2	74.2	80	4
Serine	106.2	60.2	80	4
Alanine	90.2	44.2	80	4
Glycine	76.2	30.1	80	1
Proline	116.2	70.2	90	12
Cystine	241.1	74.2	100	24
Arginine	175.2	70.2	110	20
Histidine	156.1	110.1	100	8
Ornithine	133.2	70.3	80	14
Lysine	147.1	84.2	80	12
Glutamic acid	148.1	84.2	80	12
Leucine	132.2	43.3	100	24
Isoleucine	132.2	69.2	100	14
Valine	118.2	72.2	80	4

Table 1b. MRM transitions of amino acids and conditions

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	FV (v)	CE (v)
Phenylalanine IS	175.1	129.1	100	8
Tyrosine IS	192.1	145.2	80	8
Methionine IS	153.1	107.2	80	6
Aspartic acid IS	137.1	91.2	90	5
Threonine IS	121.1	75	80	6
Serine IS	109.1	63	90	8
Alanine IS	94.1	48.2	90	6
Glycine IS	78.2	31.3	90	4
Proline IS	122.1	75.2	90	14
Cystine IS	244.9	153.9	90	8
Arginine IS	177.2	70.2	110	20
3-Methyl histidine IS	173.2	127.2	80	10
Ornithine IS	138.2	74.2	80	16
Lysine IS	151.1	88.1	90	16
Glutamic acid IS	150.1	85.2	80	12
Leucine IS	142.2	96.3	120	6
Valine IS	126.1	80.2	80	8

Statistical Analysis

One-way ANOVA was used to determine the amino acids difference in different tissues of whiting. The statistical analyses were done using the software package PAST version 1.94b (Hammer *et al.*, 2001). Averages of significant variance sources were compared using Tukey's pair-wise comparisons test at a statistical significant level of 0.05.

Results

A total of 19 amino acids (AA) were detected in the different tissues of whiting samples (Table 2, Fig. 1). The essential amino acids (EAA) are Arg, Cys, His, Iso, Leu, Lys, Met, Phe, Thr, Tyr and Val. These EAA in different tissues of whiting constituted approximately 55.9% in meat, 54.8% in ovary and 51.7% in liver of total amino acids. The non-essential amino acids (NEAA) are Ala, Asp, Glu, Gly, Orn, Pro, Ser and Tau. The NEAA of whiting constituted about 44.1 per cent in meat, 45.2 per cent in ovary and 48.3 per cent in liver of total amino acids.

The EAA and the NEAA contents in different tissues of whiting are generally $AA_{Meat} \geq AA_{Ovary} > AA_{Liver}$ (Fig. 1). Namely, the NEAA; Asp, Glu, Ala and the EAA; Lys, Leu, Iso, Arg values were determined the higher values in meat > ovary > liver. Similar trends were obtained for other the EAA and the NEAA values (Fig. 1). It was found out that the most abundant the NEAA in meat, ovary and liver were Glu, Asp, Ala and Gly. Moreover, the most abundant the EAA in different tissues were determined as Lys, Leu, Iso, Arg and Val (Table 2). The amino acid contents except for Arg, Glu, Pro and Tau in meat and ovary of whiting determined statistically close to each other ($P > 0.05$), but the AA contents except for Cys, Tyr, Asp, Orn and Tau of these two tissues were determined statistically higher than AA contents of liver ($P < 0.05$) (Table 2).

Table 2. The mean values ± SE of amino acid in different tissues (meat, ovary and liver) on raw weight of Whiting (*Merlangus merlangus euxinus*) in the Black Sea

EAA/NEAA	Amino acids	Amino acid values in tissues (g/100 g)		
		Meat	Ovary	Liver
EAA	Arginine	1.29±0.010[a]	1.22±0.005[b]	0.44±0.005[c]
	Cystine	0.15±0.005[a]	0.14±0.030[a]	0.06±0.005[a]
	Histidine	0.00±0.000	0.28±0.280	0.00±0.000
	Isoleucine	0.97±0.135[a]	0.84±0.120[a]	0.28±0.030[b]
	Leucine	1.76±0.185[a]	1.74±0.145[a]	0.64±0.045[b]
	Lysine	2.25±0.270[a]	1.57±0.180[a]	0.55±0.095[b]
	Methionine	0.71±0.085[a]	0.61±0.070[a]	0.26±0.045[b]
	Phenylalanine	0.85±0.025[a]	0.92±0.025[a]	0.38±0.005[b]
	Threonine	0.88±0.040[a]	0.87±0.130[a]	0.27±0.065[b]
	Tyrosine	0.98±0.185[a]	0.72±0.135[a]	0.26±0.045[a]
	Valine	1.06±0.050[a]	1.05±0.020[a]	0.51±0.020[b]
Total (EAA)		11.695	10.89	3.915
NEAA	Alanine	1.34±0.030[a]	1.17±0.045[a]	0.46±0.010[b]
	Aspartic acid*	2.43±0.530[a]	2.11±0.375[a]	0.75±0.150[a]
	Glutamic acid*	3.34±0.075[a]	2.86±0.055[b]	1.08±0.020[c]
	Glycine	0.94±0.085[a]	1.15±0.040[a]	0.55±0.020[b]
	Ornitine	0.11±0.005[a]	0.12±0.000[a]	0.11±0.000[a]
	Proline	0.78±0.010[a]	1.06±0.010[b]	0.43±0.005[c]
	Serine	0.83±0.045[a]	0.95±0.050[a]	0.31±0.025[b]
	Taurine	0.29±0.020[a]	0.52±0.025[b]	0.29±0.005[a]
Total (NEAA)		9.215	8.970	3.660
Total (AA)		20.91	19.86	7.575

SE: standard error, AA: amino acid, EAA: essential amino acid, NEAA: non- essential amino acid. Values with different superscripts in same row are significantly different ($P < 0.05$). * Under the condition of acidic hydrolysis, glutamine and asparagine are entirely converted to glutamic acid and aspartic acid respectively

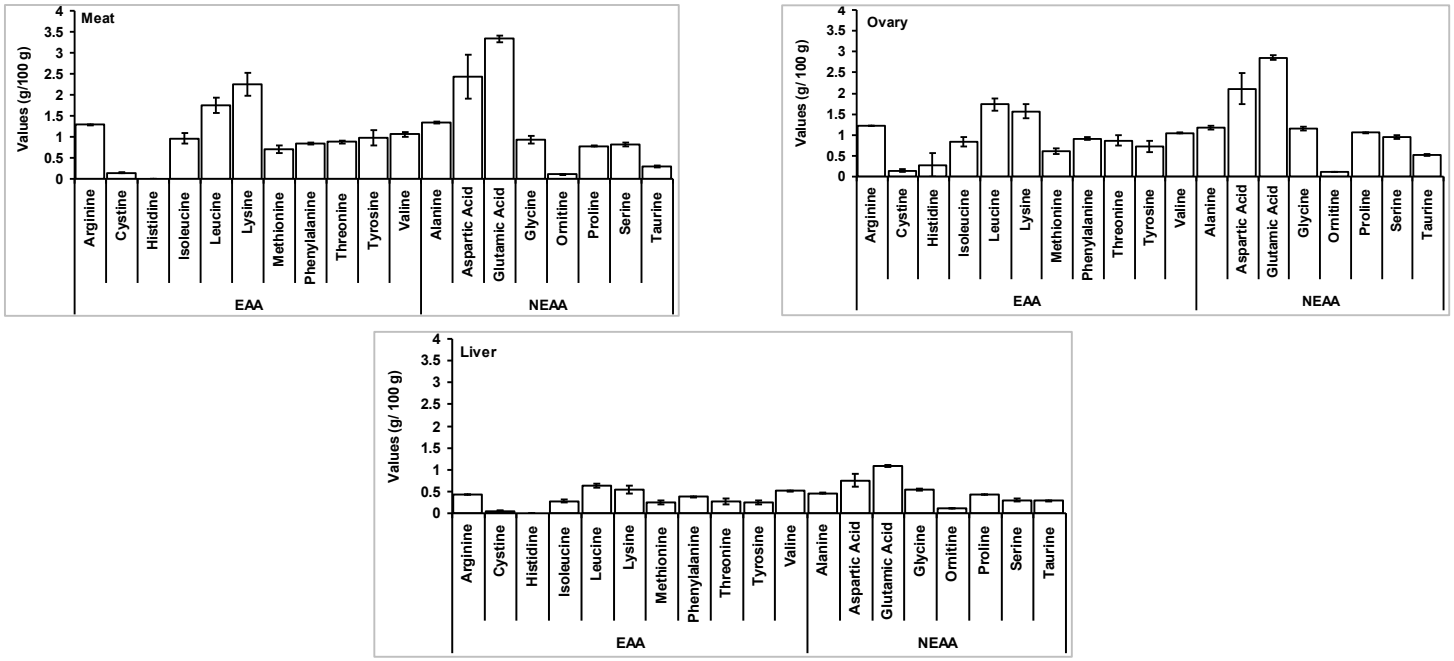

Figure 1. The amino acid composition in different tissues (meat, ovary and liver) on raw weight of whiting (*Merlangus merlangus euxinus*) in the Black Sea

Table 3. The five most abundant amino acids in muscle of different fish species from different geographical areas

Species	Origin	Five most abundant amino acids (AAs)	Ref.
Pleuronectes ferruginea	Aquaculture	Glutamic acid > aspartic acid > lysine > glycine > leucine	1
Hippoglossus hippoglossus	Aquaculture	Glutamic acid > aspartic acid > lysine > leucine > arginine	1
Paralichthys olivaceus	Aquaculture	Glutamic acid > aspartic acid > lysine > leucine > arginine	1
Pseudosciaena crocea	Xiamen aBay of China	Glutamic acid > aspartic acid > lysine > leucine > arginine	2
Lateolabrax japonicas	Xiamen Bay of China	Glutamic acid > aspartic acid > lysine > leucine > arginine	2
Pagrosomus major	Xiamen Bay of China	Glutamic acid > aspartic acid > lysine > leucine > arginine	2
Seriola dumerili	Xiamen Bay of China	Glutamic acid > aspartic acid > lysine > leucine > arginine	2
Hapalogenys nitens	Xiamen Bay of China	Glutamic acid > aspartic acid > leucine > lysine > arginine	2
Clarias anguillarias	Market in Ado Ekiti, Nigeria	Glutamic acid > aspartic acid > leucine > lysine > arginine	3
Oreochromis niloticus	Market in Ado Ekiti, Nigeria	Glutamic acid > aspartic acid > leucine > lysine > arginine	3
Cynoglossus senegalensis	Market in Ado Ekiti, Nigeria	Glutamic acid > aspartic acid > leucine > arginine > lysine	3
Trachurus trachurus	Market in Istanbul, Turkey	Glutamic acid > aspartic acid > lysine > leucine > valine	4
Zues faber	Aegean Sea	Glutamic acid > aspartic acid > lysine > leucine > alanine	5
Trigla lucerna	Marmara Sea	Glutamic acid > phenylalanine > aspartic acid > lysine > alanine	5
Scorpaena scrofa	Marmara Sea	Glutamic acid > lysine > aspartic acid > arginine > leucine	5
Scorpaena porcus	Marmara Sea	Proline > phenylalanine > glutamic acid > lysine > leucine	5
Merluccius merluccius	Marmara Sea	Proline > phenylalanine > glutamic acid > lysine > leucine	5
Lophius piscotorius	Marmara Sea	Proline > glutamic acid > phenylalanine > lysine > leucine	5
Trachinus draco	Marmara Sea	Proline > phenylalanine > glutamic acid > lysine > leucine	5
Esox lucius	Edirne Lake in Turkey	Proline > glutamic acid > phenylalanine > aspartic acid > lysine	5
Psetta maxima	Black Sea	Phenylalanine > glutamic acid > aspartic acid > lysine > leucine	5
Upeneus moluccensis	Antalya Gulf of Turkey	Lysine > leucine > aspartic acid > glutamic acid > alanine	6
Engraulis encrasicolus	Market in Istanbul, Turkey	Lysine > leucine > arginine > glutamic acid > aspartic acid	7
Pomatomus saltatrix	Market in Istanbul, Turkey	Lysine > leucine > arginine > glutamic acid > aspartic acid	7
Sarda sarda	Market in Istanbul, Turkey	Lysine > leucine > arginine > glutamic acid > aspartic acid	7
Murlus surmelutus	Market in Istanbul, Turkey	Lysine > leucine > arginine > glutamic acid > aspartic acid	7
Merlangius merlangus	Market in Istanbul, Turkey	Lysine > leucine > arginine > glutamic acid > aspartic acid	7

[1]: Kinm and Lall (2000); [2]: Limin *et al*. (2006); [3]: Adeyeye (2009); [4]: Erkan *et al*. (2010a); [5]: Özden and Erkan (2011); [6]: Doğan and Ertan (2017); [7]: Erkan *et al*. (2010b)

Discussion

Five the most abundant amino acids in muscle of different fish species from different geographical areas are shown in Table 3. In the previous studies Glu, Asp, Lys, Gly and Leu are determined as the most abundant five amino acids in muscle of different fish species such as *Pleuronectes ferruginea* (Storer, 1839), *Hippoglossus hippoglossus* (Linnaeus, 1758), *Paralichthys olivaceus* (Temminck & Schlegel, 1846), *Pseudosciaena crocea* (Richardson, 1846), *Lateolabrax japonicus* (Cuvier, 1828), *Pagrosomus major* (Temminck & Schlegel, 1843), *Seriola dumerili* (Risso, 1810), *Hapalogenys nitens* (Temminck & Schlegel, 1843), *Clarias anguillarias* (Linnaeus, 1758) and *Oreochromis niloticus* (Linnaeus, 1758) (Kinm and Lall, 2000; Limin *et al*., 2006; Adeyeye, 2009). Therefore, Özden and Erkan (2011) reported this classification as proline, Phe or Glu, Lys or Leu in different marine and inland water fish species such as *Scorpaena porcus* Linnaeus, 1758, *Merluccius merluccius* (Linnaeus, 1758), *Lophius piscotorius* Linnaeus, 1758, *Trachinus draco* Linnaeus, 1758 and *Esox lucius* Linnaeus, 1758 from different geographical areas. Erkan *et al*. (2010b) reported that Lys, Leu, Arg, Glu and Asp are the most abundant amino acids in different raw marine fish species (*Engraulis encrasicolus* (Linnaeus, 1758), *Pomatomus saltatrix* (Linnaeus, 1766), *Sarda sarda* (Bloch, 1793), *Mullus surmelutus* Linnaeus, 1758 and *Merlangius merlangus* (Linnaeus, 1758)). Moreover, Phe and Lys reported as the most abundant AAs for *Psetta maxima* (Linnaeus, 1758) in the Black Sea (Özden and Erkan, 2011) and *Upeneus moluccensis* (Bleeker, 1855) in the Mediterranean (Doğan and Ertan, 2017).

We did not detect any study about AAs contents in different tissues of whiting. For that reason, we create Table 3 with different fish species and we found generally similar results for the most abundant amino acid contents (e.g. NEAA: Asp, Glu; EAA: Leu and Lys). Our data showed that the amino acid contents including Arg, Glu, Pro and Tau in meat and ovary of whiting was statistically different each other ($P < 0.05$), and also the AA contents except for Cys, Tyr, Asp, Orn and Tau of these two tissues were statistically higher than AA contents of liver ($P < 0.05$). In general, when looking at figure 2, it can be seen that the AAs contents in different tissues of whiting are $AA_{Meat} \geq AA_{Ovary} > AA_{Liver}$. These results showed that whiting ovary approximately have AAs content as much as meat AAs content.

The EAA of whiting constituted approximately 55.9% in meat, 54.8% in ovary and 51.7% in liver of total amino acids. The NEAA of whiting constituted about 44.1% in meat, 45.2% in ovary and 48.3% in liver of total amino acids. These ratios for the EAA values were reported between 42 - 57% for 9 fish species, 37 - 47% for 6 crustaceans and 34 - 56% for 6 mollusc species (Özden and Erkan, 2011).

The above values demonstrated that considerable variations in amino acid levels can be obtained from the different and/or same fish species. Such variations are possibly a result of several factors including differences in feeding,

season, species, sex, stage of maturity, nutritional, and environmental condition as well as methods used for AA determination (Kinm and Lall, 2000; Limin *et al*., 2006; Adeyeye, 2009; Erkan *et al*., 2010ab; Özden and Erkan, 2011; Doğan and Ertan, 2017). In the classical method (HPLC), the sample is derivatized while being made ready for analysis. However, there is no derivatization in the LC-MS/MS method and the sample is directly hydrolyzed. Also, with the LC-MS/MS method, it is possible to work with a much lower sample (e.g. 0.5 g) than the classical methods.

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RESEARCH ARTICLE

Effect of *Spirulina platensis* (Gomont) Geitler Extract on Seed Germination of Wheat and Barley

Füsun Akgül*

Mehmet Akif Ersoy University, Faculty of Science and Arts, Department of Molecular Biology and Genetic, Burdur/Turkey

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ABSTRACT

Cyanobacteria has a highly diversity group that consists of photosynthetic prokaryotic microorganisms. Cyanobacteria that produce lots of metabolites such as amino acids, proteins, vitamins etc. have a wide spread. In this study, the effects of different concentrations of *Spirulina platensis* extracts on the germination of wheat and barley seeds and root-stem length, lateral root number and fresh-dry weight were investigated. The application of S5 (100% cell extract) showed an inhibitory effect on seed germination on both wheat and barley. S2 (25% cell extract) and S4 (75% cell extract) applications had a positive effect on germination and seedling development in wheat. In barley, S2 (25% cell extract) application activated germination and seedling growth and other concentration applications did not create a positive effect. As a result; cyanobacterial extract has positive effects on seed germination and plant growth-development and it is possible to produce a commercial and ecological biostimulant by developing different extract concentrations.

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Introduction

The increase in the world population forced the agricultural countries to take more products from the unit area and made it necessary to carry out studies to increase this. However, while carrying out such studies, practices have been carried out giving serious harmful to aquatic and terrestrial ecosystems. The amount of artificial fertilizers using that the most harmful of these applications is increasing day by day, when the unconscious using is added to this process, the ecosystem has irreversible damage. Scientists who take these effects into consideration, have started to work on the production of nature-friendly, biofertilizer-bitostimulant and its use. For this purpose, bacteria, cyanobacteria and algae have been used extensively and effectively.

In the countries such as Norway, Ireland, France and the United States located in the seaside, have been looked for the ways to benefit from the algae and to use them as biofertilizer (Özdemir et. al., 2015). In recent years, numerous studies have been carried out in which cyanobacteria and microalgae have been used as biofertilizer-biostimulants and promising results have been obtained from these studies. Mogor et al. (2017) investigated the effects of *Arthrospira platensis* (*S. platensis*) hydrolysate on the growth of lettuce plants and found that hydrolysates showed cytokine-like effects in lettuce seedlings. Grzesik et al. (2017); researched the foliar applications effects of the biofertilizer that consists of *Microcystis aeruginosa*, *Anabaena* sp., and *Chlorella* sp. species cultures on willow and they detected that increased the plant growth and decreased the amount of artificial fertilizers needing. Muñoz-Rojas et al. (2018) who did the restoration studies with local plant species

* Corresponding author
E-mail address: fakgul@mehmetakif.edu.tr

of Australia, they covered the seeds of these plants with cyanobacteria and then planted them in the soil. They noted a significant increase in the germination percentage of seeds compared to those not covered with cyanobacteria.

S. platensis is a member of a filamentous cyanobacteria and can be used as a food additive in terms of its high protein content and nutritive properties. *Spirulina* sp. has a natural distribution in alkaline environment generally, which prevents its easy contamination (Olguín et al., 1997). In this way, it becomes a suitable organism for use in ecological studies. In addition, *Spirulina* sp. is a good protein supplement in animal nutrition as well as an alternative to chemical fertilizers (Habib et al., 2008). The fact that approximately 60% of the *S. platensis* biomass is protein allows this biomass to be used to obtain protein hydrolysates containing valuable biomarkers. Decarboxylation of these molecules provides polyamine synthesis and the presence of polyamine enables cyanobacteria to be used effectively in plant growth (Kim et al., 2014; Zhang and Zhang, 2013; Lisboa et al., 2016).

In this study; *S. platensis* was cultured continuously and then biomass was extracted and different concentrations of solutions were prepared. The effects of these extracts on the germination of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) seeds which are the most produced 2 monocotyl species in our country were investigated. Germinated seed number, germination percentage, germination energy, body shoot length, total root length, lateral root number and fresh-dry weight parameters have been determined. It has been investigated whether these extracts have a stimulatory effect on seed germination and it is aimed to produce an alternative organic biostimulant from these extracts.

Materials and Methods

Cultivation and Harvesting

S. platensis, obtained from Mehmet Akif Ersoy University, Algal Biotechnology Laboratory, was cultivated flasks using standard Zarrouk culture medium (Zarrouk, 1966), bubbled with air. The biomass was harvested by centrifugation at day 20 of cultivation. The biomass dried in an oven at 45°C for 24 hours and then powdered with a grinder and stored at +4°C.

Cell Extract

Dried biomass was suspended in distilled water (DIW) at a concentration of 150 g L^{-1}. For obtaining the intracellular extracts, the suspension was extracted with a sonicator. The suspension centrifuged at 22°C, 6000×g for 6 minutes for removing biomass residue. To minimize potential degradation, the resulting extract supernatant was collected in a flask covered with aluminum foil and stored in a cold room at 4 °C. Five different concentration solution were prepared with cell extract. S1, Control, 0% extract (10 mL DIW); S2, 25% (2,5 mL extract, 7,5mL DIW); S3, 50% (5 mL extract, 5mL DIW); S4, 75% (7,5 mL extract, 2,5mL DIW); S5, 100% (10 mL extract). The biomass residue was also stored in the cold room for potential future use.

Seed Experiment

All solutions were replicated three times with ten seeds per replicate. The seeds were sterilized with 10 mL of 5 % solution of sodium hypochlorite for 10 min, rinsed twice with DIW, transferred to sterile Petri plates, and soaked in 10 mL of the S1, S2, S3, S4, S5 solutions for 24 h. Following the 24-h soaking period, the seeds were placed between two 42.5-mm Whatman no. 1 filter papers and allowed to dry for 24 h at room temperature (21 °C). Then, the seeds were transferred to a sterile 100-mm Petri plate containing a moist 75-mm Whatman no. 1 filter, which was soaked with 3 mL of DIW. The plates were incubated at 21 °C under a 16-h light/8-h dark cycle. Seed germination was checked at 24-h intervals for 10 days and counted as germinated if at least 2 mm of the radicle had emerged. The filter paper for all treatments was saturated as needed with 3 mL of DIW to maintain moisture. Root, shoot, and leaf lengths (mm) were measured with a caliper. Total root length consists of main and lateral roots and total shoot length consists of main and lateral branches. And also number of lateral roots measured and germination percentage (GP), and germination energy (GE) were calculated.

Germination percentage (GP) was calculated as

GP= (number of germinated seeds/total number of seeds) ×100

Germination energy (GE) was calculated according to Hernández Herrera et al. (2013),

GE= (number of germinating seeds on X. day/number of total seeds) ×100

In this research GE of 3., 5. and 7. days were calculated.

Results and Discussion

When the germination percentage graph of wheat seeds examined (Figure 1); It is clear that S2 and S4 applications have a positive effect on seed germination. S2 and S4 applications have 93% germination percentage, this value is 13% more than control group. The application of S5 with 100% extract was able to produce a germination percentage of 23% by creating a germination inhibitory effect.

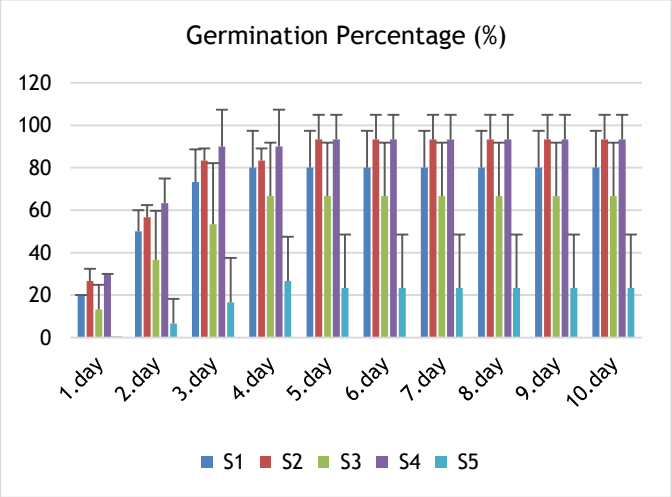

Figure 1. Germination percentage of wheat seeds according to the applications and days

Germination energy is a value indicating the germination rate and it is generally evaluated according to the germination rate of the seeds on the 3rd, 5th and 7th days. It is seen that S4 application in wheat seeds rapidly reaches 90% germination rate on day 3, and S2 application has achieved a rate of 83%. In S2 and S4 applications, a germination energy ratio of 93% is seen on day 5 (Figure 2). In other concentration applications, such a high rate could not be achieved.

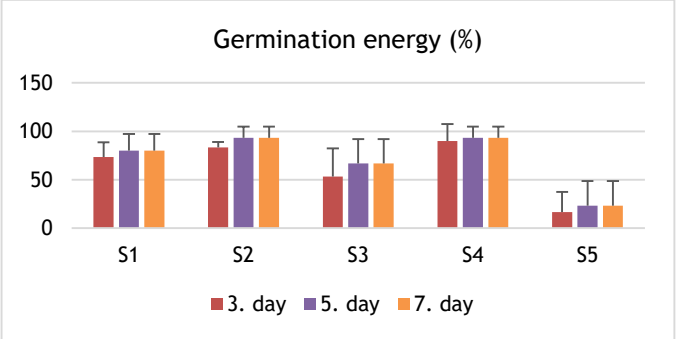

Figure 2. Germination energies of wheat seeds on the 3th, 5th and 7th days according to the applications

When germination percentage and germination rate data are examined, it is seen that S2 and S4 applications have activator effect on germination of wheat seeds.

The graph of wheat total root length (Figure 3) shows that S2 application has more extension, approximately 100 cm according to control group. The S5 application seems to slow down root growth, but S4 seems to be effective even if it is not as effective as S2 in root growth.

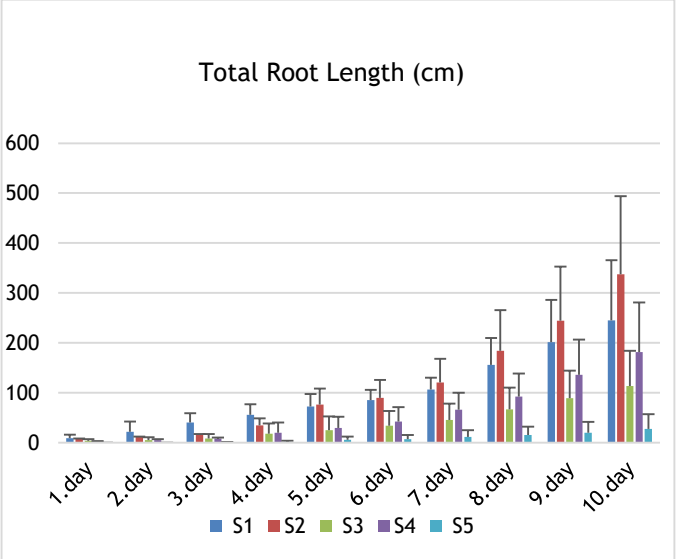

Figure 3. Total root length of wheat seedlings according to the applications and days (cm)

It is seen that the effects of the applications in the body shoot length are similar to the root length effects. The S2 application provided an extension of about 15 cm more than the control group. Again, S4 application almost extended the S1 (control) application (Figure 4). However, S5 application had an inhibitory effect on shoot extension.

When the lateral root number graph was examined (Figure 5), S2 and S4 applications were caused to more lateral root formation than the control group. And this result shows that this extracts have a stimulating effect on plant growth.

Dry weight data revealed that S2 and S4 applications had positive effects (Figure 6).

When all these data are examined; it is seen that S2 (25%) and S4 (75%) applications have a stimulating effect on germination and seedling development of wheat germ. Much better germination and development results than the S1 (control) group shows that *S. platensis* extract can be used as a biostimulant in seed germination.

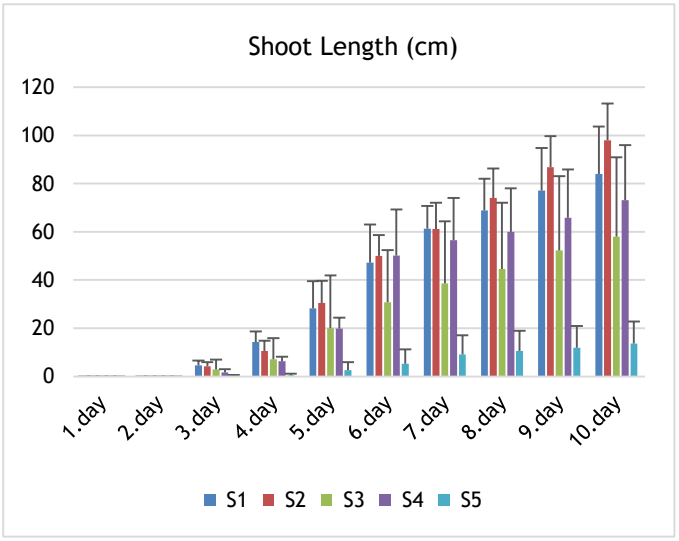

Figure 4. Shoot length of wheat seedlings according to the applications and days (cm)

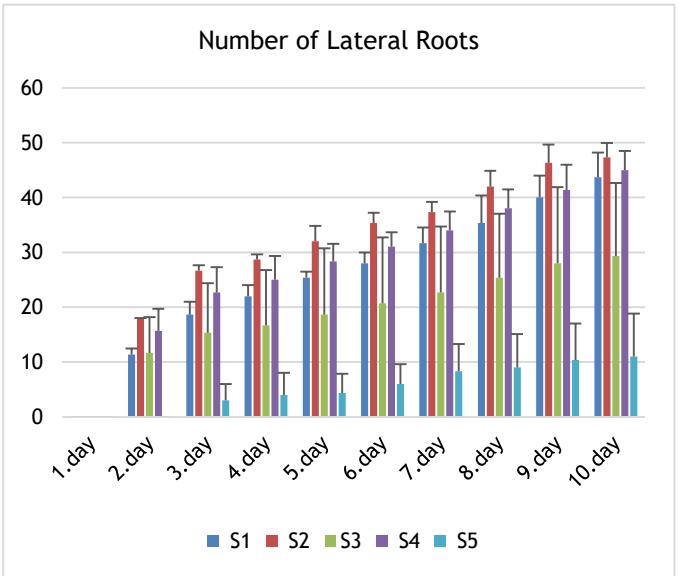

Figure 5. Number of lateral roots of wheat seedlings according to the applications and days (piece)

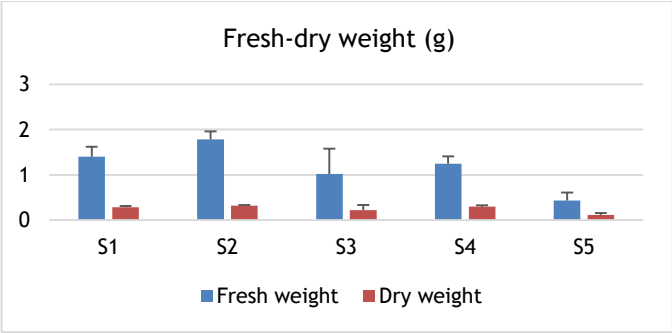

Figure 6. Fresh and dry weights of wheat seedlings according to applications (g)

Similar to this result; Michalak et al. (2016); investigated the effects of some algae and *S. platensis* extracts on wheat development, especially in the *S. platensis* extract group, the number of grains in the wheat ear and the length of the stem were significantly higher than the others. In addition; Khushwaha and Banerjee (2015); the effect of the addition of cyanobacterial culture on chickpea, rice and wheat seed germination on the low temperature, in the laboratory conditions were investigated and the germination percentages, root-stem length, number of leaves, chlorophyll amount found more than the control group that without cyanobacteria culture. Shariatmadari et al. (2015); have applied the extracts of different cyanobacterial members to wheat seeds and they noticed that germination percentage does not change much, but the seed and root length of the seedlings more extended than the control group. In another study, Gahlout et al. (2017); researched that the effects of the extracts of different cyanobacteria on wheat and mung bean seed germination and seedling development in their studies and they detected that extracts increased the percentage of seed germination (at the end of the 3rd day, more germination percentage -70% excess-was observed than the control group) and extract has a positive effect on seedling development. We found similar results in Kumar and Kaur (2014); *Anabaena variabilis*, *Nostoc muscorum*, *Aulosira fertilissima* and *Tolypothrix tenuis* filtrates applied to wheat seeds, and they found a positive effect on seedling growth compared to those without filtrate.

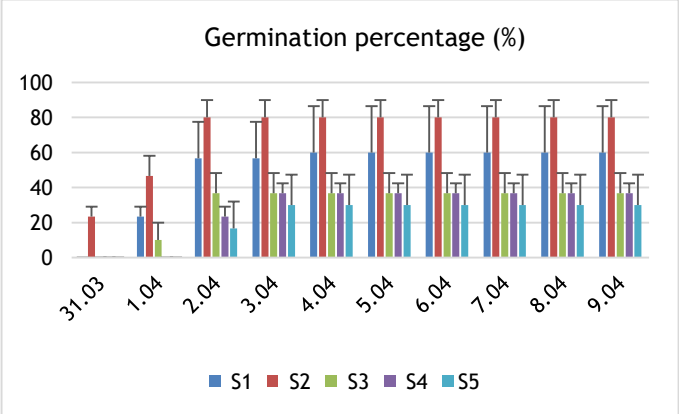

Figure 7. Germination percentage of barley seeds according to the applications and days (%)

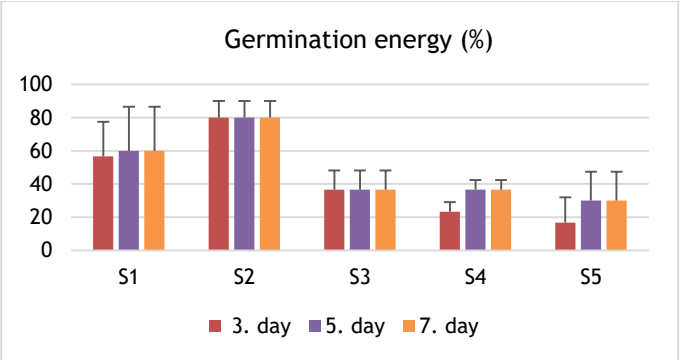

Figure 8. Germination energies of barley seeds on the 3th, 5th and 7th days according to the applications (%)

When the germination percentage and energy of barley seeds are examined (Figure 7 and 8); It is seen that S2 application has a positive effect on germination of barley seeds and accelerates germination.

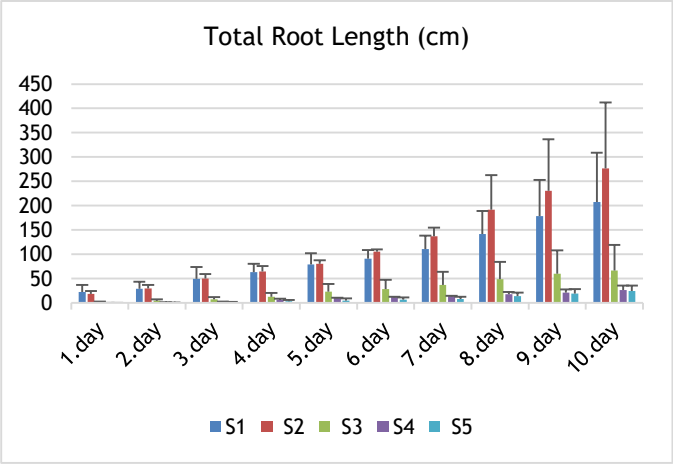

Figure 9. Total root length of barley seedlings according to the applications and days (cm)

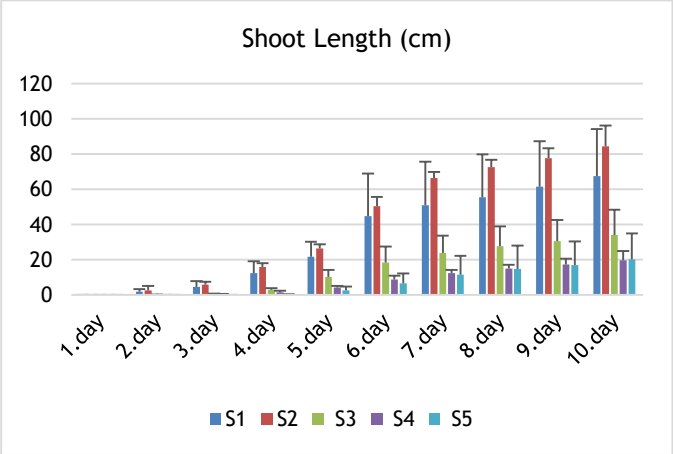

Figure 10. Shoot length of barley seedlings according to the applications and days (cm)

Total root length and shoot length graphs of barley seedlings were examined (Figure 9 and Figure 10); S2 application has a positive effect on root and shoot development and compared to S1 (control) application;

approximately 70 cm in root length, 17 cm in body length more elongation were observed.

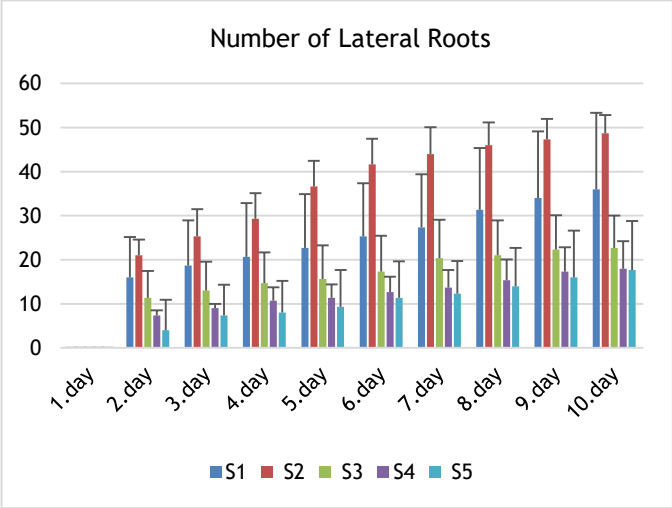

Figure 11. Number of lateral roots of barley seedlings according to the applications and days (piece)

Figure 11 shows that S2 application significantly increases the number of lateral roots in barley compared to S1 (control) application and it affects the seedling development positively. The total number of lateral roots in the seedlings at the end of the 10-day; was 36 in the S1 (control) application and 48.6 in the S2 application.

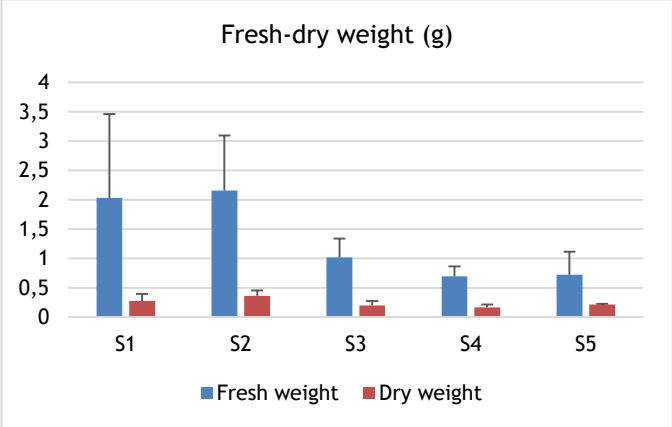

Figure 12. Fresh and dry weights of barley seedlings according to applications (g)

At the end of 10 days, the fresh and dry weight graph of barley seedlings (Figure 12) also showed that S2 application had a positive effect on biomass increase.

When all the data related to barley seeds are evaluated; it is observed that S2 (25%) application has positive effects on seed germination and seedling development. It can be said that *S. platensis* extract accelerates the seed germination and have biostimulant effect in plant growth. S4 and S5 applications showed a slowing effect as close to each other compared to S1 (control) application. The use of intensive extracts has an inhibitory effect but the extracts in certain doses activate the growth.

Ismail and Hamad (2017); used *Anabaena variabilis* extracts in their work and stated that this extract increases the germination percentage, root-shoot length and fresh-dry weight of the seeds of *Hordeum vulgare* and *Trigonella foenum-graecum* L. Shariatmadari et al. (2013); used the extracts of *Anabaena* and *Nostoc* genus in the development of rice plants and stated that they affected plant growth positively. Essa et al. (2015); investigated the effects of some cyanobacterial exudates on sorghum and sunflower seed germination and seedling development and found that *Anabaena oryzae* has been very effective on seed germination and root-shoot development. Grzesik and Romanowska-Duda (2014); applied *Microcystis aeruginosa*, *Anabaena* sp. (Cyanobacteria), and *Chlorella* sp. cultures to commercial corn plants; and detected positive effects on seed germination and seedling growth. Win et al. (2018); stated in their review study that algal biofertilizers should be used strictly for sustainable agriculture.

Conclusion

All these data indicate that the bio-fertilizers containing cyanobacterium extracts that are natural and dissolve in nature spontaneously should be preferred instead of artificial fertilizers which cause bioaccumulation in living systems, because of their positive effects on plant growth and non-destructive properties.

Although there are many benefits of this organic biofertilizer, some obstacles in the process of commercialization. These obstacles can be overcome by explaining the biofertilizer to the farmer very well. In the future studies, the chemical compositions of microalgae and cyanobacterial extracts should be characterized in detail and the most effective concentration and composition should be developed and this product should be placed in the fertilizer sector by emphasizing the ecological and economic importance of this product. Besides; similar trials should be carried out in the field and the results should be discussed carefully.

Acknowledgements

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RESEARCH ARTICLE

Enhanced Egg Weight, Egg Production and Shell Breaking Strength in Late Laying Period of Hens Fed a Diet Containing a Eubiotic Mixture

Şaziye Canan Bölükbaşı Aktaş[1*] • Sulhattin Yaşar[2] • Fatih Muhammed Yıldırım[1] • Hafiz Ghulam Qutab Ud Din[1]

[1]*Atatürk University, Faculty of Agriculture, Department of Animal Science, Erzurum/Turkey*
[2]*Iğdır University, Faculty of Agriculture, Department of Animal Science, Iğdır/Turkey*

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ABSTRACT

The effects of different levels of an eubiotic on laying performance, egg quality parameters, serum enzymes and antioxidant levels, and egg yolk fatty acid composition were examined in the present study. Six diets were formulated to contain 0, 200, 400, 600, 800 and 1000 mg/kg EFA. Each diet was randomly fed to a group of 24 hens for 10 weeks, housed in 6 separate cages (4 hens per cage). Average egg weight was remarkably increased as an effect of EFA dietary supplementation. In comparison to the control group (89.2%), significantly higher egg production rates of 93.7 and 96.7% were observed in the groups of hens fed diets supplemented with 800 and 1000 mg/kg EFA, respectively. An improvement of 5 to 11% in FCR of EFA supplemented groups was found. Concerning the other examined parameters, only shell breaking strength was increased by 30-36% in EFA supplemented groups at the level of 200, 800 and 1000 mg/kg, whereas no significant differences in egg yolk fatty acid composition, serum enzymes and antioxidant levels were observed among groups. In conclusion, an improved hen performance at late laying phase could be achieved as an effect of EFA dietary supplementation at the level of 1000 mg/kg.

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Introduction

The use of antibiotic growth promoters (AGPs) in animal nutrition was banned in 2006 by the EU. Since then a wide range of feed additives such as organic acids, probiotics, prebiotics, essential oil compounds (EOC), minerals and trace elements have been used as alternatives to AGPs with positive claims of an improved gut microflora and animal performance (Huyghebaert et al., 2011; Gadde et al., 2017).

Active agents from certain feed additives have been recently isolated and combined in single products to offer improved animal health and performance. A dietary supplementation of a mixture containing EOC (thymol, eugenol and piperine) and an organic acid at the level of 300 mg/kg enhanced performance parameters in broiler chickens (Weber et al., 2012) and turkey poults (Giannenas et al., 2014). The purpose of mixing such active agents is to create a eubiosis synergistic effect to optimally balance gut microbiota in the absence of AGPs. It was recently shown that a mixture of EOC and organic acid increased live weight gains and reduced *E. coli* fecal shedding in piglets (Torallardona et al., 2016). A possible improvement in hen performance during late laying

* Corresponding author
E-mail address: canan@atauni.edu.tr

period as an effect of such dietary interventions is of great importance in sustainable animal production systems. The effects of single or multiple mixture of functional feed materials or active substances (symbiotic/eubiotic effect) have already been examined in laying hens: Supplementing the diets with dry whey powder at level of 60g/kg for 13 weeks significantly reduced ceacal counts of *Clostridium perfringens* and increased egg production from 75% to 82.5% in late laying phase (Pineda-Quiroga et al., 2017). However, dietary supplementation with xylooligosaccharides known as xylose polymer or prebiotic did not affect laying performance, but improved eggshell quality and digestibility of dietary calcium as well as reduced plasma cholesterol level (Li et al., 2017). A diet containing 4% of a mixture of red seaweeds, *Chondrus crispus* known as Carrageen and a prebiotic had a protective effect against *Salmonella* Enteritis colonisation in laying hens (Kulshreshtha et al., 2017). The humate (humic, fulvic, ulmic, and humatomelanic acids) and probiotic together in the diets of laying hens during late laying period increased egg production and improved feed conversion efficiency (FCR) without any effect on egg quality (Yoruk et al., 2004). A mixture of additives containing both *Aspergillus awamori* and lactic acid bacteria had no effects on the performance of laying hens, but significantly increased unsaturated and reduced saturated fatty acid contents in the egg yolk (Saleh et al., 2017). Finally, dietary supplementation with selenium enriched yeast products could be more efficient in increasing selenium bioavailability of whole egg compared to the inorganic selenium sources (Chantiratikul et al., 2017).

Feed materials/additives that are produced through fermentation processes contain several active substances and have been reported to improve performance and health in broiler and Japanese quails (Yasar and Gok, 2014; Yasar et al., 2016). An EFA produced at our laboratory through a solid-state fermentation (SSF) process containing enzymes, organic acids and live yeast cells successfully induced a growth promoting effect (GPE) in broiler chicken (Yasar and Yegen, 2017), and a similar SSF product containing fermented whey, fermented grain and fermented fruit pomace had a significant positive effect on health status and performance in both calves and lambs with severe diarrhea at a daily dose of 15 g for a period of 2 to 5 days (Yasar et al., 2017). Many studies with dietary supplements have already been implemented with the intention to improve laying performance of hens in late laying period (Yoruk et al., 2004 and Bolukbasi et al., 2010; Kaya et al., 2014a; Świątkiewicz et al., 2018). To the best of our knowledge, no studies exist concerning the effects of a SSF additive containing several active agents on the productive parameters of in laying hens.

Therefore, this study aimed at highlighting the effects of various dietary levels of a eubiotic feed additive (EFA) containing organic acids, probiotic yeast/bacteria, enzymes and rosemary dry powder on performance, egg quality, egg yolk fatty acid composition, serum enzymes and antioxidant levels in 75 weeks old Lohman LSL hens.

Materials and Methods

Animal and Housing

The research was conducted at the poultry research station of Atatürk University, Erzurum, Turkey in accordance with EU Directive 2010/63/EU. A total of 144, 75 week-old Lohman hens were randomly allocated into 6 treatment groups. Each group included 6 cage replicates of 4 hens (50 x 46 x 46). The hens were previously fed with the control diet (Table 1) and subjected to a lighting regime of 16 h per day prior to the trial.

Table 1. Composition of basal diet

Ingredients (ground)	Composition (g per kg)
Corn	520.00
Soybean meal	240.00
Wheat	118.63
Limestone	90.80
Vegetable oil	10.30
Dicalcium phosphate	11.54
Vit+Min mixture*	2.00
NaCl	5.00
DL-Methionine (99% purity)	1.23
L-lysine	0.50
Total	1000.00
Chemical Analysis (g per kg)	
Crude protein	167.00
Calcium	37.80
Total phosphorous	6.75
Calculated Analysis (g per kg)	
Metabolisable Energy (Kcal per kg)	2752
Lysine	9.00
Met+Cys	6.20

* Supplied per kilogram of diet: 12 000 IU vitamin A; 2 500 IU vitamin D3; 30 IU vitamin E; 4 mg vitamin K3; 3 mg vitamin B1; 6 mg vitamin B2; 30 mg niacin; 10 mg calcium D-pantothenate; 5 mg vitamin B6; 0.015 mg vitamin B12; 1 mg folic acid; 0.050 mg D-biotin; 50 mg vitamin C; 300 mg choline chloride; 80 mg manganese; 60 mg iron; 60 mg zinc; 5 mg copper; 0.5 mg cobalt; 2 mg iodine; 0.15 mg selenium.

Dietary Treatments, Sample Collection and Analysis

The additive used in the study was developed by a SSF process of agricultural co-products at the department of Animal Science, Agricultural Faculty of Igdir University, Turkey. Each g of the product contained 12.5 mg of finely powdered leaves of rosemary (*Rosmarinus officinalis*), $3.1x10^9$ c.f.u (colony forming unit) of *Saccharomyces cerevisiae*, a $3.7x10^9$ c.f.u of *Streprococcus thermophiles*, a $1.1x10^8$ c.f.u of *Lactobacillus spp.*, an activity of 40 IU of amylase, 845 IU of betaglucanase, 2000 of IU arabinoxylanase and 15% of organic acids (mostly acetate, lactate and citrate). The EFA has a pH of 3.6-3.9, an average particle size of 1-2 mm, and a light orange colour. Six experimental diets (mash) were prepared in the present study. Control diet (Table 1) was formulated to meet the nutrient requirements of laying hens (NRC, 1994). Experimental diets were further supplemented with 200, 400, 600, 800 and 1000 mg of EFA. The trial lasted 70 days, and during this period all the hens had free access to water and feed and were subjected to a photoperiod of 16 h per day.

Feed intake, egg weight, egg production, FCR (g feed per g egg produced per group) were daily recorded for each cage, while the egg weight, Haugh unit, shell breaking strength, shell thickness, shape index and ratio of albumen, yolk and shell were determined biweekly using six eggs from each dietary treatment. At the end of the study, blood samples (n = 6/group) were collected from wing vena into additive free blood tubes. The levels of glutathione peroxidase (GPx), malondialdehyde (MDA), total antioxidant (TAS) and total oxidants (TOS) in the blood serum of the hens were analysed by the use of commercial kits (Roche) in auto-analyser (Cobas 6000, Japan). In addition, the fatty acid composition was examined in the samples of egg yolk (Folc, 1957; AOAC 2000). Fatty acid methyl esters were separated using gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA) equipped with a fused silica capillary column (25 m by 0.2 mm) with 5% cross-linked phenylmethyl silicone. Fatty acid methyl ester profiles of the samples were identified by comparing the commercial Eucary database with the MIS software package (MIS ver. No 3.8, Microbial ID, Inc., Newark, Delaware). Individual fatty acid methyl esters were expressed as percentage of total FA.

Statistical Analysis

Data was analysed by using a general linear model (GLM) to test the effects of EFA supplementation on the laying performance, egg and egg-shell quality parameters, serum enzyme activities and antioxidant capacity, and fatty acid composition of yolk. Differences among treatments means were detected by Duncan's multiple comparisons test at 0.05 significance level using SPSS version 21 for Windows.

Results

As indicated in Table 2, the effect of EFA supplementation on daily feed intake (FI) was not significant (P > 0.05). The mean daily FI ranged from 149.02 to 150.41 g in the present study. The effect of EFA supplementation on egg production was significant (P < 0.05). Egg production rates were increased in the 800 and 1000 mg/kg EFA supplemented groups (93.75 and 96.66%, respectively) compared to the controls (89.23%). Egg production rates of the other groups did not vary. The egg weight of the control group (64.13 g) was significantly lower than that of the 200 and 1000 mg/kg EFA supplemented groups (69.25 and 67.34 g, respectively) (P < 0.05). Egg weight of the other EFA groups was not significantly different compared to the controls. FCR was significantly improved (P < 0.05) in 200 and 1000 mg/kg EFA supplemented groups (2.39 and 2.32, respectively) (Table 2).

In the present study, the effect of EFA supplementation on shape index, eggshell index, egg yolk index, egg-albumin index, shell thickness and Haugh unit was not significant (P > 0.05). However, egg-shell breaking strength was significantly (P < 0.05) increased as an effect of EFA dietary supplementation (Table 3; from 1.10 in the controls to 1.46, 1.50 and 1.42 kg/cm^3 in the 200, 800 and 1000 mg/kg EFA supplemented groups, respectively). Values of serum GPx, MDA, TAS and TOS levels were not influenced by EFA dietary supplementation (Table 4). As indicated in Table 5, EFA dietary supplementation did not also induce significant effects on fatty acid composition of egg yolk.

Table 2. Effects of varying supplementation levels of EFA on laying performance

EFA, mg/kg	Feed intake (g/hen)	Egg weight (g/hen)	Egg production (% of kept hens)	FCR (g:g)
0	149.02	64.13^{c}	89.23^{c}	2.62^{a}
200	148.77	69.25^{a}	90.41^{bc}	2.39^{bc}
400	148.11	65.58^{bc}	92.70^{bc}	2.45^{bc}
600	148.15	64.77^{c}	92.08^{bc}	2.49^{ab}
800	148.54	64.70^{c}	93.75^{ab}	2.46^{bc}
1000	150.41	67.34^{ab}	96.66^{a}	2.32^{c}
SEM[1]	0.24	0.31	0.51	0.02
P-value	>0.05	=0.000	=0.000	=0.000

[a-c] Values in the same column indicate significant (P<0.05) differences.
[1] SEM; Standard error of means.

Table 3. Effects of varying supplementation levels of EFA on egg quality

EFA (mg/kg)	Shape index (%)	Egg shell index (%)	Egg yolk index (%)	Albumin index (%)	Eggshell breaking strength (kg/mc^3)	Shell-thickness (µm)	Haugh Unit
0	75.75	10.92	29.62	59.45	1.10^{b}	344.50	91.48
200	74.37	10.80	26.33	62.85	1.46^{a}	332.88	93.54
400	76.25	10.89	25.29	63.81	1.30^{ab}	332.54	89.95
600	75.31	11.03	26.57	62.39	1.35^{ab}	334.00	89.30
800	74.5	11.02	26.76	62.20	1.50^{a}	331.25	86.94
1000	74.41	10.43	26.02	63.54	1.42^{a}	336.66	94.58
SEM[1]	0.33	0.17	0.50	0.48	0.04	1.89	0.91
P-value	>0.05	>0.05	>0.05	>0.05	=0.047	>0.05	>0.05

[a-c] Values in the same column indicate significant (P<0.05) differences.
[1] SEM; Standard error of means.

Table 4. Effects of varying supplementation levels of EFA on serum glutathione peroxidase (GPx), malondialdehyde (MDA), total antioxidant (TAS, trolox equivalent) and total oxidants (TOS) levels

EFA (mg/kg)	GPx (nmol/ml)	MDA (nmol/ml)	TAS (µmol/ml)	TOS (µmole/L)
0	3.10	15.69	0.61	35.93
200	3.01	19.12	0.63	26.68
400	3.54	20.38	0.67	32.31
600	3.46	19.65	0.67	30.50
800	3.70	17.58	0.55	30.45
1000	3.13	17.05	0.49	30.68
SEM[1]	0.14	0.76	0.03	3.78
P-value	>0.05	>0.05	>0.05	>0.05

[a-c] Values in the same column indicate significant (P<0.05) differences.
[1] SEM; Standard error of means.

Table 5. Effect of dietary supplementation of EFA on fatty acid composition of yolk

Fatty acids	Control	EFA 200 mg/kg	EFA 400 mg/kg	EFA 600 mg/kg	EFA 800 mg/kg	EFA 1000 mg/kg	SEM[1]	*P-value*
Miristic	1.24	1.23	1.29	1.21	1.18	1.30	0.04	>0.05
Palmitic	28.73	29.46	32.08	32.90	29.78	29.46	1.01	>0.05
Palmitoleic	5.13	4.92	4.36	4.19	4.92	5.03	0.13	>0.05
Stearic	12.06	13.19	14.33	14.65	15.57	15.24	0.44	>0.05
Oleic	32.77	30.73	27.79	27.33	27.61	29.77	0.73	>0.05
Linoleic	13.28	14.10	14.17	13.75	14.50	12.91	0.55	>0.05
Linolenic	0.64	0.61	0.59	0.60	0.58	0.59	0.02	>0.05
Arachidonic	2.37	2.41	2.31	2.25	2.46	2.40	0.05	>0.05
EPA	1.09	0.85	0.76	0.75	0.85	0.71	0.04	>0.05
DHA	0.96	0.99	0.82	0.79	0.79	0.84	0.03	>0.05
SFA	42.03	43.89	47.70	48.77	46.53	46.01	0.53	>0.05
MUFA	37.90	35.66	32.16	31.53	32.54	34.80	0.81	>0.05
PUFA	18.34	18.97	18.67	18.14	19.20	17.46	0.60	>0.05

[1] SEM; Standard error of means.

Discussion

Feed intake of hens was not affected by EFA dietary supplementation. Several studies demonstrated that the beneficial effects of microbial feed additives (probiotics/eubiotics) in several poultry species were not regulated by a change in voluntary feed intake (Yoruk et al., 2004; Yasar and Yegen, 2017). On the other hand, the use of feed additives in subclinical infected birds remarkably increased FI and performance by improving gut health and the rate of nutrient digestion and assimilation at the digestive sites (Jiang et al., 2010; Kulshreshtha et al., 2017).

It is most likely that the enhanced egg productions and egg weight as well as egg breaking strength due to the EFA dietary supplementation could be a result of a combined eubiosis effect of organic acids, enzymes and probiotics that are present in the examined feed additive. It was previously reported that the dietary supplementation with probiotics till the level of 150 mg/kg diet has a positive quadratic effect on egg production (Mohan et al., 1995). The use of probiotics, synbiotics and eubiotics with or without organic acids under normal or subclinical infection conditions has been shown to provide an optimal balance of gut microbiota, which, in turn, is responsible for an improved performance in laying hens (Yoruk et al., 2004; Gaggìa et al., 2010;Kaya et al., 2014b; Torallardona et al., 2016; Gadde et al., 2017; Pineda-Quiroga et al., 2017). The use of exogenous enzymes in laying hen diets is often recommended in order to efficiently utilize the cell wall polysaccharide contents, and positive effects on FCR, egg production and egg yield are generally reported (Mirzaie et al., 2012). It can be concluded that the daily administration of probiotics and organic acids from EFA in our study might contributed in a healthier gut, and the exogenous enzymes caused a greater breakdown of cell-wall constituents and other nutrients with a further positive effect on laying performance.

Several herbal extracts significantly improved egg production and FCR (Cabuk et al., 2006; Kaya et al., 2013) even in hens reared under extreme cold conditions (Akbari et al., 2016). Egg mass and egg weight also appeared to increase after EO supplementation (Olgun, 2016). The EFA used in the present study contained a powder derived from rosemary leaves. Powder of rosemary leaves used as feed additive at a rate of 0.3 to 0.9% significantly increased both egg weight and egg production (Alagawany and Abd El-Hack, 2015). The products of rosemary powders or extracts are rich in phenolic compounds (carnosol and carnosic acids with antioxidant effects) and are shown to enhance the performance of poultry (Bozin et al., 2006; Yasar et al., 2017). Rosemary essential oils were previously found to reduce yolk percentage and Haugh unit (Bolukbasi et al., 2008), whereas the rosemary powder increases the values of these parameters (Alagawany and Abd El-Hack, 2015). These discrepancies may be related with the dose or the different forms of rosemary (oils or powdered raw leaves). Both forms of rosemary were found to lower serum triglyceride and total cholesterol levels (Bolukbasi et al., 2008; Alagawany and Abd El-Hack, 2015) as well as increase serum superoxide dismutase activity (Alagawany and Abd El-Hack, 2015) in laying hens. The use of bergamot oil (Citrus bergamia) in the diet of laying hens increased fatty acid composition (EPA and DHA) in the yolk (Bolukbasi et al., 2010). In our experiment, EFA dietary supplementation (containing powdered rosemary leave) did not affect serum antioxidant levels and egg yolk fatty acid composition.

Supplementing the diets at the levels of 0.5 and 1.0% with a similar EFA as that used in the study of Yasar and Yegen (2017) has a growth promoting effect in broiler chickens. In our trial, the egg production, egg weight and eggshell breaking strength were increased by addition of EFA into the diets of laying hens. No effects of EFA supplementations were observed on yolk's fatty acid composition and serum GPx, MDA, TAS and TOS levels.

These results were strongly supported by recent findings (Świątkiewicz et al., 2018). Dietary supplementation of hens at late laying period with sodium butyrate, probiotic bacteria, a blend of herb extracts or chitosan induced significant increases in laying rate, egg shell thickness and breaking strength with no effects on egg yolk fatty acid composition. In our study, a combined effect of several active agents from the EFA resulted in a cumulative beneficial effect on hen performance and egg quality. Moreover, the improvement of laying performance is greater in our study than that observed in the study of Świątkiewicz et al. (2018). Addition of antioxidant compounds including a source of herbal additive to the diet of hens during late laying period did not induce any changes in the body antioxidant level, but rather caused an increase in the egg yolk content of antioxidant (Loetscher et al. 2014). In our study, the blood serum levels of antioxidants were not influenced by the EFA addition, and it is likely that the antioxidant compounds available in the EFA was not sufficiently enough to induce such changes.

Conclusion

All the above indicated that the level of 1000 mg/kg EFA supplementation can be optimally be recommended for the diets of laying hens in order to enhance egg weight, egg production and eggshell breaking strength during late laying phase.

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RESEARCH ARTICLE

Maximum Length Record of Common Two-banded Seabream (*Diplodus vulgaris* Geoffroy Saint-Hilaire, 1817) for Aegean Sea with Turkish Waters

Özgür Cengiz[1] • Şükrü Şenol Paruğ[2*] • Bayram Kızılkaya[3]

[1]*Van Yüzüncü Yıl University, Faculty of Fisheries, Van/Turkey*
[2]*Kastamonu University, Faculty of Fisheries, Kastamonu/Turkey*
[3]*Çanakkale Onsekiz Mart University, Faculty of Marine Sciences and Technology, Çanakkale/Turkey*

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ABSTRACT

The maximum length, weight, and age information of living things in an ecosystem are necessary for population dynamics and stock assessment studies. Hence, the recording of such data may be beneficial for scientific databases for life history and fisheries science. The common two-banded seabream (*Diplodus vulgaris* Geoffroy Saint-Hilaire, 1817) is a widespread demersal marine fish, which belongs to the Sparidae family and inhabits down to 90 m depth. Because it is a demanded seafood, it has commercial importance and usually available in the fish market almost every month of the year in Turkey. A single specimen of common two-banded seabream with 31.9 cm in total length and 467.00 g in total weight, which was caught off İbrice Bight (Saroz Bay) with handline by a professional fisherman on 12 June 2015, was obtained from a fishmonger. This study aims to present the maximum size record of this species for the Aegean Sea with Turkish waters.

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Introduction

The common two-banded seabream (*Diplodus vulgaris* Geoffroy Saint-Hilaire, 1817) is a demersal marine fish, which inhabits inshore waters on rocky or sandy bottoms and posidoniabeds down to 90 m depth. It is a common fish with a wide distribution range in the Eastern Atlantic, from the Bay of Biscay to the Cap Verde Islands and around the Madeira and the Canary Islands, and from Angola to South Africa. It is also present throughout the Mediterranean Sea and in the Black Sea (Bauchot and Hureau 1986; Mouine et al. 2010).

Maximum length and weight are important parameters used in life history studies and fishery science. These measurements are applied directly or indirectly in most stock assessment models (Borges 2001; Cengiz 2014). Therefore, it is important to regularly update the maximum size of commercially important species (Navarro et al. 2012; Cengiz et al. 2019a). This study presents the maximum size record of the species for the Aegean Sea with Turkish waters.

Materials and Methods

Saroz Bay, which is situated in the Northeastern Aegean Sea, is connected to the North Aegean with a depth of approximately 600 m to the west. The shelf extends at a water depth of 90-120 m. The length of the bay is about 61 km and

* Corresponding author
E-mail address: senolparug@gmail.com

the width at the opening to the Aegean Sea is about 36 km (Eronat and Sayın 2014; Cengiz et al. 2019b). As Saroz Bay had been closed to bottom trawl fishing since 2000 (Cengiz et al. 2011) and no industrial activity was prevalent in the area (Sarı and Çağatay 2001), the bay can be considered as a pristine environment (Cengiz et al. 2013; 2019c; 2019d).

A single specimen of *D. vulgaris* was caught off İbrice Bight (Saroz Bay) (Fig. 1) at 15 m depth with handline by fisherman on 12 June 2015. In legal regulations of Turkey (communique no: 2016/35), the total length is expressed as the projection length between the front end of the fish head and the end point of the longest ray of the caudal fin when the mouth is closed. Hereby, the specimen was subsequently measured to the nearest mm and weighted to the nearest g. Some morphometric and meristic characters were measured. Unfortunately, the specimen was not preserved as it was sold by a professional fisherman at the fish market.

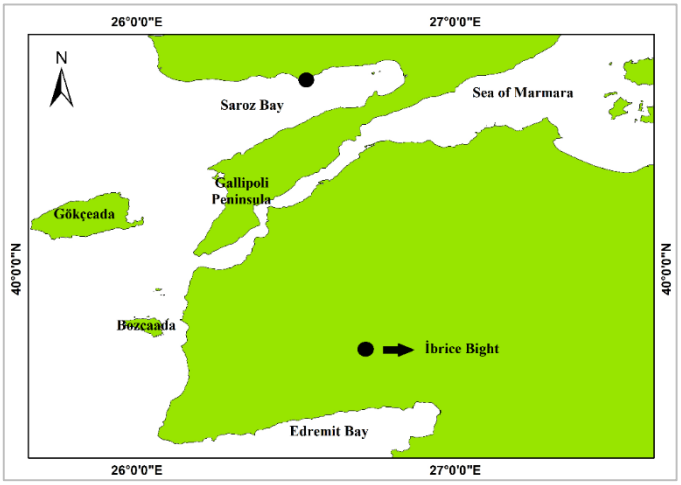

Figure 1. Saroz Bay and sampling station

Results and Discussion

The captured common two-banded seabream was 31.9 cm in total length and 467.00 g in total weight (Fig. 2). Some morphometric and meristic characters for *D. vulgaris* is presented in Table 1. The comparison of the maximum lengths and weights recorded for *D. vulgaris* in the Aegean Sea with Turkish waters is given in Table 2.

Figure 2. The common two-banded seabream with 31.9 cm TL and 467.00 g TW

Table 1. Some morphometric and meristic characters for *D. vulgaris* captured in the İbrice Bight (Saros Bay)

Morphometric characters	Value
Weight (g)	467.0
Total length (mm)	319.0
Fork lenght (mm)	302.0
Standard length (mm)	269.4
Body depth (mm)	100.0
Head length (mm)	70.9
Snout length (mm)	23.4
Eye diameter (mm)	17.4
Dorsal fin base length (mm)	135.4
Anal fin base length (mm)	57.8
Pectoral fin base length (mm)	98.5
Prepectoral length (mm)	105.6
Postorbital length (mm)	30.5
Caudal peduncle (mm)	48.2
Meristic characters	
Dorsal fin rays	XII - 15
Anal fin rays	III - 14
Pectoral fin rays	15

Table 2. The comparison of the maximum lengths and weights recorded for *D. vulgaris* in the Aegean Sea with Turkish waters

Author(s)	Area	N	L_{max} (cm)	W_{max} (g)
Petrakis and Stergiou (1995)	Euboikos Gulf, Greece	28	14.7	-
Can et al. (2002)	İskenderun Bay, Turkey	105	27.0	-
Moutopoulos and Stergiou (2002)	Cyclades, Greece	122	29.6	-
Karakulak et al. (2006)	Gökçeada Island, Turkey	93	25.0	-
Özaydın and Taşkavak (2006)	İzmir Bay, Turkey	63	15.4	80.00
Akyol et al. (2007)	Gökova Bay, Turkey	69	26.5	-
Gökçe et al. (2007)	North Aegean, Turkey	18	13.3	28.00
Gökçe et al. (2010)	İskenderun Bay, Turkey	22	17.9	91.77
İşmen et al. (2007)	Saroz Bay, Turkey	23	19.1	104.00
Özaydın et al. (2007)	İzmir Bay, Turkey	1615	23.1	-
İlkyaz et al. (2008)	İzmir Bay, Turkey	242	18.7	-
Karachle and Stergiou (2008)	Thermaikos Gulf, Greece	50	16.7	-
Acarli et al. (2009)	Homa Lagoon, Turkey	68	14.1	45.83
Gürkan et al. (2010)	Candarli Bay, Turkey	119	10.1	11.60
Cengiz (2013)	Gallipoli Peninsula, Turkey	50	28.4	347.08
Acarli et al. (2014)	Homa Lagoon, Turkey	81	15.2	52.90
Bilge et al. (2014)	Southern Aegean , Turkey	1893	23.1	-
Altın et al. (2015)	Gökçeada Island, Turkey	334	22.6	160.60
Kara et al. (2017)	Gediz Estuary, Turkey	87	13.0	31.80
This study	Saroz Bay, Turkey	1	31.9	467.00

As well known, the individuals in populations exposed to high levels fishing pressure will respond by reproducing at smaller average sizes and ages and so reached maximum

lengths may getting and getting smaller. But, the one individual that subjected to no overfishing pressure could be reached that kind of length (Filiz 2011; Cengiz et al. 2019e). On the other hand, any factor that might possibly influence growth has been shown to have an effect, including nutrient availability, feeding, light regime, oxygen, salinity, temperature, pollutants, current speed, nutrient concentration, predator density, intra-specific social interactions and genetics (Helfman et al. 2009; Acarli et al. 2018). In conclusion, the present study proves that this species can grow above the previous maximum data reported in the Aegean Sea with Turkish waters. The information presented here may be used to compare the similar parameters in ongoing fishery studies all over world by providing the scientific support to the fisheries scientists.

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RESEARCH ARTICLE

Plant-Space Relationship: An Example of Mosque Courtyard

Çiğdem Sakıcı* • Yasemin Pişkin

Kastamonu University, Faculty of Engineering and Architecture, Department of Landscape Architecture, Kastamonu/Turkey

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ABSTRACT

The most important spatial element which helps understanding and defining a place are the type of plants which give a certain character or symbol. Although it is difficult to reveal the perception of space and plant interactions by humans, it can be achieved through experimental studies. In this study, visual impressions of the users in evaluating the perception of plants with spaces were determined by experimental study and the survey technique was used. The study was conducted in Istanbul, and over 500 people including 100 primary school students, 100 secondary school students, 100 high school students, 100 university students and 100 university graduates participated in the survey. In this study, 28 plants, which are frequently seen and familiar with outdoor areas, were used. In this study, it was aimed to reveal the opinions about which of these plants were associated with the mosque courtyard and which characteristic of the plants were emphasized. Gender and educational level differences were investigated and results revealed that gender and educational levels effected participants' preferences. According to the results, flower bushes were preferred primarily for the mosque courtyard, and rose, pine, tulip, violet and buxus plants were preferred as the first choices respectively.

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Introduction

Cities are places with identities and soul (Tekeli, 1991). Places also have identities and soul. The first image presented by the places is a combination of both natural and structural elements; This space is the projection and connotations of us (Kalın, 1997; Williamson, 2001). This is the spatial elements and components that make up the places (Yalım, 2017). The most important of these elements and components are plants (Özbilen and Kalın, 2001). Plants are an important spatial component in terms of understanding our physical environment. Plants form the living structure of open-green areas (Tyson, 1998). It is a well known fact that plants have many contributions to open-green spaces and to the community in aesthetic and functional terms. However, eventhough plants have many contributions to the society from a psychological point of view (Sakıcı, 2014; Söderback et al., 2004), the symbolic meanings of plants are neglected by everyone (Kalın, 1997; Guiraud, 1990). With the help of this study, when plants are used in open green areas, it will be emphasized that plant preference should be made specifically depending on the property of the place. In addition, it will be determined evoke meanings of plants, mental stimulation through these meanings, history revival property of plants and preservation of urban identity with the help of appropriate plant preferences. The relationship between the place and the plant will be revealed, an example of the courtyard of the mosque.

It is important and meaningful to symbolize a place, to recall, to announce, to promote, to embrace the entire scope of that place, such as its history, its special position in the society and its activities (Emin, 2012). That symbol is identical to that place. When it is said the space, it should be

* Corresponding author
E-mail address: csakici@kastamonu.edu.tr

understood as an icon, when the icon will be seen it should be recognised (Sakıcı, 2017). The symbolic value of the place is also important for the cultural continuity of the place (Tanyeli, 1988). It is argued that plant and spaces can be matched or identified with the help of this study.

Materials and Methods

While it is difficult to determine the perception of space-plant interactions by humans, it is possible to obtain targeted data with the help of experimental studies. In this study, the visual impressions of the users in the evaluation of the perception of plants with the spaces was revealed with an experimental study. For this purpose, the survey technique was used (Özbilen and Kalın, 2001). In order to determine the plants identified with the mosque courtyard, they were asked to write the first three plants in the courtyard of the mosque. In the survey, a table which consist of the visuals and names of the 28 plants which is considered to be the most widely recognized by the public and commonly used in Istanbul was created and the first three plants were determined with the help of this table (Table 1). As a result of the study, the prominent plant species in the courtyard of the mosque, the characteristic features of the prominent plants and the prominent plant dimensions were determined and the plants were identified with the courtyard of the mosque. In addition, chi-square analysis was used to determine whether educational levels and gender differences have an impact on plant space identification. For this research, Suleymaniye Mosque Courtyard in Istanbul was chosen and plant species used in this area were determined and studied.

Table 1. Plants used in the study

Picea (Spruce tree)	*Acacia* (Acacia tree)
Pinus (Pine tree)	*Nerium* (Oleander)
Cupressus (Cypress Tree)	*Olea* (Olive-tree)
Platanus (Plane tree)	*Elaeagnus* (Oleaster Tree)
Salix (Willow tree)	*Robinia* (Round Acacia)
Tilia (Linden)	*Thuja* (Thuja)
Magnolia (Magnolia)	*Buxus* (Boxwood)
Populus (Poplar)	*Euonymus* (Spindle Tree)
Cercis (Redbud Tree)	*Rosa* (Rose)
Lagerstroemia (Needle Tree)	*Tamarix* (Tamarix)
Viburnum (Guelder Rose)	*Bougainvillea* (Bougainvillea)
Jasminum (Jasmine)	*Tulipa* (Tulip)
Lonicera (Honeysuckle)	*Papaver* (Poppy)
Vitis (Grapevine)	*Viola* (Violet)

In the determination of the plants to be used for this study, it was paid attention that the plants consisted of five different measure groups (trees, small trees, bushes, climbers and ground covers) and to be preferred from the plants we often see in our environment. According to their characteristic properties, these plants are divided into nine groups. Table 2 shows these both groupings according to characteristic properties and size of the plants.

Table 2. Grouping of the plants used in the study according to the characteristics properties and size

SIZE OF PLANTS	GROUP NAMES	CHARACTERISTICS PROPERTIES OF PLANTS	PLANTS
TREES	1.Group	Coniferous Trees	Picea, Pinus, Cupressus
	2.Group	Wide Leaf Trees	Platanus, Salix, Tilia, Magnolia, Populus
SMALL TREES	3.Group	Flowering Small Trees	Cercis, Lagerstroemia, Acacia, Nerium
	4.Group	Gray Colored Fruity Small Trees	Olea, Elaeagnus,
	5.Group	Widely Used Tijli Small Trees	Round Robinia
BUSHES	6.Group	Bushes Used for Live Fence	Thuja, Buxus, Euonymus
	7.Group	Flowering Bushes	Rosa, Tamarix, Viburnum, Jasminum
CLIMBER	8.Group	Clinging, Climber Plants	Lonicera, Vitis, Bougainvillea
GRANDCOVER	9.Group	Flowers	Tulipa, Papaver, Viola

Results and Discussion

Demographic Characteristics of Participants

The surveys were conducted on 100 participants from each education level in Istanbul. A total of 500 people were surveyed from primary, secondary, high school, university and university graduates. In total, 299 of the participants were female and 201 were male. Gender distribution by educational level is shown in Table 3.

Recommended Plants for Mosque Courtyard

The distribution of preferences of the all participants according to the different education level of participants is shown in Table 4, in order to determine the plants identified with the mosque courtyard. According to the results, the first 5 most preferred plants for the Mosque Courtyard were rosa (52% preference), pinus (25% preference), tulipa (25% preference), viola (20% preference) and buxus (18% preference). When we look at the distribution of preferences according to education levels, elementary school students rosa (62%), tulipa (38%) and viola (32%), secondary school students rosa (57%), viola (24%) and pinus (23%), high school students rosa (46%), salix (25%) and tulipa (25%), university students rosa (50%), pinus (24%) and platanus (23%), university graduates rosa (45%), pinus (36%) and cupressus (34%) were preferred and the first choice in each education level group was rose. Kalin (1997) was revealed that the most preferred plants for the Mosque Courtyard were Cupressus and Platanus in his study, but in this study, Cupressus and Platanus was preferred in tenth and seventh, respectively.

We divided the plants into 9 groups according to the characteristic properties of the plants. The distribution of preferences according to these groups is given in Table 5. According to the results, the most preferred group were flowering bushes (Group 7) with 342 preference, 274 preferred flowers (Group 9) and coniferous trees (Group 1) with 218

preferences. According to the results of statistical analysis, there was a difference between the preferences of the groups depending on the level of education (p = 0.000) and the primary school students preferred the most flowers for the mosque courtyard (81 Preference), while the secondary school (81 preference), high school (65 Preference) and university students (68 Preference) preferred flowering bushes and university graduates (78 Preference) preferred coniferous trees. There was also a difference between the preferences of the groups depending on the gender (p = 0.004) and the first choice for the mosque courtyard was flowering bushes for both women (198 Preference) and men (144 Preference). Table 6 shows the distribution of preferences depending on gender.

Table 3. Gender distribution according to the educational level of the participants

Educational Level		Primary School	Secondary School	High School	University	University Graduate	Total
Gender	Female	46	44	88	59	62	299
	Male	54	56	12	41	38	201
Total		100	100	100	100	100	500

Table 4. Distribution of plant preferences for mosque courtyard according to educational level

		MOSQUE COURTYARD (p=0,000)					
Number	**Plant**	**Primary School**	**Secondary School**	**High School**	**University**	**University Graduates**	**Total**
1	Rosa	**62**	**57**	**46**	**50**	**45**	260
2	Pinus	21	**23**	21	**24**	**36**	125
3	Tulipa	**38**	22	**25**	21	18	124
4	Viola	**32**	**24**	14	13	15	98
5	Buxus	16	21	14	15	24	90
6	Thuja	16	16	12	20	19	83
7	Platanus	13	17	13	**23**	13	79
8	Vitis	11	21	24	15	8	79
9	Salix	8	11	**25**	17	12	73
10	Cupressus	9	4	10	11	**34**	68
11	Lonicera	7	5	24	11	9	56
12	Papaver	11	16	9	8	8	52
13	Jasminum	11	15	12	10	1	49
14	Cercis	10	2	2	8	9	31
15	Populus	5	4	4	11	4	28
16	Lagerstroemia	7	4	4	6	7	28
17	Bougainvillea	4	6	4	7	4	25
18	Picea	5	1	8	3	8	25
19	Nerium	1	4	4	9	4	22
20	Tamarix	1	5	6	5	4	21
21	Euonymus	5	4	3	2	1	15
22	Magnolia	1	1	2	3	7	14
23	Viburnum	2	4	1	3	2	12
24	Tilia	1	3	2	3	3	12
25	Elaeagnus	2	1	5	1	1	10
26	Acacia	1	3	3	0	1	8
27	Olea	0	2	3	0	2	7
28	RoundRobinia	0	4	0	1	1	6

Table 5. Preference distribution according to the education level of plant groups according to the characteristic properties of the plants for the mosque courtyard

Plant Groups Depending on characteristic property	All		Group										
			Primary School		**Secondary School**		**High School**		**University**		**University Graduates**		
	n	%	n	%	n	%	n	%	n	%	n	%	*p*
7.Group	342	22,8	76	*25,3*	***81***	***27,0***	***65***	***21,7***	***68***	***22,7***	52	*17,3*	
9.Group	274	18,3	***81***	***27,0***	62	*20,7*	48	*16,0*	42	*14,0*	41	*13,7*	
1.Group	218	14,5	35	*11,7*	28	*9,3*	39	*13,0*	38	*12,7*	***78***	***26,0***	
2.Group	206	13,7	28	*9,3*	36	*12,0*	46	*15,3*	57	*19,0*	39	*13,0*	
6.Group	188	12,5	37	*12,3*	41	*13,7*	29	*9,7*	37	*12,3*	44	*14,7*	**0,000**
8.Group	160	10,7	22	*7,3*	32	*10,7*	52	*17,3*	33	*11,0*	21	*7,0*	
3.Group	89	5,9	19	*6,3*	13	*4,3*	13	*4,3*	23	*7,7*	21	*7,0*	
4.Group	17	1,1	2	*0,7*	3	*1,0*	8	*2,7*	1	*0,3*	3	*1,0*	
5.Group	6	0,4	0	*0,0*	4	*1,3*	0	*0,0*	1	*0,3*	1	*0,3*	

Table 6. Preference distribution according to the gender distribution of plant groups according to the characteristic properties of the plants for the mosque courtyard

Plant Groups Depending on Characteristic Properties	Gender				
	Female		Male		
	n	%	n	%	p
Coniferous Trees (1.Group)	143	*15,9*	75	*12,4*	
Wide Leaf Trees (2.Group)	136	*15,2*	70	*11,6*	
Flowering Small Trees (3.Group)	53	*5,9*	36	*6,0*	
Gray Colored Fruity Small Trees (4.Group)	13	*1,4*	4	*0,7*	
Widely Used Tijli Small Trees (5.Group)	3	*0,3*	3	*0,5*	**0,004**
Bushes Used for Live Fence (6.Group)	94	*10,5*	94	*15,6*	
Flowering Bushes (7. Group)	***198***	***22,1***	***144***	***23,9***	
Clinging, Climber Plants (8.Group)	107	*11,9*	53	*8,8*	
Flowers (9.Group)	150	*16,7*	124	*20,6*	

Table 7. Preference distribution according to the education level of plant groups according to the size of the plants for the mosque courtyard

Plant Groups (In terms of size)	All		Group										
			Primary School		Secondary School		High School		University		University Graduates		
	n	%	n	%	n	%	n	%	n	%	n	%	p
Trees	424	28,3	63	*21,0*	64	*21,3*	85	*28,3*	95	*31,7*	***117***	***39,0***	
Small Trees	112	7,5	21	*7,0*	20	*6,7*	21	*7,0*	25	*8,3*	25	*8,3*	
Bushes	***530***	***35,3***	***113***	***37,7***	***122***	***40,7***	***94***	***31,3***	***105***	***35,0***	96	*32,0*	**0,000**
Climber	160	10,7	22	*7,3*	32	*10,7*	52	*17,3*	33	*11,0*	21	*7,0*	
Grandcover	274	18,3	81	*27,0*	62	*20,7*	48	*16,0*	42	*14,0*	41	*13,7*	

The distribution of preferences according to the grouping based on the size of the plants is shown in Table 7. According to the results, bushes (530 Preference) and trees (424 Preference) were more preferred for the mosque courtyard. Preference distributions according to the level of education were shown diversity (p = 0.000), primary, secondary, high school and University prefered bushes, but university graduates preferred trees. Depending on the gender, there was a difference between the preferences of the groups (p = 0.001) and females (292 Preference) and males (238 Preference) were the first group of bushes for the mosque courtyard. Table 8 shows the distribution of preferences depending on gender.

Table 8. Preference distribution according to the gender of plant groups according to the size of the plants for the mosque courtyard

Plant Groups (In terms of size)	Gender				
	Female		Male		
	n	%	n	%	p
Trees	279	*31,1*	145	*24,0*	
Small Trees	69	*7,7*	43	*7,1*	
Bushes	**292**	***32,6***	**238**	***39,5***	**0,001**
Climbers	107	*11,9*	53	*8,8*	
Grandcovers	150	*16,7*	124	*20,6*	

Conclusion

With the help of this work, It was revealed that certain plants were preferred more amongst others for the mosque courtyard. This situation reveals that places can be identified with plants. The best five plants rosa, pinus, tulipa, viola and buxus were preferred for the mosque courtyard and there were differences in preferences according to the education level. However, people with different levels of education first preferred the rosa plant for the mosque courtyard. When we look at the distribution of preference according to the characteristic properties of the preferred plants for the mosque courtyard, firstly 'Flowering Bushes', second 'Flowers' and third 'Coniferous Trees' are preferred.'Widely Used Tijli Small Trees' and 'Gray Colored Fruity Small Trees' are not preferred.

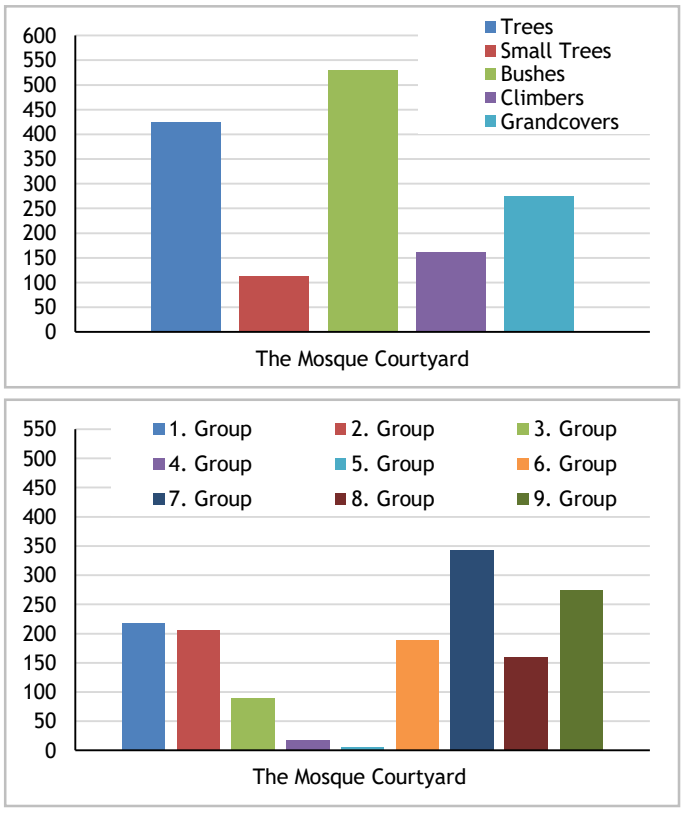

Figure 1. Preferred plants according to its size and characteristic properties for the mosque courtyard

When we look at the plants according to size grouping, respectively bushes and the trees were preferred for the courtyard of the mosque as a result of the study. The distribution of preferences of participants according to groups is shown in Figure 1. In addition, it was determined that the level of education and gender differences create differences in terms of preference distribution in both groups.

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RESEARCH ARTICLE

Association between the Crab, *Nepinnotheres pinnotheres* (Linnaeus, 1758), and the Endangered Species Fan Mussel, *Pinna nobilis* (Linnaeus, 1758), from the Aegean Sea, Turkey

Sefa Acarlı[1] • Pervin Vural[2] • Ahmet Öktener[3*]

[1]*Çanakkale Onsekiz Mart University, Faculty of Marine Sciences and Technology, Çanakkale/Turkey*
[2]*Çanakkale Onsekiz Mart University, Bayramiç Vocational School, Department of Aquaculture, Çanakkale/Turkey*
[3]*Sheep Research Institute, Department of Fisheries, Çanakkale Street 7km, Bandırma, Balıkesir/Turkey*

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ABSTRACT

Pinna nobilis, commonly known as the fan mussel or pen shell, is an endemic species in the Mediterranean Sea. The fan mussel population has recently been significantly endangered along the Mediterranean coast, mainly due to diseases. However, it has a critical role in the ecological system of the throughout the coasts of the Mediterranean. Therefore, it is important to investigate the interactions between *P. nobilis* and their enemies, parasites, symbiont in the ecological environment. Pea crabs are small crustacean and symbionts in a variety of invertebrates. They inhabit the mantle cavities of bivalve. The association between the pea crab, *Nepinnotheres pinnotheres* (Decapoda), and *P. nobilis* (Bivalvia) from the Aegean Sea, Turkey was examined in this study. The crab samples were collected off Urla Karantina Island, Izmir Bay, Aegean Sea. The biometric characteristics of bivalve and crab in this coexistence were analyzed. 80% of *P. nobilis* was occupied by *N. pinnotheres*. The weight of pea crabs was recorded between 0.01 g and 3.87 g.

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Introduction

Commonly known as fan mussel, pen shell, or wings shell, *Pinna nobilis* is the largest bivalve (shell length growing up to 120 cm) and a species endemic to the Mediterranean (Zavodnik et al., 1991). Fan mussel shell length usually varies between 20 and 40 cm (Fischer et al., 1987). They live at depths from 0.5 to 60 m (Zavodnik et al., 1991; Theodorou et al., 2017) and have a long life (27 years) (Galinou-Mitsoudi et al., 2006). It is an infaunal distributed in mud, sandy mud, silt, rocks bottom, and holds on to small gravel or pieces of shells by its byssus (Tebble, 1966; Theodorou et al., 2015). Furthermore, *Posidonia oceanica* meadows can be seen intensively in the habitats of fan mussel (García-March et al., 2006). *P. nobilis* is filter feeding organisms that feed on suspended organic matter, inorganic matter, zooplankton, phytoplankton, bacteria, and viruses from the water column (Gosling, 2003; Davenport et al., 2011). Thus, it contributes to an increase in water quality. Because of this, it has a vital role in the ecological system of the Mediterranean Sea (Natalotto et al., 2015).

The fan mussel population has recently become dramatically reduced on the Mediterranean seashore, particularly in countries such as France, Greece, Spain, Italy, and Croatia (Vicente and Moretau, 1991; Catanese et al.,

* Corresponding author
E-mail address: ahmetoktener@yahoo.com

2018). In some regions, the population density has dropped from 17 individuals per 100 m^2 (Richardson et al., 1999) to 1 individual per 100 m^2 (Katsanevakis and Thessalou-Legaki, 2009). Nowadays, in some areas in the western Mediterranean and the Tyrrhenian Sea, Italy, 100% of deaths occurred (Catanese et al., 2018; Carella et al., 2019). Moreover, Katsanevakis et al. (2019) carried out a study about the mass mortalities of the *Pinna nobilis* in the Lesvos Island, northern Aegean Sea. They identified the occurrence of parasite *Haplosporidium pinnae* with 100% prevalence as histopathological and molecular analysis examinations. *P. nobilis* is under threat of extinction due to damage to coastal habitat, increased water pollution, the destruction caused by fishing activities, harvesting for the decorative use, the damage made by dragging anchors of recreational boats, the presence of a haplosporidianum parasite in the digestive tract, and anthropological activities (Vincente and Moreteau, 1991; Ayaz et al., 2006; Hendriks et al., 2013; Deudero et al., 2015; Capó et al., 2015; Dariba, 2017). Due to these causes, *P. nobilis* has been listed as an endangered species in the Mediterranean ecosystem and has been under strict protection according to Annex II of the Barcelona Convention (SPA/BD Protocol 1995) and the European Council Directive 92/43/EEC (Annex IV). In Turkey, *P. nobilis* has been under protection since 1998 when Directorate of Food and Control, Ministry of Food Agriculture and Livestock issued a regulation, circulation No. 32/1, on Marine and Inland Commercial Fisheries.

Pea crabs live either parasitic or in commensal association with an array of marine organisms and are ubiquitous throughout the world. They inhabit the mantle cavity of bivalves (Palmer, 1995) and gastropods (Geiger and Martin, 1999), in the tubes of polychaetous worms (Grove and Woodin, 1996), in the gut cloaca, and respiratory trees of holothurians (Hamel et al., 1999), the oral surface of sea urchins, and pharyngeal cavity of tunicates (Reeves and Brooks, 2001). Pinnotherid crabs are used as a permanent shelter and often as a food source by the breeding female (Becker and Türkay, 2017).

Nepinnotheres pinnotheres (described as *Cancer pinnotheres* Linnaeus, 1758) is known as pinna pea crab with brown color. Carapace average length is 7 mm for male and 12 mm for female (Hayward and Ryland, 1995). This species is parasitic and found in the mantle cavity of the *P. nobilis* and also in ascidians (Becker and Türkay, 2017). It is thought that *N. pinnotheres* live in *P. nobilis* as an impermanent refuge. Therefore, more than one male is encountered in the same *P. nobilis* (Rabaoui et al., 2008).

At present, the population of *P. nobilis* is under significant threat on the Mediterranean coasts. Moreover, little information on the behavior, morphology, ecology, enemies, and diseases of *P. nobilis* is known and there is need of detailed investigations. This study examines the association between the fan mussel, *P. nobilis* and the pea crab, *N. pinnotheres*.

Materials and Methods

This study was carried out with individuals sampled during the investigation of reproductive cycle biochemical composition of *P. nobilis* between March 2008 and February 2009 by Acarlı et al. (2018). The materials were obtained monthly and a total of one hundred and fifteen individuals of fan mussels were examined throughout the study. The study area is shallow coastal water with a depth from 5 to 20 m. The bottom is primarily composed of muddy sand and covered with meadows (Figure 1).

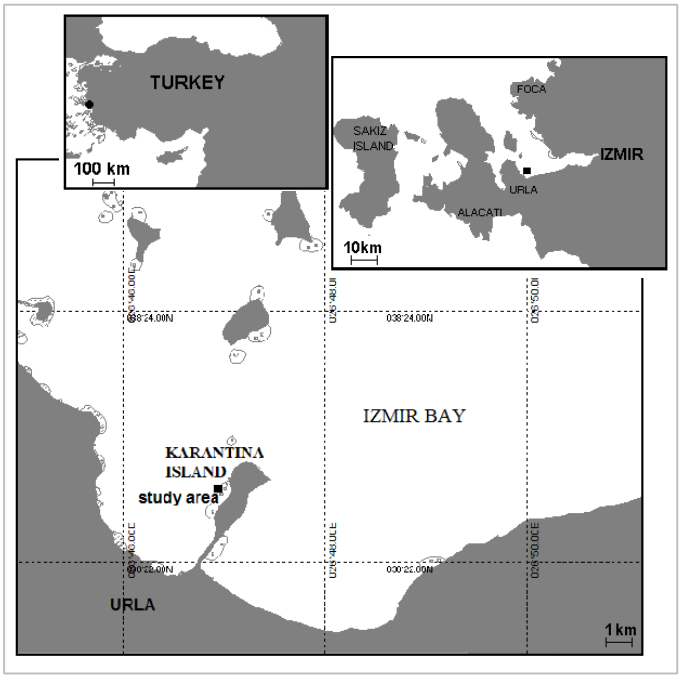

Figure 1. Map showing the study area

Fan mussels were transported to the Shellfish Laboratory of Fisheries Faculty (Ege University, Izmir, Turkey). Biofouling and other adherence were entirely removed from samples that then were for biological parameters such as shell length and total weight. Total weight and shell length were measured with a caliper and an electronic scale, respectively. Anterior and posterior adductors of *P. nobilis* were cut by a knife, and then its shells were opened. Careful examination for pea crabs was made inside fan mussels, and species were determined using the key of Silas and Alagarswami (1965). The monthly distribution of infested individuals was identified during the study period. Each pea crab was weighed during the study period. The Kolmogorov-Smirnov Test was applied to determine the normality of the data. According to the results of this test, Pearson's correlation analysis was used to assess the degree of association between host size and weight of pea crab. Data were analyzed using SPSS 13.0 for Windows.

Results and Discussion

The shell length of *P. nobilis* from the Aegean Sea was recorded as 51.45 cm (min. 36.5- max. 73.5), and width as 17.36 cm (min.12.5- max.24.5). The weight and thickness were recorded as 2076.4 g (min.864.9- max.4533.9) and 6.11 cm (min.3.9- max.12), respectively.

The crab samples were found in the mantle of the fan mussel (Figure 2). A total of 93 pea crabs were found in 84 *P. nobilis* individuals. 73% of the population was infected.

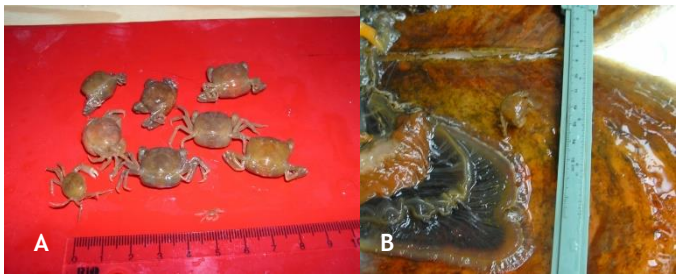

Figure 2. Photographs showing pea crabs (A) and pea crab on the *P. nobilis* shell (B)

Seventy-eight fan mussels were occupied by one crab, one fan mussel by three crabs, four fan mussels by two crabs, and one fan mussel by four crabs. The weight of pea crabs was recorded as between 0.01 g and 3.87 g (Figure 3). The shell length of infested fan mussels ranged between 38 cm and 73.5 cm (Figure 4). When the association rate of *N. pinnotheres* and *P. nobilis* seasonally is examined, infested fan mussels were observed throughout the year, reaching the highest value with %100 in April. The lowest value was found to be in October with 70% infestation (Figure 5). No correlation was found between the weight of the pea crab and host size (P>0.05) (Figure 6).

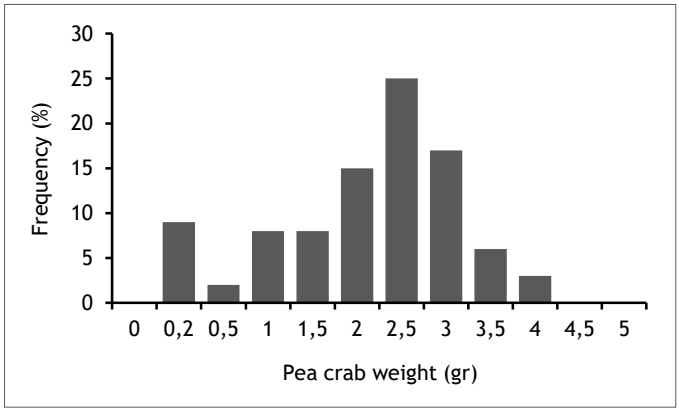

Figure 3. Weight frequency of pea crab, *Nepinnotheres pinnotheres*

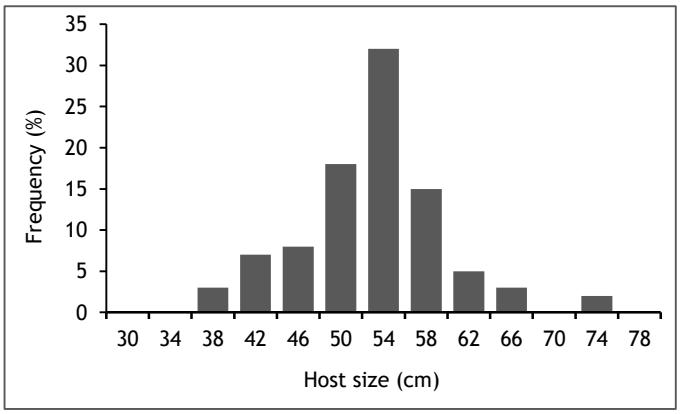

Figure 4. Length frequency of infested *Pinna nobilis* population of Urla Karantina Island

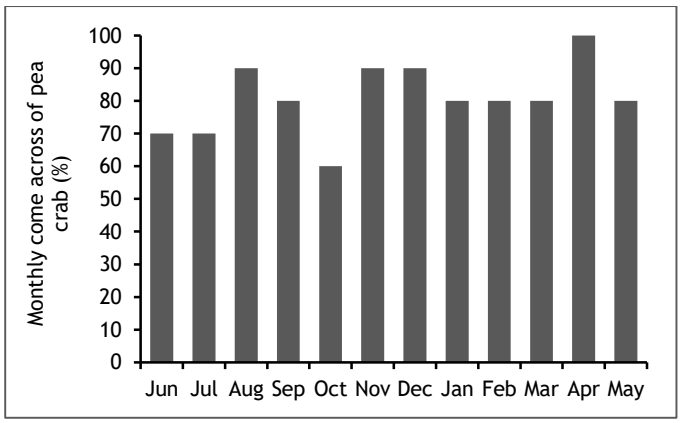

Figure 5. Monthly variation of infested *Pinna nobilis* (%)

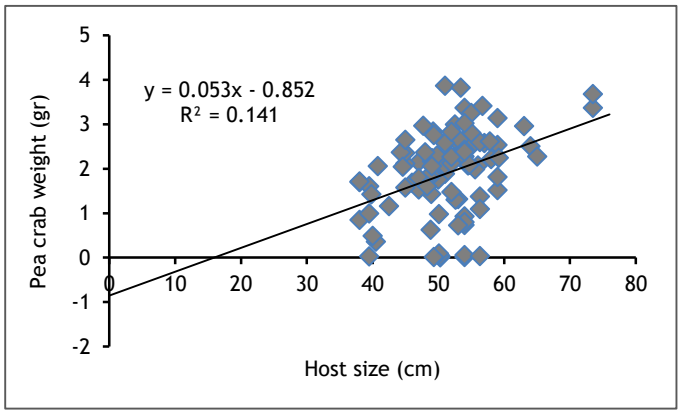

Figure 6. Relationship between infested *Pinna nobilis* (cm) and pea crab, *Nepinnotheres pinnotheres*

N. pinnotheres were reported especially on fan mussel *Pinna nobilis* (Rabaoui et al., 2008; Becker and Türkay, 2010; Cabanellas-Reboredo et al., 2010; Akyol and Ulaş, 2015; Trigos and Vicente, 2018), *Atrina pectinata* (Delongueville and Scaillet, 2002), *Perna canalicus, Mytilus edulis, Mactra ocata* (Stevens, 1990), and ascidians such as *Halocynthiapapillosa*, *Microcosmus* spp. (Becker and Türkay, 2017), and *Ascidia mentula* (Lutzen, 1967; Becker and Türkay 2017).

There is no detailed study on the association between the crab, *N. pinnotheres* and *P. nobilis*. Only, Rabaoui et al. (2008) found an association of 56.7% between *N. pinnotheres* and *P. nobilis* from StahJaber. Cabanellas-Reboredo et al. (2010) identified *N. pinnotheres* in 8.3% of the fan mussel samples they examined. The association rate of *N. pinnotheres* in *P. nobilis* in the Aegean Sea is found to be higher than those reported in those studies. This difference possibly may be explained with environmental factors such as tide, flow, sediment structure, amount of nutrition, mollusk status (length of buried shell and age of mussel), or amount of fan mussel examined by researchers.

There are several studies about epibionts (Giacobbe, 2002, Cosentino and Giacobbe 2007, Rabaoui et al., 2009, Addis et al., 2009) and commensals (Richardson et al., 1997; Kennedy et al., 2001, Calafiore et al., 1991, Laganà et al., 2007; Rabaoui et al., 2008, Rada and Milat, 2009; Cabanellas-Reboredo et al., 2010, Akyol and Ulaş, 2015) of fan mussel. Acarlı et al. (2010) and Basso et al. (2015) stated that fan

mussel has a rich biodiversity that is composed of macrobenthos, epibionts, and commensal species.

Va´zquez-Luis et al. (2015) examined the mass mortalities of the bivalve *P. nobilis* with mortality rates up to 100% in the Spanish Mediterranean Sea. Their histological examinations indicated that a haplosporidian-like parasite living in the digestive gland is responsible for these mortalities. Darriba (2017) reported that a parasite belonging to the haplosporidian group infected *P. nobilis* and caused mortality in Alicante (Spain, Western Mediterranean). On the Spanish coasts of the Western Mediterranean Sea, *Haplosporidium pinnae* are most likely responsible for the mass mortality of *P. nobilis* (Catanese et al., 2018). In Campania and Sicily, large scale mortality of *P. nobilis* population was noticed pen shella of all sizes because of mycobacterial diseases (Carella et al., 2019).

Trottier (2012) indicated that pea crab *Nepinnotheres novaezelandiae* (Filhol, 1885) caused a significant loss of production on aquaculture in New Zealand green-lipped mussels, *Perna canaliculus*. Pea crabs lead to a variety of negative effects such as gill damage, shell distortion, nodule formation, and decrease in condition index to their host (Trottier, 2014). Becker and Türkay (2017) indicated that larger hosts were probably preferred due to more significant food resources. Consequently, it might be expected that *P. nobilis* is a highly infested host. There was a relationship between damage to hosts and host size. Thus, small individuals infested with pinnotherids may be more severely affected than older individuals.

Conclusion

In this study, the damage to the tissue of the infested fan mussel was inconspicuous during the whole study period. We think that *N. pinnotheres* is not directly effective on the healthy *P. nobilis* population. On the other hand, with adverse environmental conditions or the spread of infectious diseases of bivalve such as a mycobacterial and a haplosporidian parasite, it may threaten its physical condition.

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RESEARCH ARTICLE

The Variability of the Predominant Culturable Plant Growth-Promoting Rhizobacterial Diversity in the Acidic Tea Rhizosphere Soils in the Eastern Black Sea Region

Ramazan Çakmakçı*

Çanakkale Onsekiz Mart University, Faculty of Agriculture, Department of Field Crops, Çanakkale/Turkey

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ABSTRACT

The purpose of this study was to investigate the diversity of cultivable nitrogen fixing, phosphate solubilising and total bacteria originated from 580 rhizospheric acidic soils samples of tea plants grown at 62 locations. Based on FAME profiles of over 1428 rhizoplane bacteria, 63 bacterial genera were identified with a similarity index > 0.3, but 56.4% of the identified isolates belonged to six genera: *Bacillus* (37.02%), *Pseudomonas* (12.67%), *Stenotrophomonas* (5.71%), *Paenibacillus* (6.58%), *Arthrobacter* (4.35%) and *Brevibacillus* (3.98%). Most of the total, N_2-fixing and P-solubilizing bacteria isolated were Gram positive (59.9, 58.8 and 56.3%) and Gram negative constituted only 40.1, 41.2 and 43.7%. Among different groups, *Firmicutes*, *Gammaproteobacteria* and *Actinobacteria* comprised the largest groups contributing to about 50.3 and 46.6%, 30.8 and 32.5%, and 8.3 and 9.6% of the total N_2-fixing and P-solubilizing isolates, respectively. *B. cereus*, *P. fluorescens*, *B. megaterium*, *S. maltophilia*, *P. putida*, *B. licheniformis*, *B. pumilus*, *B. subtilis* and *P. polymyxa* were the most frequent N_2-fixing and P-solubilizing species in the acidic tea rhizosohere soils. In these studies were evaluated to represent the dominant culturable diversity of diazotrophs and phosphobacteria, and thus potentially beneficial to the growth and survival of tea plants in that specific acidic ecosystem of eastern Black Sea region.

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Introduction

The composition of the bacterial community associated with plant roots is influenced by a variety of plant and environmental factors (Poonguzhali et al. 2006). Soil and plant species affect the indigenous bacterial soil communities. Some studies conclude that plant species have the greatest effect on community structure (Costa et al. 2006) whereas others shown that soil type have the greatest effect (Fierer and Jackson 2006). Rhizosphere microorganisms in turn having a great impact on root biology, influence plant growth, nutrition and development. Microorganisms colonizing the rhizosphere can affect plant growth both positively and negatively, the term plant growth promoting rhizobacteria (PGPR) often describes beneficial rhizobacteria that stimulate plant growth (Asghar et al. 2002). Selection of an efficient PGPR requires an understanding of the composition and diversity of the root-associated bacteria, and characterization of its plant growth promotion-related properties. For this reason, there has been considerable interest in examining the effect of soil type, plant species and root zone location on bacterial community structure in the rhizosphere (Varmazyari and Çakmakçı, 2018). Furthermore, a good selection of PGPR strain requires an understanding the dynamic and composition of the bacterial

* Corresponding author
E-mail address: rcakmakci@comu.edu.tr

communities colonizing the rhizosphere and characterization of its PGP related properties (Donate-Correa et al. 2004; Çakmakçı et al., 2010).

Tea (Camellia sinensis) the most important plant of Turkey is used in the traditional preparation of its national food and is planted widely on acidic soils. Turkey has the second largest tea market in the world. Turkish tea is produced on the eastern Black Sea coast, which has a mild climate with high precipitation and fertile soil. Tea gardens are usually grown as a monoculture and receive considerable amounts of fertilization, root exudates, and leaf litter (Çakmakçı et al., 2010). There is very little knowledge on the rhizosphere microbiology of the tea plants (Xue et al., 2006; Çakmakçı et al., 2010). Also, little information is available regarding the microbial community characteristics in tea garden soil ecosystems (Xue et al. 2008). Apart from our studies, the effect of the tea plants on the rhizospheric bacteria has not been studied so far in this area. These pioneering studies have been carried out on the diversity and functional importance of N_2-fixing bacteria (NFB) and P-solubilizing bacteria (PSB) in the acidic tea orchard soils in the eastern black sea region. A significant portion of the research results have already been published (Çakmakçı et al., 2010) and this article briefly summarizes some of the data on bacterial diversity. As a result, the objective of this present study was to isolate and identify plant growth promoting rhizobacteria from the rhizosphere of tea grown in eastern Black Sea region, and characterize them for phosphate solubilization and and nitrogen fixation.

Materials and Methods

Soil Samples, Isolation and Identification of Bacteria

The field of rhizosphere microbiology of tea garden soil ecosystems unexplored and in this work, we have isolated the bacterial population from the rhizosphere of tea plants production zones of various agro climatic regions of Rize and Trabzon during June–September, 2006-2016. The study area is located on the eastern Black Sea region, most of the country in Rize, between 40°50' and 41°20' N and 38°49' and 41°28' E. The tea gardens were established between 1938 -1944 years and now all of them are between 65-70 years old. It was found that the acidity of tea soils are too high depends on the quality and quantity of used nitrogen and in 80% of the soils the P content is low or too low. The tea plantations have been surveyed and 580 acidic soils samples from 62 locations were collected.

Rhizosphere soil samples were collected from healthy field-grown plant. Ten gram of the soil for each individual tea plant adhering to the roots, considered the rhizospheric soil was mixed and used for the bacterial isolation procedures (Çakmakçı et al., 2010; Karagöz et al., 2012). Uprooted plants along with a good amount of non-rhizosphere soil were brought immediately to the laboratory in polythene bags and air-dried. The non-rhizosphere soil was removed by gentle shaking whereas the soil adhering strongly to the root was referred to as rhizosphere soil. Rhizobacteria isolates were randomly selected from agar-solidified trypticase soy broth, and identified using fatty acid methyl ester (FAME) profiles. The method was carried out according to the described procedure already (Çakmakçı et al., 2010; Karagöz et al., 2012). Only strains with the similarity index (SIM) ≥0.3 were considered a good match (Oka et al. 2000). Fatty acid methyl ester (FAME) analysis is a well-established method for bacterial identification based on whole cellular fatty acids derivatized to methyl esters, analyzed by gas chromatography (Poonguzhali et al. 2006).

Nitrogen Fixation and Phosphate Solubilisation

Isolation and purification of N_2-fixing strains were carried out in an N-free solid malate-sucrose medium (NFMM) modified from Döbereiner (19899. Modified NFMM medium per liter distilled water (sucrose, 10.0 g; L-malic acid, 5.0 g; $MgSO_4$, H_2O, 0.2 g; $FeCl_3$, 0.01 g; NaCl, 0.1 g; $CaCl_2.2\ H_2O$, 0.02 g; K_2HPO_4, 0.1 g; KH_2PO_4, 0.4 g; $Na_2MoO_4.H_2O$, 0.002 g) with 18 g agar for solid mediumwas used for isolation. The medium adjusted to pH 7.2 with 1 N NaOH prior to agar addition and was then sterilized at 121 ºC for 20 min in an autoclave (Xie et al., 2003). N-free medium was used in order to obtain nitrogen fixing PGPR (Piromyou et al., 2011). Phosphate solubilization activity of the bacterial isolates was detected on Pikovskaya (1948) and National Botanical Research Institute's phosphate growth medium (NBRIP-BPB). NBRIP-BPB contained (per liter): glucose, 20 g; $Ca_3(PO_4)_2$, 10 g; $MgCl_2\ 6H_2O$, 5 g; $MgSO_4\ 7H2O$, 0.25 g; KCl, 0.2 g; $(NH_4)_2SO_4$, 0.1 g, and BPB, 0.025 g. To compare the reproducibility of the halo formation, pH indicator bromophenol blue was supplemented phosphate growth medium. Phosphate solubilization was was carried out according to the described procedure already (Çakmakçı et al., 2010).

Results

In our studies, a total of 1428 colonies were selected from the acidic tea rhizosphere. Over 1428 rhizoplane bacteria were randomly selected from agar-solidified trypticase soy broth, and identified using fatty acid methyl ester (FAME) profiles. The MIDI system identified (SIM > 0.3) 56.4% (805 out of 1428) of the bacteria isolated from the rhizosphere of tea and 38.9% (556 out of 1428) of the bacteria fixed nitrogen and 31.2% (446 out of 1428) solubilized P from insoluble calcium phosphate on NBRIP medium (Table 1). These isolates showed significant differences in their phosphate solubilizing potential, their extend of phosphate solubilization ranged between 22.9–172.8 mg L^{-1} liquid medium. Eight hundred five dominant, morphologically distinct rhizobacteria were purified, which belonged to 63 genera and 122 species. Furthermore, it assigned an additional 30.2% (431 out of 1428) of the tea isolates a taxonomic name based on a similarity index of less than 0.3. About 30.2% of the bacterial isolates could not be classified to genus since their similarity indices were <0.3 indicating no close matches. The majority of them were identified as belonging to the *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Stenotrophomonas*, *Arthrobacter* and *Brevibacillus* genus. The analysis of FAME profiles for the

total bacteria isolated, facilitated their classification under four bacterial divisions: *Bacteroidetes* (1.1%), γ, β and α-subdivisions of *Proteobacteria* (29.6%, 6.2% and 3.2%, respectively), *Firmicutes* (50.1%), and *Actinobacteria* (9.8%). Also about 13.4% of the isolates were classified as flagged since there were no matches or the analysis was of unacceptable quality (Table 1).

Table 1. Diversity of culturable P-solubilizing and N_2-fixing bacteria in the acidic tea rhizosphere soils

Taxonomic identification	Order	Bacterial strain FAME identification	Number of isolates[a]	N_2-fixing isolates	P-solubilizing isolates
Alphaproteobacteria	*Rhizobiales*	*Rhizobium radiobacter*	8	6	4
		Rhizobium rubi	1	1	1
		Phyllobacterium rubiacearum	2	1	1
		Roseomonas fauriae	6	5	4
		Ochrobactrum anthropi	2	2	1
	Rhodobacterales	*Paracoccus denitrificans*	2	2	1
Betaproteobacteria	*Burkholderiales*	*Burkholderia cepacia*	11	8	7
		Burkholderia pyrrocinia	4	4	3
		Ralstonia eutropha	3	2	2
		Ralstonia pickettii	1	1	1
		Ac. xylosoxidans denitrificans	6	5	3
		Acidovorax facilis	3	1	3
		Acidovorax konjaci	1		1
		Alcaligenes faecalis	16	9	8
Gammaproteobacteria	*Xanthomonadales*	*Lysobacter enz. enzymogenes*	15	12	10
		Pseudoxanthomonas sp	5	4	3
		Stenotrophomonas acidaminiphila	10	6	6
		Stenotrophomonas maltophilia	36	28	21
	Pseudomonadales	*Pseudomonas alcaligenes*	7	5	4
		Pseudomonas agarici	5	3	2
		Pseudomonas aurantiaca	2		1
		Pseudomonas chlororaphis	3	2	1
		Pseudomonas fluorescens	39	28	26
		Pseudomonas mucidolens	1	1	
		Pseudomonas putida	28	22	19
		Pseudomonas pseudoalcaligenes	1		1
		Pseudomonas stutzeri	3	3	1
		Pseudomonas syringae maculicola	3	3	2
		Pseudomonas atrofaciens	3	2	1
		Pseudomonas sp.	7	4	3
		Acinetobacter calcoaceticus	15	9	7
		Acinetobacter lwoffii	5	3	2
	Alteromonadales	*Pseudoalteromonas tetraodonis*	1	1	1
		Aeromonas hydrophila	1	1	1
	Enterobacteriales	*Cedecea davisae*	1	1	1
		Enterobacter intermedius	2	1	1
		Citrobacter freundii	2	1	2
		Ewingella Americana	2	1	1
		Erwinia crysanthemi	3	1	3
		Hafnia alvei	4	3	4
		Photorhabdus luminescens	5	4	3
		Proteus vulgaris	5	4	3
		Rahnella aquatilis	3	2	2
		Providencia alcalifaciens	1		1
		Serratia fonticola	4	3	3
		Serratia marcescens	4	4	3
		Serratia plymuthica	2	2	1
		Pantoea agglomerans	3	3	3
Firmicutes	*Bacillales*	*Bacillus amyloliquefaciens*	2	1	
		Bacillus atrophaeus	10	9	8
		Bacillus badius	1	1	1
		Bacillus alcalophılus	2	1	1
		Bacillus cereus	78	55	28
		Bacillus coagulans	8	2	5

Taxonomic identification	Order	Bacterial strain FAME identification	Number of isolates[a]	N_2-fixing isolates	P-solubilizing isolates
		Bacillus globisporus	1	1	
		Bacillus laevolacticus	20	17	12
		Bacillus licheniformis	27	23	18
		Bacillus lentus	4	2	1
		Bacillus megaterium	37	28	25
		Bacillus mycoides	11	7	4
		Bacillus parabrevis	1	1	1
		Bacillus pumilus	26	20	16
		Bacillus simplex	6	3	2
		Bacillus sp	14	8	7
		Bacillus sphaericus	26	13	8
		Bacillus subtilis	24	20	16
		Bacillus thuringiensis	2	1	
		Paenibacillus alvei	1	1	1
		Paenibacillus azotofixans	2	2	1
		Paenibacillus larvae	3	2	2
		Paenibacillus lentimorbus	6	4	3
		Paenibacillus macquariensis	7	5	6
		Paenibacillus polymyxa	20	17	12
		Paenibacillus validus	14	11	8
		Brevibacillus choshinensis	12	8	5
		Brevibacillus centrosporus	8	4	3
		Brevibacillus parabrevis	3	2	1
		Brevibacillus reuszeri	9	3	4
		Geobacillus stearothermophilus	1	1	1
		Kurthia gibsonii	1	1	1
		Kurthia sibirica	11	3	5
Actinobacteria	*Actinomycetales*	*Arthrobacter pascens*	2	1	1
		Arthrobacter agilis	4	3	2
		Arthrobacter aurescens	3	1	1
		Arthrobacter crystallopoites	1	1	1
		Arthrobacter citreus	2	1	2
		Arthrobacter globiformis	6	5	4
		Arthrobacter mysorens	4	1	2
		Arthrobacter viscosus	13	6	5
		Kocuria rosea	6	4	4
		Micrococcus lylae	5	4	4
		Micrococcus luteus	11	6	7
		Brevibacterium liquefaciens	4	2	1
		Microbacterium chocolatum	1	1	1
		Rhodococcus erythropolis	11	8	6
Bacteroidetes	*Flavobacteriales*	*Chryseobacterium indologenes*	5	3	3
Others[a]			32	18	13
No library match			192		
Unidentified[b]			431		
Total			1428	556	446

[a]Others includes the genera: *Brevundimonas*, *Methylobacterium, Rhodobacter, Xanthobacter*, *Comamonas, Kingella*, *Variovorax, Xanthomonas, Raoultella*, *Yersinia, Photobacterium, Clostridium*, *Enterococcus*, *Sporosarcina*, *Staphylococcus*, *Cellulomonas, Curtobacterium*, *Nocardia*, *Bergeyella, Flavobacterium Sphingobacterium*, N_2-fixing and P-solubilizing bacteria in these genera were only detected once or twice.
[b]Isolates named with a similarity index < 0.3.

The bacterial community of tea rhizosphere was composed by 40.1% Gram-negative, 59.9% of Gram-positive bacteria. The identified Gram-positive groups, comprising the community, were classified into four orders; *Actinomycetales, Bacillales, Clostridiales* and *Lactobacillales*. The order *Bacillales* was the most diverse, and was composed of seven different genera; *Bacillus*, *Paenibacillus*, *Brevibacillus*, *Kurthia*, *Geobacillus*, *Staphylococcus* and *Sporosarcina*. The order *Actinomycetales* was represented by eight genera: *Arthrobacter*, *Rhodococcus*, *Kocuria*, *Brevibacterium*, *Micrococcus*, *Cellulomonas, Curtobacterium* and *Microbacterium*; while the order *Clostridiales* and *Lactobacillales* was represented by the genera *Clostridium and* Enterococcus (Table 1).

Among non-enteric Gram-negative *Pseudomonads* and *Xanthomonads* group were the most abundant with three species identified (*Pseudomonas fluorescens* 12.1%, *Pseudomonas putida* 8.7% and *Stenotrophomonas maltophilia* 11.1%). Among Gram-negative bacteria, sixteen strains of

Alcaligenes faecalis, fifteen strain each of *Lysobacter enzymogenes enzymogenes* and *Acinetobacter calcoaceticus*, eleven strains of *Burkholderia cepacia*, eight strains of *Rhizobium radiobacter*, seven strains each of *Pseudomonas alcaligenes* and *Pseudomonas* sp. and six strains each of *Roseomonas fauriae and Achromobacter xylosoxidans denitrificans* were identified. The genus *Bacillus* was the 61.8% of the Gram-positive population (298 out of 482), with a prevalence of *B. cereus* (26.1%), followed by *B. megaterium* (12.4%), *B. licheniformis* (9.1%), *B. pumilus* and *B. sphaericus* (8.7%) and *B. subtilis* (8.1%). Also, the *Bacillus* group was the most abundant with five other species identified (*B. laevolacticus*, *Bacillus* sp., *B. mycoides*, *B. atrophaeus*, *B. coagulans*). Gram-positive *Paenibacillus* genus was the second most abundant (11.0%) with seven species identified (*P. polymyxa*, *P. validus*, *P. lentimorbus*, *P. macquariensis P. larvae*, *P. azotofixans* and *P. alvei*). Among the other Gram-positive bacteria, twelve strain of *Brevibacillus choshinensis*, thirteen strain of *Arthrobacter viscosus*, eleven strain each of *Kurthia sibirica*, *Micrococcus luteus* and *Rhodococcus erythropolis*, nine strains of *Brevibacillus reuszeri* and seven strain of *Brevibacillus centrosporu* were identified. *Arthrobacter* (7.3%) included the species *A. viscosus*, *A. mysorens*, *A. globiformis*, *A. agilis*, *A. aurescens*, *A. citreus*, *A. Pascens* and *A. crystallopoites*.

We selected two hundred and fourteen different potential PSB from a pool of 805 rhizobacterial isolates obtained from the tea rhizosphere on the basis of their P-solubilizing and N_2-fixing ability on NBRIP and N-free solid malate sucrose medium (NFMM) medium. Table 1 shows that 446 and 556 out of the 805 tested isolates had potential for P-solubilization and N_2-fixation, which 52 different known bacterial genera represented by *Bacillus* (34.3 and 38.1%), *Pseudomonas* (13.7 and 13.1%), *Paenibacillus* (7.4 and 7.6%), *Stenotrophomonas* (6.1 and 6.1%), *Arthrobacter* (3.8 and 3.2%), *Brevibacillus* (2.9 and 3.1%) as the predominant genera. Among different groups, *Firmicutes*, *Gammaproteobacteria* and *Actinobacteria* comprised the largest groups contributing to about 46.6 and 50.5%, 32.5 and 30.7 % and 9.6 and 8.3% of the total P-solubilizing and N_2-fixing isolates, respectively.

Discussion

The taxonomic identities of 63 genera from approximately 1428 rhizospheric root-associated bacteria isolated from 580 rhizospheric soil samples of tea, grown at 62 sites were determined. Of these 1428 isolates, 13.4% (192 isolates) could not be identified by the MIDI system since there were no matches or the analysis was of unacceptable quality. Also about 30.2% of the isolates (431/1428) were identified with a SIM <0.3 which indicates a tentative identification, and were not included in further analysis. Identification of the bacterial isolates was more successful in the tea rhizosphere samples expressing an overall identification of about 56.4% of the total isolates. *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Stenotrophomonas*, *Arthrobacter* and *Brevibacillus* genera were the most prominent N_2-fixing and P-solubilizing groups in the rhizosphere and soil populations analysed.

Characterization of the isolates on the basis of their FAME profiles revealed the presence of both Gram-positive and Gram-negative bacteria within the tea rhizosphere soils although larger number was that of Gram-positive. Most of the rhizospheric bacteria isolated were Gram-positive (59.9%) and Gram-negative constituted only 40.1%. Out of a total of 805 isolates, 323 belonged to Gram-negative, which included 238 γ-proteobacteria, 50 ß-proteobacteria, 26 α-proteobacteria,; 9 isolate belonged to the Bacteroidetes group. Major α-proteobacterial genera recovered from tea rhizospheres included several species of *Rhizobium*, *Roseomonas*, *Phyllobacterium* and *Paracoccus*. Major ß-proteobacterial included *Alcaligenes*, *Burkholderia* and *Achromobacter*, while *Pseudomonas*, *Stenotrophomonas*, *Acinetobacter*, and *Lysobacter* dominated the γ-proteobacterial genera. The 556 Gram-positive isolates included 403 *Firmicutes* and 79 *Actinobacteria*. The data obtained show a greater abundance of Gram-positive bacteria in the tea rhizosphere, in agreement with previous studies (Xue et al., 2008; Rau et al. 2009; Çakmakçı et al., 2010, Karagöz et al., 2012; Varmazyari and Çakmakçı 2018) that show a higher level of Gram-positive *Bacillus* and *Paenibacillus* species in the in the tea garden soils. Also the tea rhizoplane was dominated by *Bacillus* (37% or 298/805 identified isolates). The widely studied *Bacillus*, *Pseudomonas* and *Paenibacillus* genus represents one of the most diverse genera in the plant rhizosphere and soil populations (Beneduzi et al., 2008; Çakmakçı et al., 2010) and these species can be characterized with the ability to tolerate unfavourable conditions (Borsodi et al., 2007). Bacterial identification by the MIDI system indicated that *Bacillus*, *Pseudomonas* and *Paenibacillus* genera inhabit the rhizosphere of tea, and soil pH was the characteristic most closely related with their diversity.

We conducted a survey of dominant culturable N_2-fixing and P-solubilising bacteria naturally colonizing a mild climate with high precipitation and acidic soil in the eastern Black Sea biogeographical tea growing regions. The highest percentage of NFB and PSB was recorded *Firmicutes* in, followed by the *Gammaproteobacteria*. The results obtained indicated that *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Stenotrophomonas*, *Brevibacillus* and *Arthrobacter* genera were the most prominent culturable groups in the rhizosphere and soil populations. Bacteria from these genera are generally regarded as good phosphate solubilisers, nitrogen fixers and plant growth promoters (Xie et al., 2003; Şahin et al., 2004; Çakmakçı et al., 2006, 2007; Chen et al. 2006; Poonguzhali et al. 2006; Beneduzi et al. 2008; Hariprasad and Niranjana 2009; Rau et al. 2009; Varmazyari and Çakmakçı 2018). *B. cereus* was the most dominant culturable NFB and PSB in the acidic tea rhizosphere, followed by *P. fluorescens*, *B. megaterium*, *S. maltophilia*, *P. putida*, *B. licheniformis*, *B. pumilus*, *B. subtilis* and *P. polymyxa*. The ability of a few soil microorganisms to convert insoluble forms of phosphorus to an accessible form is an important trait in plant growth-promoting bacteria for increasing plant yields. We demonstrate that the natural acidic soil supports a diverse group of potential PSB. These PSB could serve as efficient biofertilizer candidates for improving the P-nutrition of crop plants. The advantage of using natural soil isolates over the genetically manipulated or the one which has

been isolated from a different environmental set up is the easier adaptation and succession when inoculated into the plant rhizosphere (Chen et al. 2006). Use of these acid tolerant P-solubilizing and N_2-fixing bacteria as bio-inoculants will increase the available N and P in soil and the N and P uptake by plants, helps to minimize the mineral fertilizer application, reduces environmental pollution and promotes sustainable agriculture. This strain could be useful in the formulation of new inoculants, improving the cropping systems into which it can be most profitably applied. The identification and the isolation of PGPR from acidic and P-deficient soils, which combine the ability to fix nitrogen and solubilize phosphate, could also significantly increase the productivity of crops in acidic soil.

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RESEARCH ARTICLE

The Effects of Different Intensity of Thinning on the Development in Scots Pine (*Pinus sylvestris* L.) Stands in Kazakh Uplands

Andrei V. Ebel[1] • Yekaterina I. Ebel[2] • Sergei V. Zalesov[3] • Sezgin Ayan[4*]

[1]*S.Seifullin Kazakh Agro Technical University, Faculty of Agronomy, Nur-Sultan/Kazakhstan*
[2]*Kokshetau Forest Breeding Center (KFBC), Cordon "School Forestry", Nur-Sultan/Kazakhstan*
[3]*Ural State Forest Engineering University (USFEU), Yekaterinburg/Russian Federation*
[4]*Kastamonu University, Faculty of Forestry, Department of Silviculture, Kastamonu/Turkey*

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ABSTRACT

The search for more accurate methods of predicting the growth and development of forest stands became the most urgent task set for foresters of Kazakhstan to determine the permissible interventions in the natural course of the life of plantings, provide high durability and resilience in forests. The aim of the study was to identify the effects of diameter and density of Scots pine stands of Kazakh Uplands on their growth and productivity and the related productivity of single plantation stands taking into account the conditions of growth and development of internal factors as well as further study of the methodology for assessing the forestry cost-effectiveness and improvement thinning. To achieve this aim, effects of varied felling intensities on Scots pine stands were studied. The most common two forest types in upland Scots pine forests were chosen as permanent sample plots; the dead pine-lichen and moss pine-grass. The results showed that improvement thinning of moderate and severe intensity which are more profitable should be done in Scots pine forests of Kazakh Upland as well as carrying out such thinning increases the yield of the larger logs and increases the value of the left stand.

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Introduction

To satisfy the growing demand for wood from local forests, keeping their best soil and water protection and shelterbelts functions, ensuring increased productivity of forests by at least 10-15% is the pioneer task of the Republic of Kazakhstan foresters. Zhukov (1976) points out: "*The cardinal solution to enhance the productivity of forests is closely related to a deep identification of patterns of development, the establishment of the relationship between the individual components of forest ecosystems and the formation of the structural features of plants in different environments*" to emphasize the main directions of forest biology science. Lebkov (1965, 1967), Plotnikov (1979) and many other studies highlighted the structure of forest stands in their researches.

Prevailing soil and climatic conditions become particular importance in the Scots pine forests of Kazakh Upland. Furthermore, taking into account the great value and increase in demand for Scots pine wood the importance of the further cultivation of this species become more visible in local forests. Besides this recent forest inventory data indicates a significant

* Corresponding author
E-mail address: sezginayan@gmail.com

change of distribution of Scots pine plantations more than that of little value aspen and birch. The prevalence of different tree species depends on their biological and environmental sustainability in these conditions, as well as on the level of forest management.

The predominance of Scots pine in the stand composition can be provided with the forestry measures as well as it can be significantly improved the condition and growth of plants. The search of more accurate methods of predicting the growth and development of forest stands become more urgent in accordance with the new challenges of forest cultivation for determining the extent measure of permissible interference in the natural processes; adjust their course and provide high durability and resilience of forest ecosystems. One of these methods which can contribute to the direction of growth of the trees in the woods is forest felling. Timofeev and Georgievski (1957) denotes that for various purposes improvement thinning can be used as a main silvicultural method of cultivation of healthy and highly productive forests.

Decreasing in workload of forestry in terms of felling is observed currently. The state institutions for the protection of forests and fauna have difficulties on carrying out forestry works because of current economic conditions in Kazakhstan. There is no exception in the felling of forests, because timber obtained during felling operations is one of the sources of their own funds, so it is important not to turn the felling of forests in the "cutting income" for receiving the wood will not become the main purpose, and in the process of care it will be not broken silvicultural principle of trees selection for felling. This is especially important during the felling, as it can lead not only to a decrease in growing stock in the age of final felling, but also to the loss of vegetation stability. At the same time, compliance with the requirements of silvicultural felling in general and migratory logging in particular can significantly increase the productivity of cultivated stands (Sennov 1977, 1984, 1999, 2005, Zalesov and Luganski 1989, Makarenko and Mukanov 2002). Thus, nowadays in economic environment it becomes more urgent to find ways of optimizing silvicultural and economic indicators of improvement felling. The aim of the research was to determine the effects of diameter and density of Scots pine (*Pinus sylvestris* L.) stands on growth and productivity at intermediate felling activities in Kazakh uplands.

Materials and Methods

The area of research is characterized by a sharply continental climate conditions on the background of a lack of moisture in the summer when evaporation is almost twice the amount of precipitation. In difficult soil and climatic conditions, Scots pine forests of Kazakh Upland have great soil and water protection, field and climate-regulating value because it has that geographical position. The amount of Scots pine forest is about 33.8 % of the total forest land area (Table 1).

Table 1. Distribution of forested land by dominant species and age groups for growth class III (by Taxation Description Government National Nature Park "Burabay")

Predominance species	Covered with forest area		Distribution of forest area by age groups, th. ha					
			Young stand		Middle aged	Maturing	Ripe and overripe	
	th. ha	%	I class	II class			total	including maturing
Pine	100,3	33,8	24,4	13,1	50,9	9,4	2,5	-
Larch	0,4	0,2	0,3	0,1	-	-	-	-
Maple	0,3	0,1	0,2	0,1	-	-	-	-
Elm	2,7	0,9	1,7	1,0	-	-	-	-
Birch	160,9	53,9	16,0	11,0	74,1	36,3	23,5	-
Aspen	29,4	9,9	3,5	4,9	7,9	7,1	6,0	-
Poplar	1,1	0,4	0,5	0,6	-	-	-	-
Willow	1,5	0,5	-	-	-	-	1,5	1,4
Bushes	0,8	0,3	-	-	-	-	0,8	0,5
Total	297,4	100	46,6	30,8	132,9	52,8	34,3	1,9

To measure effects of diameter and density of Scots pine stands of Kazakh Uplands on their growth and productivity, the study area was chosen from two forest types in most common pine forests of Kazakh uplands as called dead pine-lichen forest and moss pine-grass forest. The number of trees on each permanent sample plot with an area of 0.005 to 5 hectares were decreased at least 150-200 trees after felling where the initial number of trees were among 400 to 2000. Each sample area was divided into several sections, one section remained as a control plot and thinning of stands with varied intensity carried out on the rest. Ranking of felling changes among weak, moderate, strong and very strong where the selected stocks are 15, 16-25, 26-35 and > 35 %, respectively of the growing stock (Danchenko and Danchenko 2004).

Processing and description of sample plots were carried out according to the procedure adopted in forest management with the account of instructions (1995) to meet the requirements of OST 56-60-83 and methodological developments of Georgievski (1953), Molchanov (1967), Anuchina (1977, 1982), Verhunova (1979), Atrohina and Yeviņ (1985). Primary enumeration on sample plots allocated for felling was used for the study of growth patterns of thickened pine.

Sections on sample plots were laid a square or rectangular shape (Operating rules, 1995). In the corners of all the sections of permanent sample plots it was established posts, in accordance with the requirements of OST 56-44-80. Measuring the diameter was done with a caliper with an accuracy of up to 1 mm in two directions: N-S and E- W. When enumeration, trees are divided into categories of technical validity and by Craft's classes. Forest growth site was established by Orlov's scale (Orlov 1927), the fullness of the table of growth progress of pine layer continuum of Kazakh Upland was established by Makarenko's scale et al. (1980). Accounting of deadwood was done separately.

Identification of the impact on the completeness of the stands to the taxation rates was conducted by comparing a number of plots of the same age but with different fulfillment in different forest types. Stand density was determined by dividing the amount of space in the test section of the stand on the proper amount of space in the normal section of the stand for a certain "local tables total basal area and stocks of trees and shrubs in the fullness of 1.0" (Makarenko et al. 1980).

To determine the average height, it was measured the height of the altimeter BH-1 with an accuracy of 10 cm, followed by the construction of curves heights. To determine the total basal area of the stand it was held caliper measurement of the diameter up to 1 mm in two directions: NS and EW. Then, according to the enumeration of trees of diameter classes it was calculated cross-sectional area in each diameter and using the "*assortment tables of pine stands of Kazakh upland*" (Makarenko et al. 1987) and it was determined the amount of space per section and per 1 hectare. The average diameter was defined as the diameter of a circle, the area of which is equal to the estimated average cross-sectional area of one tree.

All digital material of fieldwork was processed by the traditional methods of mathematical statistics (Zdvorik, 1952, Svalov 1977, Gromyko 1981, Zaicev 1984). In carrying out the research work the generally accepted techniques used in forest inventory, forestry, soil science and biocenology were used. The experimental data was obtained by re-enumeration on the permanent sample plots. Taxation stands was performed instrumentally.

Results and Discussion

During the work it was revealed that Scots pine forests of Kazakh Upland is characterized with its considerable heterogeneity of relative completeness in both dead-lichen and moss-grass forest types. The dead pine-moss forest type is characterized with an increase in the completeness with increasing age tree stands. Pine stands of moss-grassy type of forest with the age of older than 60 years are characterized by the opposite behavior. High rates of relative density of stands of all ages recorded in the number of permanent sample plots indicate the need to clarify the standard tables for Scots pine of Kazakh Upland.

After analyzing the data of Table 2 it can be concluded that by the age 105 year dependent of natural mortality of the initial density of stand becomes more apparent. The higher the initial density of the stand, the higher the proportion of mortality among the trees. On the experimental plot №3 at the age of 93 the density makes up by the following sections: K-1 - 5500; K-2 - 6860; K-3 - 7000; K-4 - 6900 / ha, and the share of apostasy: K-1 - 88.5; K-2 - 85.1 K-3 - 74.8; K-4 - 88.0%.

Table 2. Changes in density of Scots pine stands of Kazakh Upland with age

Experimental plot №2

Section	Density, pieces (ps)/ha			Mortality			
				during 12 years		during 58 years	
	in the age of 47	in the age of 58	in the age of 105	ps/ha	%	ps/ha	%
K-1	11710	9384	3660	2326	19,9	8050	68,7
K-2	19150	13990	4880	5160	26,9	14270	74,5
K-3	19680	14652	5380	5028	25,5	14300	72,7
K-4	18540	16024	3920	2516	13,5	14620	78,9
Average	17270	13512	4460	3758	21,7	12810	74,2

Experimental plot №3

Section	Density, pieces (ps)/ha			Mortality			
				during 12 years		during 58 years	
	in the age of 35	in the age of 47	in the age of 93	ps/ha	%	ps/ha	%
K-1	47900	23974	5500	23926	49,9	42400	88,5
K-2	46000	29333	6860	16667	36,2	39140	85,1
K-3	27800	19504	7000	9296	32,2	20800	74,8
K-4	57700	30547	6900	27153	47,0	50800	88,0
Average	45100	25839	6565	19261	42,7	38535	85,4

The values of the original density in stands older than 35 years in the range of 0.6 - 1.5 does not have a significant impact on the average height of the stands at maturity. Thus optimum relative density in the 47 - year old pine stands of the dead-lichen forest type, in terms of the average height of the stand makes up 0.9-1.0, in the 35 years old pine stands of the same forest type it is - 0.71-0.8 and in the age of 60 - year old pine tree stand of moss-grass forest type, it is - 0.8, average difference values of 93-105-years-old pure Scots pine stands with different initial relative completeness usually does not exceed the accuracy of the determination of the taxation measure.

In the result of this research; it was revealed that in this type of forest, Scots pine forest is dead cover lichen to the age of 47, stands are with medium density and dense and almost equalize by the number of trees, and rare have lower density. By the age of 93-105 years, the density indicators in all forest stands are almost equal. It must be concluded that the higher the initial density, the more intense the natural felling of the stand and by the age of 93-105 years, regardless of the initial density in the stands is approximately equal to the number of trees.

One of the two types of Scots pine forests, moss pine-grass forest stand density is much lower than similar stands of the dead pine-lichen forest. Last pattern clearly shows that in the area of research, intensive felling in Scots pine stands of moss-grass forest type has significantly higher density than the dead pine-lichen.

Throughout the period of maximum forest growing, stock of stem wood is characterized by dense stands. At the same time in a pine forest of the dead-lichen differences in stock 35 years old and rare dense stands is 3.9 m^3/ha (5.9%), and 93 years - 101.3 m^3/ha (33.4%). The stock of mature pine stands of moss-grass exceeds that in rare and medium density, but inferior to that of the dense mature stands of the dead pine-lichen.

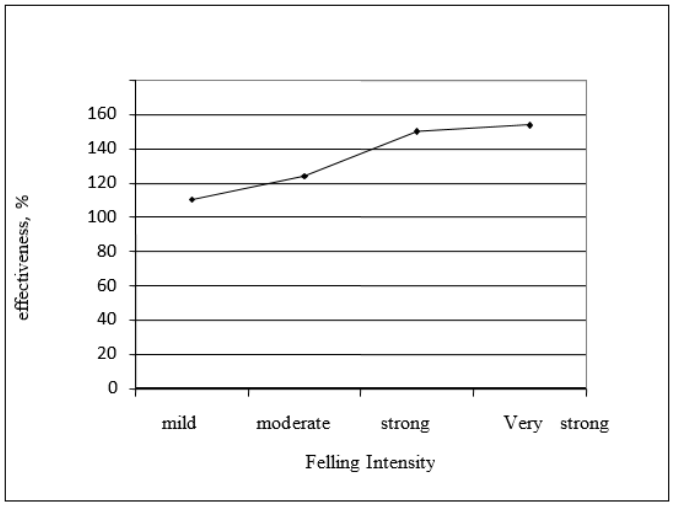

Figure 1. Distribution of overall economic efficiency, depending on the intensity of logging (Experimental plot № 3)

Figure 1 shows the analysis of the impact of logging on the value of the wood over time that there is accumulation of a larger timber on a more space areas and therefore its value increases. Demand for wood from improvement felling in the conditions of Northern Kazakhstan is quite high, especially since felling in coniferous of the Republic prohibited for 10 years on the basis of the Resolution of the Government of the Republic of Kazakhstan from 23.04.2004 № 460 "*On the prohibition of felling in coniferous and sexual plantations on the lands of the state forest fund and measures for their conservation*." At the same time, in spite of the high demand, some of the wood is related to illiquid as small firewood has no sales. The proportion of illiquid wood averages about 8% of the total blank.

Calculation of economic efficiency shows that in thickened Kazakh Upland Scots pine forests, felling should be of moderate to severe intensity which are more profitable, as well as holding such felling increases the yield of the larger logs and increases the value left on the vine growing.

There are some opposite conclusions on the effect of fullness and density of plantings on the average height of the stand. According to some studies changing the fullness and density of planting does not increase growth in the average height of the stand (Malenko 1980) but on the other site some scientists say that changing of the fullness and density of planting may increase the growth in height (Smirnov 1970) and some authors consider that greatest increase in height is inherent in forest stands with the optimum density (Pamfilov 1951), Makarenko and Mukanov (2002) states that the change in the fullness and density of plantation in Scots pine forests of older age do not have a significant impact on the value of growth of the average tree height, in the thickened stands these indicators have a positive impact on the increase in growth in height, though not in all cases.

Current study shows that 47 age of the average maximum height of the pilot area, characterized by 2 sampling areas with the relative completeness of 0.9-1.0. With increasing age, this trend does not change, that is, the imposition of the original fullness of below and above 0.9-1.0 are characterized by the absence of forest management activities, the worst performance of medium height. At the same time, it should be noted that differences in the average height of the plots with different initial relative completeness does not exceed 105 - age of 0.5 m (3.9%), i.e. the accuracy of their determination. In other words, the relative completeness of the stands in the range of 0.6-1.3 does not have a significant effect on the average height in stands older than 47 years.

As noted earlier stand density is one of the most important factors determining the productivity of forest stands. Eytingen's (1962) considers that the variability of tree height depends on the degree of interaction between them, which, other things being equal, is determined by the density of the stand. With increasing stand age, it is decreased the number of trees per unit area, i.e., planting is self-thinned. Eytingen (1962) says self-thinning stands affects the initial density of the stand, and the self-thinning dense stands is more intense than in the middle and especially in the rare ones. Morozov (1970) notes that the intensity of the process of self-thinning occurs in a variety of stands not equal and depend on the soil

and climatic conditions. In the best conditions and on the best soils self-drop thinning plantations is faster than the plants on poor soils. Great importance to the process of self-thinning also influence on the ratio of tree species to light. Tretyakov (1937) notes that dying out processes of light-loving tree species occur more rapidly than that of shade-tolerant species. Nesterov (1961) finds the better conditions of life, the more the plant survives, and woodland in favorable conditions is denser. These examples show that to the process of self-thinning in the forest is paid much attention by various scientists in their works because of all sorts of factors and environmental conditions.

Our data show that at the age of 90-100 years, there is a direct dependence of the mortality of trees and the percentage of thinning stands. It should be noted that the sample in the range 20% have the highest percentage dropping out when the sample from 30 to 40% percentage of dropping out is reduced to 0%, and high sampling rate greater than 40% leads to an increase in percent dropping out from 1 to 8%. It should be noted that both low and large thinning leads to an increase in natural mortality in the thickened Scots pine plantations, and in areas with a strong inrush observed thinning out trees from the wind.

A large number of scientific papers are devoted to the analysis of economic efficiency and silvicultural thinning (Timofeev and Georgievski 1957, Sennov 1977, 1984, 1999, Zalesov and Luganski 1989, Smirnov 1970, Izuyminski 1970, Davydov 1971, Zalesov 1986, 1988, Chibisov 1992). All researchers have noted a positive change does not affect only quality but also quantity indicators stand after thinning. Changing the density and structure of the stand after thinning, its structure, improving commodity structure, increases the stability of the stand against the adverse effects of wind and snow. Thinning contribute to an increase in radial growth of trees. It is reduced number of timber passing in mortality (Sennov 1977, 1999, Zalesov and Luganski 1989, Smirnov 1970, Izuyminski 1970, Davydov 1971).

Conclusion

In the course of our research, significant qualitative improvement of the species composition of the forest fund did not happen because the thinning was carried out in pure composition of Scots pine plantations. At the same time, cutting maintenance had a positive impact on improving the health condition of forests. When thinning, in the result of removal of part of the stand of trees, their average inventory indices changes as well. Assessing the impact of improvement felling on basic inventory indices stands makes it possible to determine silvicultural effectiveness of their implementation. The conclusions derived from the results obtained allow us to establish the optimal intensity of thinning stands for each forest type, and frequency of improvement felling.

The results of our research in the thickened Scots pine forests aged of 93-105 years show that there is no clear dependence on the weight gain of the intensity of the thinning of forest stands which confirms the results obtained on these same sites at the age of 47-59 years (Makarenko 1967). This suggests that the dynamics of inventory change with age remains stable. Calculation of economic efficiency shows that it should be thinning of moderate to severe intensity in thickened Kazakh Upland which are more profitable, as well as holding such thinning increases the yield of the larger logs and increases the value left on the vine growing.

From all the above data it can be concluded that, in accordance with the new objectives of forest growing it is urgent the search for more accurate methods of predicting the growth and development of forest stands to determine the permissible extent of interference with the natural processes that regulate their process, to ensure high durability and resilience of forest ecosystems. The practical significance of the results of this study can be used to estimate the growth and productivity of Scots pine forest stands at similar site conditions as well as to assess the economic efficiency of forestry and felling. It also can be applied in the development of programs of thinning and other economic activities in the Scots pine forests of Kazakh Uplands.

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The authors express their deep gratitude to the Candidate of Agricultural Sciences A.A. Vesmann, A.A. Makarenko for providing primary forest assessment materials for the above test area, without which our scientific work would be impossible. We consider it our duty to express deep appreciation to the Candidate of Agricultural Sciences V.N. Dudina for providing practical assistance in carrying out this work. In addition, the authors would like to thank Dr. Murat SARGINCI, Düzce University, Faculty of Forestry, in Turkey for language editing.

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RESEARCH ARTICLE

The Biology of Pomegranate Pollen: All about Formation, Morphology, Viability, Germination and Events relating to Sperm Nuclei

Hakan Engin • Zeliha Gökbayrak*

Çanakkale Onsekiz Mart University, Faculty of Agriculture, Department of Horticulture, Çanakkale/Turkey

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ABSTRACT

Investigation of pollen biology (i.e. morphology, viability, germination capacity, development of pollen tube and sperm nuclei) of pomegranates, an andromonoecious species, was aimed in this study with the aid of microscopy. Pollens were collected from the *Punica granatum* L. cv. 'Caner II' at different phenological stages. Morphological features showed that the pollen is prolate with smooth exine surface. Viability was higher in the staminate flowers but germination capacity was better in the bisexuals. Pollen germination begins after it leaves the microsporangium. During pollen tube elongation, the pollen cytoplasm, vegetative nucleus and generative cells are transported within the pollen tube. Before entering the pollen tube, the generative cell undergoes mitosis and form two haploid generative cells. Differences in pollen viability and germination ratio of the flower types were found to be insignificant. Polar length was maximum (28.5 µm) in both sexual morphs and minimum (26.8 µm) in the perfect flower. The width of pollen grains ranged from 15.9 µm to 17.1 µm in both types. Perfect and functional male flowers had small pollen (15.9-27.3 µm) with grooves on the surface without perforations. The surface displayed regular continuities where it was between the protrusions. The surface of pollen grains from both of flowers was striate, with more parallel longitudinal ridges in functional male flower. The pollen from both sexes is about the same size. These findings not only provide information on basic features of pomegranate pollen and its pollination biology, but also can help understand breeding and decide strategies to develop better cultivars.

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Introduction

One of the first domesticated fruit species is pomegranate and its cultivation is widespread between subtropical and tropical areas in the world. Although the number of varieties known to the human is around 500, it is said that only 10% is commonly grown (Hancock, 2004). Turkey is not only one of the pomegranate producing countries following Iran, India and Spain, but also has wide selections of germplasm located in different parts of the country.

It is only rational to assume that over the thousands of years of its domestication, pomegranates have gathered many genetical changes in their genome, allowing the species to spread to and successfully grow in different ecological conditions. Pollens in these terms have significant contribution to its genetic makeup. Due to the fact that they are genetically preserved and not under the influence of environmental conditions (Shangshang et al., 2015), their biology has been scientifically investigated in pomegranates (Yang et al., 2013)

* Corresponding author
E-mail address: zgokbayrak@comu.edu.tr

as well as in other plant species. Variations observed in morphology in terms of shape, size and surface characteristics using microscopy present a tool for taxonomic classification (Maiti et al., 2016).

Literature regarding pomegranate pollen studies mainly involve determining pollen morphology (Erdtman, 1971; Zhao and Xiao, 1996; Yin et al., 2011), pollen tube growth (Gadze et al., 2011), in vitro germination capability (Derin and Eti, 2001; Engin and Hepaksoy, 2003, Engin and Gökbayrak, 2017), in vitro germination with different hormones (Engin and Gökbayrak, 2015; Engin and Gökbayrak, 2016), in situ viability (Gökbayrak and Engin, 2018). Yet, no study has ever been done to fully and completely explore pollen's structure, viability, germination capacity, pollen tube development and the events relating to germ nuclei in the anthers. This research aimed to accomplish this goal using microscopy (i.e. light, stereo zoom and scanning electron) in one of the pomegranate cultivar bred in Turkey through selection.

Materials and Methods

Laboratory Analyses

Pollens obtained from the flowers of pomegranate (*Punica granatum* L.) cv. 'Caner II' were used as the material for this study. The plants have been grown at the experimental field of Çanakkale Onsekiz Mart University's Horticulture Farm, 5 m above sea level. The trees were 13 years old, planted at 3x5 m spacing. Since this research was to plan to serve four different purposes (pollen formation, pollen size and shape, viability and in vitro germination, and events relating to the germ cell inside a pollen), every step towards accomplishing them was explained separately.

Pollen formation was determined in the flowers buds at different stages every week from the beginning of April 2018 to the beginning of flowering (May 20, 2018) at 9.00-11.00 am from healthy plants. To avoid physiological deterioration, the flower buds were immediately stored in a polystyrene box with ice. Carnoy's solution (absolute ethanol and acetic acid at 4:1) was used to fix buds for 48 h, then transferred to 70% (v/v) ethanol before putting in a refrigerator. Anthers were slowly detached from the flowers using a forceps and arrow headed needles under Olympus SZX7 stereo zoom microscope (Olympus Corp., Japan). Later, they were fixed in FAA (37% formaldehyde, 70% ethanol, 98% acetic acid in a ratio of 10:80:10 v/v) for 24 h and then preserved in 70% ethanol. Anthers were then washed three times with distilled water and dehydrated in a graded ethanol series (75, 85 and 96%). Samples were prepared for light microscopy using standard methods of the squash technique in acetocarmine (1%) (Johansen, 1940). Stained samples were examined under light microscopy (Olympus CX-41). Microphotographs were photographed using a microscope camera (LC20, Olympus Corp., Japan) mountable on the microscope.

Flowers before the open petal stage were collected to assess pollen size and shape. Forceps were used to detach anthers. Moisture in the anthers were removed by keeping them for about 12-18 hours at room temperature (22°C), which enabled them to split and release pollen. After following further dehydration of pollens for another 10-12 hours under the same conditions, they were placed in brown glass vials and stored in a refrigerator at +4°C until examined. After preparation of the pollens, the method described by Engin and Unal (2007) for scanning electron microscopy (SEM, Jeol JSM-7100F, Tokyo, Japan) was used with thirty pollen grains from both perfect and functional male flowers. Morphological aspects examined were shape, length of the polar axis (*P*), length of the equatorial diameter (*E*) and ratio of polar axis to equatorial axis (*P*/*E*).

A colorimetric test of 2,3,5-triphenyl tetrazolium chloride (TTC, 1%) were utilized to determine the viability of the pollens derived from the flowers collected at the open petal stage. The separation of the pollens as viable or not viable was performed based on color tonality (darker meant viable) under a light microscope after a waiting period of two hours. Ratio calculations (%) were made with viable pollens divided to total pollen number. Additionally, the pollen grains were sawn over the medium (20% sucrose and 1% agar) at 26±1°C under 8 hours dark and 16 hours daylight conditions to assess the in vitro germination ability. Twenty-four hours later, germinated and not germinated pollens were counted using a light microscopy (Olympus CX-41) at 10x magnification from a random selection of six-field views and the ratio of germination (%) was calculated.

SEM Analysis

Pollen grains to observe pollen tube development were cultured in a medium containing 20% sucrose in a petri dish. They were kept at room temperature. After waiting for five, ten and twenty hours, samples were put into brown glass bottles, fixed with ethanol (98% purity) and stored in a refrigerator until examined. Before SEM examination, the samples were further dried at room temperature for 4-6 h. For the SEM study, samples were mounted directly on metallic stubs using double-sided adhesive tape and coated with gold in a sputtering chamber (Bal-Tec SCD 005 Sputter Coater). Observation of the prepared samples was carried out with a scanning electron microscope (SEM) Jeol JSM-7100F (Tokyo, Japan) at 15 kV.

Statistical Analysis

The first analysis was performed on the data obtained relating the shape (*P*, *E* and *P*/*E* ratio), which were from the 10 pollens in 3 replicates. The second analysis was on the viability and in vitro germination ratios of the pollens, which were realized on 6 glass slides. There were minimum 100 pollens counted in each slide. The statistical analysis was performed using MINITAB statistical package software (Minitab Inc., ver.16), and the significant means were compared using Tukey's test.

Results and Discussion

In pomegranate (*Punica granatum* L.). at the beginning of its development, the anther is composed of a mass of cells that appear undifferentiated. As the anther develops, groups of sporogenous cells form. Through meiotic division of these

cells, some of them grow into nutritive cells that supply nutrition for the microspores. However, some produce mother cell (Figure 1, A). Tetrads are typically arranged as the four cells in one plane. After the formation of the four cells, a thick layer of callose, which is darkly stained, coats whole tetrads and separates the individual microspores (Figure 1, B). All tetrads in the anthers within the same size flower bud appear to be at the same developmental stage. After the formation of the four microspores, the development of the pollen grain walls begins. The callose wall is broken down and the free pollen grains are grown in size and develop their characteristic shape and form (Figure 2, A). The wall of pollen displayed two different layers, as exine and intine. The exine is often variously sculptured, and the character of the markings is of value for identifying genus, species, or cultivar. The exine, thin in nature, displays regular continuities (Figure 2, B).

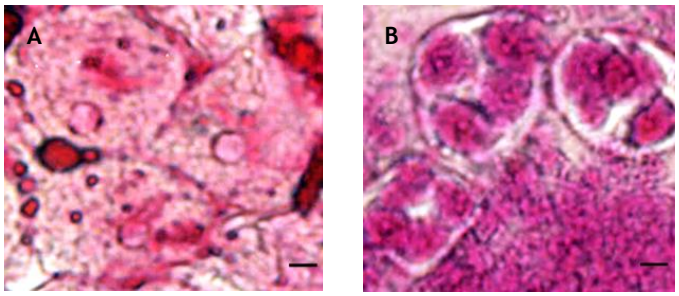

Figure 1. Light microscopy images (Stained with acetocarmine Bars = 20 µm). **A:** Pollen mother cells; **B:** Tetrad showing four microspores separated by callose wall

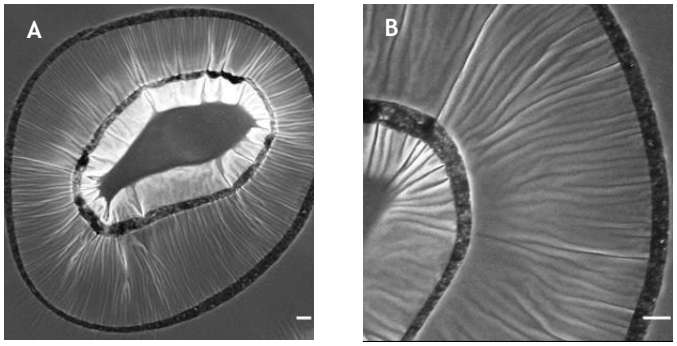

Figure 2. Scanning electron microscopy images (Bars = 1 µm). **A:** Pollen grain; **B:** Mature pollen grain showing thick exine and intine

In 'Caner II' pomegranate cultivar, the formation of pollen occurred very close to the flowering. The first pollen mother cells (PMC) were seen in flower buds which were taken on May 2. The first detection date of tetrads was May 5 and the samples of young pollen grains were also observed. The time (day) between formation of PMC, tetrads, and pollen grains and the beginning of flowering is given in Table 1.

'Caner II' had two types of flowers on the same tree: hermaphroditic and functional staminate flowers. Pollen from both perfect and male flower types is similar based on the analysis conducted by SEM (Figure 3). Pollen grains have a spheroidal shape with a smooth exine surface. The grains are considered prolate in view of the number, position and type of the apertures.

Table 1. Timing (days before flowering) of the formation of the first pollen mother cells (PMC), tetrads and pollen grains in the pomegranate cultivar

Cultivar	Days before flowering		
	PMC	Tetrads	Young pollen grains
Caner II	23	19	19

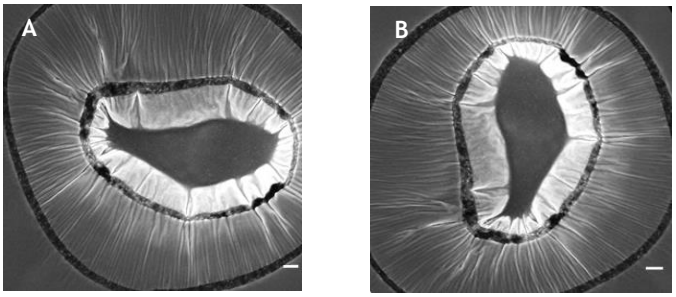

Figure 3. Scanning electron microscopy images (Bars = 1 µm). **A:** Equatorial view of pollen grain of pomegranate cultivar 'Caner II' from functional male flower; **B:** Polar view of pollen grain of pomegranate cultivar 'Caner II' from perfect flower

The values pertaining to the morphological features of the pollens were given in Table 2. The investigated pollen grains in perfect and functional male flowers of 'Caner II' cultivar did not vary in size and shape. Polar length was maximum (28.5 µm) in both sexual morphs and minimum (26.8 µm) in the perfect flower. The width of pollen grains ranged from 15.9 µm to 17.1 µm in both types. The pollen from both sexes is about the same size. According to the classification of Erdtman (1969), the pollen grain of 'Caner II' was prolate. This is in accordance with the findings of Varasteh and Arzani (2009) who characterized the shape of the pollen grains of 14 Iranian pomegranate cultivars as prolate based on *P/E* ratio. P/E ratio of 55 indigenous pomegranate cultivars was reported between 1.54 and 2.05 by Shangshang et al. (2015). Engin and Gökbayrak (2017) classified pollen of cultivar 'Caner I', another selection from 'Caner' group, also as prolate.

Table 2. Morphological characteristics of pollen grains from functional male and perfect flowers of pomegranate (*Punica granatum* L.) cultivar 'Caner II' (Mean±SE)

	Polar axis (P) µm		Equatorial axis (E) µm		P/E ratio	shape
	Variation range	Mean value	Variation range	Mean value		
Functional male	26.8-27.9	27.35±0.09	16.1-16.9	16.70±1.29	1.64±0.06	prolate
Perfect	26.8-28.5	27.34±1.01	15.9-17.1	17.06 ±1.03	1.60±0.08	prolate
Pollen (Mean)		27.35		16.89		

Pollen grains obtained from the flowers just before anthesis for scanning electron microscopy examinations show that pollen grains are spherical, and they were approximately 22 µm in size (Figure 3, A). Perfect and functional male flowers of 'Caner II' cultivar had small pollen (15.9-27.3 µm) with grooves on the surface. There were no perforations on the surface of pollen grains. The surface displayed regular continuities where it was between the protrusions. The surface of pollen grains from both of flowers was striate, with more parallel longitudinal ridges from functional male flower. The ridges were more parallel in functional male flowers and less parallel in perfect (Figure 4).

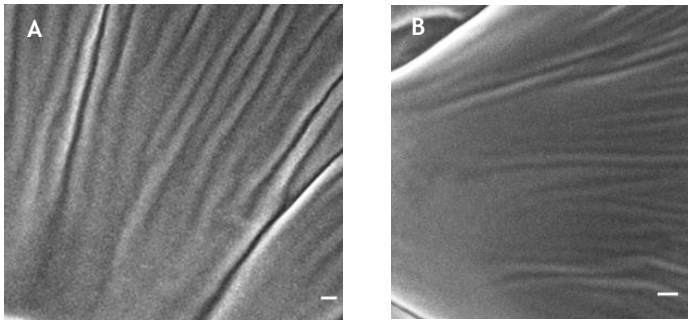

Figure 4. Scanning electron microscopy images (Bars = 1 µm). **A:** Pollen grain surface of from functional male flower; **B:** Pollen grain surface of from perfect flower

Pollen viability and in vitro germination of flowers with different sexes were tested on pomegranate variety 'Caner II' (Table 3) using the TTC test and the results did not exhibit any statistically significant differences, although staminate flowers had slightly higher viable pollens. TTC produced a distinctly clear contrast between viable and nonviable pollen grains (Figure 5). Viability of pollens considerably lower than those in other genotypes reported by Derin and Eti (2001) and Sangma and Singh (2017). Notwithstanding, germination was markedly low in both functional male and perfect flowers and there was not a significant difference in the functional male flowers compared to the perfect flowers. In contrast to the findings of Derin and Eti (2001) and in agreement with Gözlekçi and Kaynak (2000), that the two types of flowers did not produce pollens with significant differences in germination.

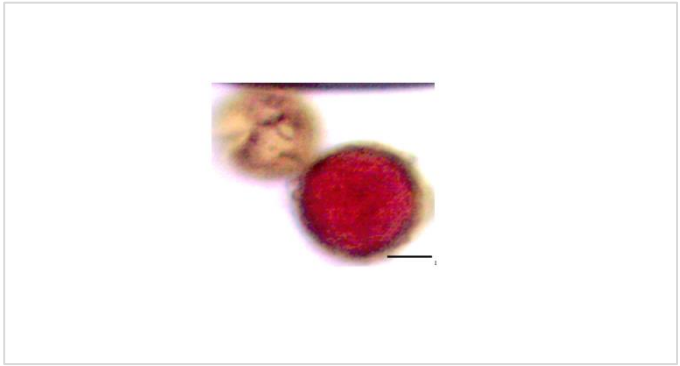

Figure 5. Non-viable (left) and viable (right) pollen grain stained with TTC using light microscopy (Bar = 10 µm)

SEM analysis of pollen grain from perfect and functional male flowers in the pomegranate 'Caner II' cultivar showed that pollen germination begins after it leaves the microsporangium (Figure 6) But in some flowering plants, this might happen before it departs the microsporangium (Carol et al., 2001). During pollen tube formation, a defined area in the pollen plasma membrane promotes a directional growth (Figure 6). Following formation, the longitudinal growth takes place very quickly (Figure 7).

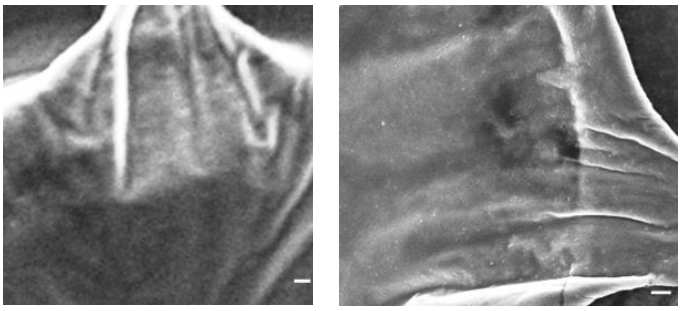

Figure 6. Scanning electron microscopy images (Bars = 1 µm) of pollen tube formation

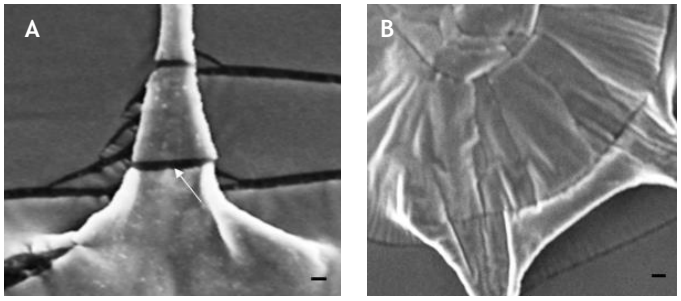

Figure 7. Scanning electron microscopy images (Bars = 1 µm). **A:** Pollen tube elongation (arrow = callose plug); **B:** Two pollen tube elongations from a pollen grain

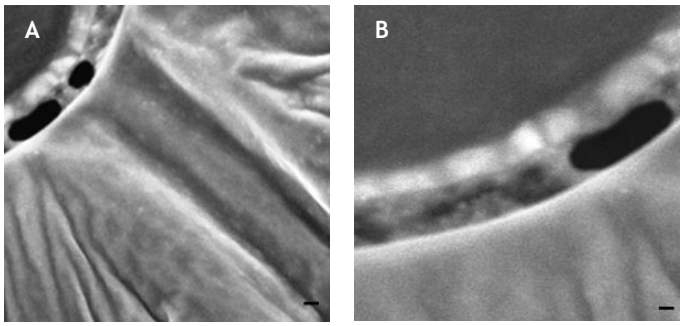

Figure 8. Scanning electron microscopy images (Bars = 1 µm). **A:** Appearance of vegetative and elongated generative cells; **B:** Close-up view of generative cell, going through a mitosis to form two generative nuclei

Barnabas and Fridvalszky (1984) reported pollen tube growth in maize at 1 cm per hour. It is generally accepted that one pollen tube grows out of a pollen but rarely two defined areas in the pollen plasma membrane initiate a directional growth of pollen tubes (Figure 7, B).

Table 3. Comparison of pollen sources (perfect and functional male flowers) in the pomegranate cultivar 'Caner II' for pollen viability and *in vitro* germination ratio (%)

Cultivar	Pollen viability (%)			Pollen germination (%)		
	Perfect	Functional male	*p* value	Perfect	Functional male	*p* value
Caner II	57.07	62.91	*0.314*	39.22	20.46	*0.089*

During pollen tube elongation, the pollen cytoplasm, vegetative nucleus and generative cells are transported within the pollen tube (Figure 8, A). In pomegranate before entering the pollen tube, the generative cell undergoes mitosis and form two haploid generative cells (Figure 8, B). The vegetative nucleus was approximately 4 µm in size (Figure 9, A). The generative nuclei were approximately 3 µm in size (Figure 9, B). At the end of the pollen tube, the cells were discharged, vegetative cell at the front and already degenerated due to lack of microphyll (Figure 10).

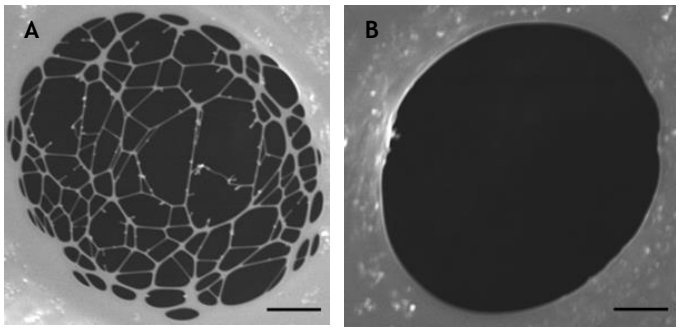

Figure 9. Scanning electron microscopy images (Bars = 1 µm). **A:** Vegetative nucleus at the brink of collapse; **B:** Generative cell

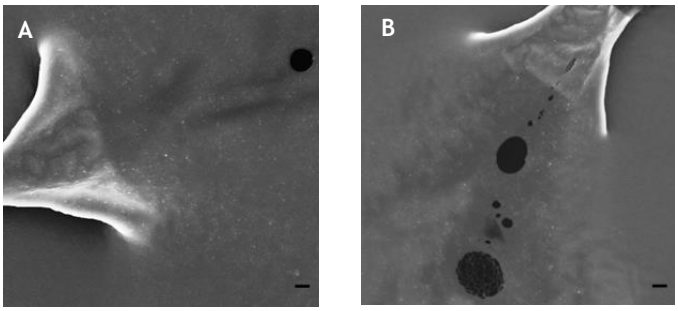

Figure 10. Scanning electron microscopy images (Bars = 1 µm). **A:** Discharge of generative cell out of the enlarged end of pollen tube; **B:** Discharge of both vegetative cell (degenerating) and generative cell from the end of the pollen tube

Conclusion

The information gathered in this study show that In pomegranate (*Punica granatum* L.). at the beginning of its development, the anther is composed of a mass of cells that appear undifferentiated and the formation of pollen occurs very close to the flowering. Scanning electron microscopy examinations show that pollen grains are spherical, approximately 22 µm in size and they have a smooth exine surface. The surface of pollen grains from both of flowers was striate, with more parallel longitudinal ridges in the staminate flower. In view of the number, position and type of the apertures, the grains are prolate. They do not differ in their size and shape in perfect and functional male flowers of 'Caner II' cultivar. Pollen viability is slightly higher in the male flowers. However, in vitro pollen germination does not differ with the sexes of the flowers. SEM analysis showed that pollen germination begins after it leaves the microsporangium. Some pollens with two pollen tube growth are also observed.

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RESEARCH ARTICLE

Effects of Dietary Fish Meal Replacement by Red Lentil Meal on Growth and Amino Acid Composition of Rainbow Trout (*Oncorhynchus mykiss*)

Keriman Yürüten Özdemir[1,2*] • Mustafa Yıldız[3]

[1]*Kastamonu University, Faculty of Fisheries, Department of Basic Sciences, Kastamonu/Turkey*
[2]*İstanbul University, Institute of Science and Technology, Department of Aquaculture, İstanbul/Turkey*
[3]*İstanbul University, Faculty of Aquatic Sciences, Department of Aquaculture, İstanbul/Turkey*

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ABSTRACT

The purpose of this study was to determine the effects of replacing fish meal with red lentil meal (RLM) as an alternative plant protein source in diets for juvenile rainbow trout (10.14±0.04 g mean initial weight) on growth performance and amino acid composition of fish. Four iso-nitrogenous and iso-lipidic experimental diets were prepared to include 15% (RLM15), 20% (RLM20) and 25% (RLM25) of fish meal. At the end of the 60 day feeding trial, the highest mean individual weight gain (30.55±0.08 g) of fish was found in control group but not significantly different from RLM15. Crude protein level of whole body/fillet gradually decreased with increase in RLM percentages in the diets. Generally, essential amino acid (EAA) profiles of whole body/fillets reflected the dietary EAA profile. EAA profile of fish fed RLM15 diet was close to control group ($P>0.05$). However, lysine levels of fish decreased with increasing dietary RLM levels. RLM20 fed fish had the highest body contents of phenylalanine ($P<0.05$). Naturally, EAA levels of fillets were higher than whole body's EAA levels. Histidine levels of fillets were highest in control group and the lowest in RLM20 group. In contrast, isoleucine levels of fillets were highest in RLM20 group whereas the control group had the least level ($P<0.05$). Leucine and valine values of fish fed the control diet were lower than the other experimental groups. Threonine level was highest in fish fed the RLM25 diet ($P<0.05$). Results of the present study showed that 15% of dietary fish meal can be replaced by RLM in diets of juvenile rainbow trout without any adverse effects on growth performance and body amino acid composition.

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Introduction

Aquaculture is one of the most rapidly developing sectors in the world (Şener & Yıldız 2003; FAO 2016). According to estimates, the capacity of global aquaculture to cope with an enhancement demand for fish meal has reached the limited supplies (Sargent & Tacon 1999; Naylor et al. 2000; FAO 2016). As a risk reduction strategy, the identification, development and use of alternatives to fish meal in aqua feeds remain a high priority (Hardy 2010). Fish meal is an excellent but costly protein source for fish feed formulation and is generally count in to 40-50% in commercial feeds for carnivorous fish species (Tacon and Metian 2008; Hardy 2010; Larsen et al. 2012).

Given the global needs of fish meal for aquaculture, there is an increasing demand for more insight into the potential of

* Corresponding author
E-mail address: k.yuruten@gmail.com

alternative protein sources in fish feeds (Kaushik et al. 2005). Among the plant protein sources, soybean meal (SBM) is considered the most cost-effective alternative for high-quality fish meal in feeds of many aquaculture animals, because of its high content of available protein with a relatively well-balanced amino acid profile, high digestibility, reasonable price, steady supply and low phosphorus content (Tan et al. 2005; Biswas et al. 2007). Although, soy protein concentrates and isolates are expensive, and the use of less soybean meals is limited in fish and other aquatic animals by anti-nutritional factors, higher crude fibre and unavailable carbohydrate concentrations (Brown 2008; Brown et al. 2008). Alternative plant protein sources are needed to reduce the current dependence on fish meal and soybean meal as the primary protein sources for aquatic animal diets (Reigh 2008). For this reason, numerous studies have been undertaken to examine the effects of replacing fish meal by other sources of proteins such as plant proteins or animal by-products in diets of rainbow trout (Dabrowski et al. 1989; Watanabe et al. 1993; Gomes et al. 1995; Kaushik et al. 1995; Xie & Jokumsen 1997; Ustaoglu-Tiril et al. 2009; Bilguven & Baris 2011; Øverland et al. 2013; Ouraji et al. 2013; Hauptman et al. 2014; Bahrevar & Faghani-Langroudi 2015; Dogan & Bircan 2015; Lee et al. 2015; Tenamura et al. 2016; Craft et al. 2016; Gerile & Pirhonen, 2017).

The use of grain products in aquaculture feeds is now common in the diet formulations of many aquatic animals (Gatlin et al. 2007). Among those grain raw materials frequently being used are red lentil meal. Red lentil meal is an important and inexpensive source of carbohydrate and protein for the human diet (Frias et al., 1996). However, there are no reports on the nutritional value of red lentil meal when fed to rainbow trout except for Ustaoglu-Tiril's (2009) research. Therefore, the objective of the present study was to determine the effects of dietary replacement of fish meal with red lentil meal as an alternative plant protein source on growth performance and whole body/fillets amino acid composition in rainbow trout.

Materials and Methods

Experimental Diets

Four iso-nitrogenous and iso-lipidic experimental diets were formulated to contain graded levels of red lentil meal (RLM) to replace fish meal. The control diet contained only fish meal as the main protein source. The other experimental diets RLM15, RLM20 and RLM25, contained 150, 200 and 250 g kg^{-1} of red lentil meal respectively. Wheat gluten and corn gluten in the diet were used to create a protein balance. The amino acid profile and proximate composition of protein sources in diets are presented in Table 1. Formulation and proximate compositions of experimental diets are shown in the Table 2. The amino acid composition of diets and essential amino acids requirement for rainbow trout are given in Table 3. Experimental diets (2 - 3 mm diameters) were produced at the Sapanca Inland Waters Research Center (Adapazari, Turkey) of Istanbul University as steam pressured pellets using a laboratory feed mill (KAHL-L, 173). Diets were kept in plastic storage bags at -20 °C until used.

Table 1. Amino acid and proximate compositions (dry weight basis) of fish meal, wheat gluten, corn gluten and red lentil meal

	Fish Meal	Wheat Gluten	Corn Gluten	Red Lentil Meal
Essential Amino Acids (EAA, g/100g)				
Arginine	3.74±0.04^{a}	2.47±0.03^{b}	1.85±0.23^{c}	1.73±0.18^{c}
Histidine	2.07±0.07^{a}	1.43±0.05^{b}	1.35±0.10^{b}	0.57±0.01^{c}
Isoleucine	2.40±0.00^{a}	1.99±0.00^{b}	1.86±0.11^{b}	0.73±0.08^{c}
Leucine	4.78±0.08^{b}	4.90±0.00^{b}	10.04±0.77^{a}	1.73±0.03^{c}
Lysine	5.08±0.46^{a}	1.35±0.18^{b}	1.11±0.03^{b}	1.61±0.10^{b}
Methionine	1.61±0.49^{a}	0.96±0.20^{a}	1.26±0.47^{a}	0.21±0.04^{a}
Phenylalanine	2.76±0.00^{b}	3.91±0.01^{a}	3.89±0.07^{a}	1.16±0.06^{c}
Threonine	3.07±0.03^{a}	2.04±0.03^{b}	2.52±0.27^{ab}	0.90±0.03^{c}
Tryptophan	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00
Valine	2.79±0.15^{a}	2.33±0.12^{b}	2.14±0.09^{b}	0.89±0.03^{c}
Total EAA	28.33±0.19^{a}	21.44±0.16^{c}	26.05±0.20^{b}	9.58±0.29^{d}
Non- Essential Amino Acids (NEAA, g/100g)				
Alanine	4.13±0.27^{b}	2.08±0.09^{c}	5.81±0.49^{a}	1.06±0.04^{c}
Asparagine	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00
Aspartic acid	5.71±0.01^{a}	2.19±0.18^{c}	3.55±0.27^{b}	2.62±0.22^{c}
Citrulline	0.02±0.00	0.02±0.00	0.02±0.00	0.81±0.73
Cystine	0.44±0.08^{a}	0.28±0.06^{ab}	0.33±0.00^{ab}	0.13±0.10^{b}
Glycine	3.47±0.24^{a}	2.45±0.03^{b}	1.89±0.01^{c}	1.01±0.02^{d}
Glutamic acid	9.12±0.24^{bc}	28.52±3.67^{a}	14.78±1.29^{b}	4.39±0.22^{c}
Hydroxyproline	0.26±0.00	0.03±0.00	0.03±0.00	0.03±0.00
Ornitine	0.03±0.00	0.00±0.00	0.03±0.00	0.03±0.00
Proline	3.24±0.32^{c}	11.48±0.70^{a}	7.42±0.25^{b}	1.21±0.02^{d}
Sarcosine	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00
Serine	2.50±0.04^{c}	3.45±0.07^{a}	3.17±0.00^{b}	1.21±0.03^{d}
Tyrosine	2.39±0.05^{a}	2.13±0.12^{a}	2.91±0.31^{a}	0.37±0.27^{b}
Total NEAA	31.37±0.44^{c}	52.70±4.00^{a}	39.99±1.12^{b}	12.94±0.29^{d}
Proximate composition				
Dry matter	88.06±0.00^{b}	92.70±0.00^{a}	93.61±0.00^{a}	92.23±0.01^{a}
Crude Protein	68.16±0.01^{c}	81,.9±0.00^{a}	70.03±0.01^{b}	27.24±0.00^{d}
Crude Lipid	11.25±0.00^{a}	1.53±0.00^{c}	0.67±0.00^{d}	1.95±0.00^{b}
Ash	11.32±0.00^{a}	0.52±0.00^{d}	1.69±0.00^{c}	2.15±0.00^{b}

Data are reported as mean ± SD of three replicates (n = 3). Means with different superscript letter in a row are significantly different (P<0.05).

Table 2. Ingredients and proximate composition of the four experimental diets

	Diets			
	Control	**RLM15**	**RLM20**	**RLM25**
Ingredients (g kg^{-1} dry weight)				
Fish meal	600	300	150	0
Soybean meal	125	20	0	0
Corn gluten	80	150	140	90
Wheat gluten	0	155	270	410
Lentil Meal	0	150	200	250
Gelatin	50	50	50	50
Fish oil (Anchovy oil)	85	115	130	140
Mineral premix[a]	30	30	30	30
Vitamin premix[a]	30	30	30	30
Analyzed proximate composition(g kg^{-1})				
Dry matter	90.65±0.17	92.08±0.08	92.62±0.22	93.82±0.09
Crude protein	49.31±0.53	49.36±0.52	47.24±0.24	46.55±0.27
Lipid	15.48±0.58	15.11±0.48	15.00±0.42	14.75±0.52
Ash	7.92±0.16	4.70±0.06	2.91±0.05	1.32±0.07
Crude cellulose	1.76±0.01	2.03±0.11	2.51±0.31	2.64±0.30
NFE[b]	16.88±0.42	21.56±1.22	25.79±1.54	29.45±2.27
Metabolizable energy (kJ g^{-1})	14.36±0.29	14.56±0.14	14.46±0.02	14.51±0.07

[a] Premix of vitamins and minerals according to NRC (1993) recommendations for fish.
[b] NFE: nitrogen-free extract calculated by difference.

Table 3. Amino acid composition in the four experimental diets (g/100 g protein)

	Diets			
	Control	**RLM15**	**RLM20**	**RLM25**
Essential Amino Acids (EAA, g/100g)				
Arginine	3.00±0.12^{a}	2.69±0.01^{b}	2.42±0.06^{bc}	2.26±0.05^{c}
Histidine	1.46±0.06^{a}	1.31±0.09^{ab}	1.18±0.13^{ab}	1.07±0.03^{b}
Isoleucine	1.84±0.03^{a}	1.71±0.04^{ab}	1.59±0.12^{ab}	1.44±0.07^{b}
Leucine	4.24±0.02	4.52±0.03	4.27±0.11	3.85±0.33
Lysine	3.36±0.02^{a}	2.27±0.11^{b}	1.86±0.04^{c}	1.39±0.06^{d}
Methionine	1.41±0.01^{a}	1.16±0.06^{b}	0.97±0.07^{b}	0.67±0.01^{c}
Phenylalanine	2.31±0.07	2.55±0.01	2.58±0.14	2.52±0.12
Threonine	2.42±0.26^{a}	1.96±0.05^{ab}	1.77±0.09^{b}	1.53±0.03^{b}
Tryptophan	0.34±0.04^{a}	0.02±0.00^{b}	0.02±0.00^{b}	0.02±0.00^{b}
Valine	2.16±0.01^{a}	2.02±0.02^{ab}	1.87±0.11^{b}	1.62±0.01^{c}
Total EAA	22.58±0.51^{a}	20.23±0.20^{ab}	18.55±0.88^{bc}	16.41±0.60^{c}
Alanine	3.57±0.27^{a}	3.22±0.00^{ab}	2.75±0.03^{b}	2.13±0.07^{c}
Asparagine	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00
Aspartic acid	4.77±0.42^{a}	3.75±0.03^{b}	3.11±0.12^{bc}	2.45±0.18^{c}
Citrulline	0.02±0.00	0.02±0.00	0.21±0.00	0.02±0.00
Cystine	0.25±0.18	0.28±0.25	0.32±0.30	0.36±0.38
Glycine	3.09±0.29^{a}	2.97±0.18^{a}	2.57±0.24^{a}	2.46±0.15^{a}
Glutamic acid	7.22±0.23^{c}	10.22±0.73^{b}	11.89±0.23^{b}	14.37±0.79^{a}
Hydroxyproline	0.26±0.00	0.26±0.00	0.26±0.00	0.26±0.00
Ornitine	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00
Proline	3.60±0.50^{c}	4.52±0.20^{c}	6.25±0.35^{b}	8.35±0.49^{a}
Sarcosine	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00
Serine	2.41±0.11^{a}	2.59±0.06^{a}	2.65±0.01^{a}	2.60±0.10^{a}
Tyrosine	1.77±0.05	1.84±0.06	1.64±0.15	1.53±0.08
Total NEAA	27.05±2.06^{b}	29.75±1.44^{ab}	31.75±0.36^{ab}	34.38±2.25^{a}

Data are reported as mean ± SD of three replicates (n = 3). Means with different superscript letter in a row are significantly different (P<0.05).

Experimental Conditions and Measurements

Juvenile rainbow trout (*Oncorhynchus mykiss*), with a mean initial body weight of 10.14±0.04 g, were obtained and stocked randomly (50 fish tank^{-1}) into 8 cylindro-conical tanks of 1000 L capacity in the Sapanca Inland Waters Research Center (Adapazari, Turkey). The tanks were supplied with freshwater having an average temperature of 12.3±0.2 °C. Dissolved oxygen was maintained around 9.9±0.1 mg L^{-1}. 12 h light: 12 h dark photoperiod regimen was utilized throughout the study. Before starting the experiment, fish were acclimatized to the experimental feeding regimen using a commercial diet for 2 weeks (trout commercial pellet 2 mm in diameter). During the

study, fish were fed to apparent satiation by hand twice per day at 09:00 and 17:00 h. Bulk fish live weight increments were measured every 2 weeks and feed intake was recorded daily throughout the study. At the end of the study, fish were taken individually weight and length for determining growth performance parameters. In addition, 15 fish per tank (30 fish per diet) were collected for chemical analyses. Fish samples were kept at -80 °C until proximate composition and amino acid profile analysis. Growth performance measured are listed below and the calculations were according to Ricker (1979);

Weight gain (%) = [(final weight-initial weight) / initial weight] × 100;
Specific growth rate (SGR) = [(ln final weight-ln initial weight) /days] × 100;
Condition factor (CF) = 100 x [(body weight (g) / length^3 (cm)];
Feed efficiency ratio (FER) = wet weight gain (g) / feed intake (g);
Protein efficiency ratio (PER) = wet weight gain (g) /protein intake (g);
Hepatosomatic index (HSI) = (liver weight /body weight) × 100;
Viscerosomatic index (VSI) = 100 × (viscera weight/body weight).

Chemical Analyses

Feed ingredients, experimental diets, and fish samples were analyzed for proximate composition (protein, lipid, ash and dry matter) according to standard AOAC (1998) procedures. Dry matter was obtained by weight loss after drying samples in an oven at 105ºC until constant weight. Crude protein was determined as total nitrogen (N) by using a semi-automatic Kjeldahl (Gerhardt Vapodest, 45s) technique (N×6,25). Crude lipid was extracted according to Soxhlet (Velp Scientifica Ser, 148) method with petroleum ether. Ash content was obtained from the weight loss after incineration of dried samples at 550 ºC for about 12 h in a Muffle Furnace. All samples were analyzed as triplicates.

Amino acid levels of feed ingredients, experimental diets and fish were hydrolyzed with 6 mL of 6 N HCl at 110 °C for 22 h in an evacuated sealed tube to determine amino acids composition. The hydrolysate was dried under nitrogen gas to remove HCl, re-dissolved in 0.1 N HCl loading buffer, and filtered through a 0.22 µm polyethersulfone ultrafiltration membrane. The filtrate was loaded on a high-performance liquid chromatography system (LC1200, Bilim Laboratory A.S., Istanbul) equipped with an Agilent ZORBAX Eclipse Plus C18 column (150 × 5 µm). Signals of 16 amino acids were detected after derivatization with ophthaldialdehyde. Asparagine, glutamine, proline, and tryptophan were notwithin the determination range. The HPLC conditions followed the protocol for the Agilent ZORBAX Eclipse Plus C18 column.

Statistical Analyses

Statistical analyses of data were subjected to one-way ANOVA, and a subsequent comparison of means by Tukey's multiple range test. All of the above mentioned statistical analyses were performed using SPSS (Version 10 for Windows). Differences were considered statistically significant at $P<0.05$.

Results

Amino Acid Composition of Dietary Ingredients and Experimental Diets

EAA levels of red lentil meal were significantly lower than EAA levels of fish meal, wheat gluten and corn gluten (Table 1). EAA levels of fish meal were higher than other protein sources ($P<0.05$).

The amino acid composition of experimental diets is presented in Table 2. Amino acid composition changes among the experimental diets reflected the replacement of fish meal with RLM. EAA levels were gradually decreased with increasing dietary levels of fish meal replacement except for leucine and phenylalanine. In contrast, NEAA levels of diets increased with increasing RLM inclusion ($P<0.05$).

Growth Performance

At the end of the experiment, final weight of the control group was higher than the other experimental fish groups ($P<0.05$). However, the final weight of RLM15 fish group were close to control group ($P>0.05$). Fish weights were similar in the RML20 and RLM25 groups ($P>0.05$) and these were lower than the other experimental groups ($P<0.05$). Fish fed the RML25 diet had the lowest SGR and the highest PER levels ($P<0.05$). All experimental groups had similar FCR, CF and HSI values ($P>0.05$). VSI value of fish fed with control diets was lower than other experimental. However, there were no significant differences in VSI among the RLM fed fishes (Table 4; $P<0.05$).

Table 4. Growth performance values of rainbow trout fed four experimental diets

	Dietary Treatments			
	Control	**RLM15**	**RLM20**	**RLM25**
Initial weight (g fish^{-1})[1]	10.15±0.06	10.14±0.00	10.15±0.08	10.12±0.03
Final weight (g fish^{-1})[1]	40.70±0.15^a	40.00±0.12^b	39.40±0.17^c	39±0.04^c
Weight gain (%)[1]	300.98±0.08^a	294.48±0.11^b	288.18±0.25^c	285.37±0.08^c
SGR[1]	2.35±0.01^a	2.31±0.01^{ab}	2.29±0.02^{ab}	2.28±0.01^b
FCR[1]	0.95±0.04	0.93±0.03	0.93±0.04	0.91±0.01
PER[1]	1.82±0.00	1.88±0.01	1.88±0.02	2.04±0.00
CF[1]	1.12±0.03^a	1.12±0.02^a	1.12±0.03^a	1.07±0.04^b
HSI[2]	1.79±0.29^c	1.92±0.21^b	2.02±0.32^a	2.01±0.26^a
VSI[2]	18.17±1.13^b	20.59±1.96^a	20.24±2.65^a	20.25±1.41^a

Data are reported as mean ± SD of three replicates (n = 3). Means with different superscript letter in a row are significantly different ($P<0.05$).
[1] n = 60 x 2
[2] n = 20

Proximate Composition of Whole Body/Fillets

Proximate composition of whole body and fillet were significantly affected by dietary treatments (Table 5). Fish fed the RLM25 had the highest fillet dry matter levels (P<0.05). Crude protein was highest in fish fed the control diet. In particular, crude protein level of whole body and fillet gradually decreased with the increase red lentil meal percentages in the diets (P<0.05). In contrast, the crude lipid and dry matter levels of fish fillet increased with the increase red lentil meal in diets (P<0.05). However, whole body crude lipid levels were similar to fish fed the control and RLM25 diets and these groups had higher level of crude lipid than the other experimental groups (P<0.05). The crude lipid levels of the fish livers decreased with increasing red lentil meal percentages in the diets (P<0.05).

Amino Acid Composition of Whole Body/Fillets

Whole body amino acid compositions were shown Table 6. There were no significant differences EAA levels among the initial, control and the other experimental groups (P>0.05), except for lysine and phenylalanine. Lysine levels of fish fed with increased RLM in the diets were gradually decreased. Phenylalanine level was the lowest in control group and the highest in RLM20 group (P<0.05).

The amino acid composition of fillets is given in Table 7. Generally, EAA levels of fillets were higher than that of whole body. Fillet histidine concentration were highest in control group and whereas the RLM20 group had the lowest content. In contrast, isoleucine levels of fillet were highest in RLM20 group while the control group recorded the lowest fillet content (P<0.05). Leucine and valine contents of fish fed the control diet were lower than the other experimental groups. Threonine level of fish fed the RLM25 diet was the highest. There were no significant differences in total EAA levels between control and other experimental groups for both whole body and fillets. In addition EAA levels of whole bodies were lower than fillets EAA levels.

Essential amino acid requirement of rainbow trout is given in Table 8. The EEA requirement of rainbow trout levels of arginine, histine, methionine, tryptophan and valine were found higher than the four experimental diets. However, isoleucine, leucine, lysine, phenylalanine and threonine levels in the experimental diets were higher than the requirement values.

Table 5. Whole body/fillets proximate composition and crude lipid in liver of rainbow trout fed four experimental diets

	Dietary Treatments			
	Control	**RLM15**	**RLM20**	**RLM25**
Whole Body				
Dry matter	26,41±0,18^{b}	26,92±0,69^{b}	26,25±0,27^{b}	28,43±0,39^{a}
Crude Protein	16,75±0,70^{a}	15,84±0,41^{ab}	15,51±0,15^{b}	14,18±0,14^{c}
Crude Lipid	13,88±0,74^{a}	12,35±0,19^{b}	12,27±0,48^{b}	14,24±0,62^{a}
Ash	1,10±0,17^{b}	1,65±0,27^{a}	1,08±0,12^{b}	1,09±0,20^{b}
Crude lipid of liver	3,70±0,44^{a}	2,65±0,35^{ab}	2,63±0,00^{ab}	1,82±0,02^{b}
Fillet				
Dry matter	20,74±0,14^{c}	22,24±0,47^{b}	22,55±0,15^{b}	25,96±0,73^{a}
Crude Protein	19,58±0,32^{a}	18,77±0,14^{b}	18,03±0,23^{c}	16,44±0,34^{d}
Crude Lipid	3,56±0,03^{d}	4,37±0,39^{c}	5,56±0,13^{b}	7,97±0,09^{a}
Ash	0.86±0.07	0.92±0.07	0.81±0.07	0.79±0.04

Data are reported as mean ± SD of three replicates (n = 3). Means with different superscript letter in a row are significantly different (P<0.05).

Table 6. Amino acid composition (dry weight basis) in whole body of rainbow trout fed four experimental diets

Amino Acids	Fish Groups				
	Initial	**Control**	**RLM15**	**RLM20**	**RLM25**
Essential Amino Acids (EAA, g/100g)					
Arginine	0.85±0.02	0.75±0.08	0.78±0.02	0.78±0.04	0.84±0.03
Histidine	0.39±0.03	0.36±0.06	0.33±0.03	0.38±0.01	0.37±0.04
Isoleucine	0.47±0.03	0.39±0.03	0.37±0.01	0.48±0.05	0.45±0.01
Leucine	1.03±0.04	0.88±0.04	0.93±0.07	0.90±0.08	0.89±0.02
Lysine	1.05±0.03	0.89±0.04	0.93±0.08	0.72±0.10	0.71±0.01
Methionine	0.42±0.01	0.21±0.14	0.21±0.14	0.34±0.03	0.37±0.01
Phenylalanine	0.58±0.03	0.49±0.03	0.51±0.03	0.66±0.07	0.64±0.00
Threonine	0.65±0.00	0.58±0.06	0.59±0.03	0.59±0.03	0.60±0.03
Tryptophan	0.19±0.06^{b}	0.11±0.02^{b}	0.10±0.01^{b}	0.22±0.00^{a}	0.17±0.01^{b}
Valine	0.57±0.03	0.52±0.05	0.52±0.03	0.54±0.06	0.51±0.01
Total EAA	6.21±0.28	5.20±0.27	5.29±0.16	5.64±0.40	5.57±0.09
Non- Essential Amino Acids (NEAA, g/100g)					
Alanine	0.87±0.01	0,74±0,06	0,79±0,00	0.75±0.05	0.80±0.01
Asparagine	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00
Aspartic acid	1.15±0.04	0.95±0.05	0.99±0.06	1.00±0.05	1.08±0.01
Citrulline	0.26±0.34	0.02±0.00	0.02±0.00	0,04±0,03	0.02±0.00

Amino Acids	Fish Groups				
	Initial	Control	RLM15	RLM20	RLM25
Cystine	0.06±0.00	0.08±0.02	0.05±0.03	0.06±0.06	0.05±0.05
Glycine	0.93±0.04	0.74±0.06	0.85±0.09	0.66±0.03	0.75±0.01
Glutamic acid	1.43±0.05^{b}	1.21±0.06^{c}	1.28±0.06^{c}	1.75±0.11^{a}	1.78±0.03^{a}
Hydroxyproline	0.26±0.00	0.26±0.00	0.26±0.00	0.26±0.00	0.26±0.00
Ornithin	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00
Proline	1.28±0.25^{a}	0.72±0.17^{b}	0.87±0.01^{b}	0.76±0.18^{b}	0.86±0.11^{b}
Sarcosine	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00
Serine	0.70±0.01	0.61±0.03	0.66±0.03	0.60±0.04	0.63±0.02
Tyrosine	0.48±0.06	0.42±0.05	0.40±0.03	0.44±0.06	0.43±0.03
Total NEAA	7.50±0.03	5.85±0.50	6.26±0.05	6.41±0.44	6.75±0.23

Data are reported as mean ± SD of three replicates (n = 3). Means with different superscript letter in a row are significantly different (P<0.05).

Table 7. Amino acid composition (dry weight basis) in fillets of rainbow trout fed four experimental diets

Amino Acids	Fish Groups			
	Control	RLM15	RLM20	RLM25
Essential Amino Acids (EAA, g/100g)				
Arginine	1.02±0.03	1.10±0.08	1.07±0.02	1.19±0.06
Histidine	0.53±0.01^{a}	0.55±0.00^{a}	0.37±0.01^{b}	0.59±0.04^{a}
Isoleucine	0.59±0.01^{b}	0.66±0.04^{ab}	0.79±0.01^{a}	0.73±0.08^{ab}
Leucine	1.25±0.04^{b}	1.37±0.08^{ab}	1.50±0.03^{a}	1.48±0.04^{a}
Lysine	1.14±0.04	1.21±0.11	1.34±0.06	1.35±0.18
Methionine	0.36±0.32	0.61±0.01	0.57±0.09	0.36±0.39
Phenylalanine	0.84±0.02	0.94±0.06	0.94±0.01	0.98±0.07
Threonine	0.81±0.01^{b}	0.88±0.03^{ab}	0.80±0.02^{b}	0.95±0.03^{a}
Tryptophan	0.20±0.06	0.23±0.02	0.06±0.03	0.12±0.11
Valine	0.65±0.01^{b}	0.74±0.03^{ab}	0.83±0.01^{a}	0.81±0.06^{a}
Total EAA	7.41±0.56	8.32±0.43	8.30±0.07	8.58±0.35
Non- Essential Amino Acids (NEAA, g/100g)				
Alanine	1.02±0.03^{b}	1.15±0.07^{ab}	1.04±0.03^{b}	1.22±0.01^{a}
Asparagine	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.01
Aspartic acid	1.59±0.03	1.75±0.13	1,.57±0.06	1.76±0.18
Citrulline	0.02±0.00	0.02±0.00	0.11±0.11	0.38±0.46
Cystine	0.07±0.03	0.11±0.05	0.12±0.07	0.05±0.01
Glycine	0.71±0.02	0.80±0.11	0.80±0.01	0.85±0.07
Glutamic acid	2.48±0.13	2.71±0.21	2.41±0.07	2.78±0.11
Hydroxyproline	0.26±0.00	0.26±0.00	0.26±0.00	0.26±0.00
Ornithin	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00
Proline	0.72±0.06	0.87±0.03	0.69±0.04	0.98±0.32
Sarcosine	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00
Serine	0.75±0.03	0.83±0.07	0.70±0.01	0.88±0.08
Tyrosine	0.58±0.01^{b}	0.65±0.01^{ab}	0.62±0.01^{b}	0.68±0.00^{a}
Total NEAA	8.28±0.34^{b}	9.24±0.62^{ab}	8.41±0.27^{ab}	9.93±0.15^{a}

Data are reported as mean ± SD of three replicates (n = 3). Means with different superscript letter in a row are significantly different (P<0.05).

Table 8. Amino acid requirement of rainbow trout (NRC, 2011)

Amino acid requirement of rainbow trout (g/100 g protein)	
Arginine	3.3
Histidine	1.6
Isoleucine	0.9
Leucine	1.6
Lysine	2.0
Methionine	2.2
Phenylalanine	2.1
Threonine	0.9
Tryptophan	0.5
Valine	3.1

Discussion

The effects of dietary fish meal replacement by alternative plant protein sources on the growth performance, feed utilization rate and body composition were investigated in the present study. There are many studies that have evaluated the use of plant protein sources in diets for rainbow trout (Dabrowski et al. 1989; Watanabe et al. 1993; Gomes et al. 1995; Kaushik et al. 1995; Xie & Jokumsen 1997; Ustaoglu-Tiril et al. 2009; Bilguven & Baris 2011; Øverland et al. 2013; Ouraji et al. 2013; Hauptman et al. 2014; Bahrevar & Faghani-Langroudi 2015; Dogan & Bircan 2015; Lee et al. 2015; Tenamura et al. 2016; Craft et al. 2016; Gerile & Pirhonen, 2017). However, information on the dietary replacement of

fish meal by RLM is non-existent, except the study by Ustaoglu-Tiril et al. (2009).

It has been reported in several studies that the use soybean meal in dietary fish meal replacement does not negatively affect growth performance in cultured fish species (Refstie et al. 1997; Davies et al. 1997; Carter&Hauler 2000; Opstvedt et al. 2003; Zhou et al. 2011). However, some studies have reported a decrease in the growth performance of fish fed diets when fish meal was replaced by alternative protein sources other than soy meal (Xie & Jokumsen 1997; Luo et al. 2006; Palmegiano et al. 2006; Romarheim et al. 2006; Øverland et al. 2009; Slawski et al. 2011; Bullerwell et al., 2016; Anderson et al., 2018). Similarly, an increasing inclusion of RLM in diets in the present study led to a decrease in weight gain of rainbow trout. At the end of the experiment, the control group had the highest weight gain (30.55±0,08 g) while the RLM25 group had the least weight gain (28,89±0,08 g). These results indicate that fish use less lentil meal than fish meal. Congruently, Kasiga and Brown (2019) were found weight gain decreased with increased fish meal replacement by carinata (*Brassica carinata*) meal.

At the end of the present trial, feed utilization ranged from 0.91-0.95 (P>0.05). These results show that fish can use all of the experimental feeds effectively. Similar results were reported by Kasiga and Brown (2019), Glencross et al. (2011) and Cheng and Hardy (2002) in juvenile rainbow trout when dietary fish meal was replaced with pods and cotton seeds respectively. However, Ustaoglu-Tiril et al. (2009) found feed utilization rates of 1.61 for rainbow trout by feeding at 30% of red lentil meal in the test diet. Feed utilization has been reported to decrease with increasing inclusion of plant protein sources diets of rainbow trout (Xie and Jokumsen 1997; Adelizi et al. 1998; Cheng et al. 2003; Lou et al. 2006; Ustaoglu-Tiril et al. 2009).

Crude protein levels in fish fillet decreased with increasing dietary fish meal replacement. (P<0.05). The liver lipid of fish showed a gradual decrease with increasing substitution of fish meal (P<0.05). Similarly, lipid content in fish fillet increased with increasing dietary RLM inclusion (P<0.05). Previous studies have showed that protein content of fish fillet is reduced whereas fillet lipid is increased when carnivorous fish species, including rainbow trout, are fed alternative diets with plant sources providing dietary protein (Palmegiano et al. 2006; Deng et al. 2006; Shafaeipour et al. 2008; Güroy et al. 2011).

The examination of the amino acid composition of test diets showed a reduction in EAA with increasing inclusion of plant protein sources (P<0.05). It appears that the reduction in dietary EAA in experimental diets was due to the inclusion of RLM as all the other dietary ingredients from plant sources had higher EAA contents than RLM. In comparison to the EAA dietary requirements of rainbow trout, replacement of fish meal with plant protein sources led to reduced dietary levels of arginine, histidine, methionine, tryptophan and valine. RLM20 and RLM25 were found to be deficient in lysine levels in feed groups. However, the levels of phenylalanine, isoleucine, leucine and threonine in the control and in all three experimental diets were above the levels required for fish. Naturally, EAA levels in fillets were higher than whole body's. Because whole body was included all fish parts such as skin, bones, gills and skull but the fillet was only pure fish meat. Non-essential amino acids are important for rainbow trout's nutrition as in all fish. Barely, it is well known that fish can synthesized NEAA in direction of their needs. For this reason, there was not much discussion on NEAA.

The present study showed that the RML15 can be used in juvenile rainbow trout feed. In addition to we thought that RML20 and RML25 groups may be used with synthetic amino acids. Because EAA composition in experimental feeds as well as amounts of EAA that seem to be inadequate, especially due to the increase in red lentil meal. In the future, research on synthetic amino acids is expected to continue.

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SHORT COMMUNICATION

The Determination of Microbiological Properties of Rainbow Trout (*Oncorhynchus mykiss*) Applied with Black Cumin Oil in Different Concentrations

Gökhan Arslan*

Atatürk University, Faculty of Fisheries, Department of Processing Technology, Erzurum/Turkey

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ABSTRACT

In this study, the effect of black cumin oil in different concentrations (1%, 1.5% and 2%) was examined on the microbiological quality of rainbow trout (*Oncorhynchus mykiss*) fillets. The study groups were separated in four as control group (A) without containing cumin black oil, group (B) containing 1% black cumin oil, group (C) containing 1.5% black cumin oil, and group (D) containing 2% black cumin oil. The microbiological changes of (total mesophilic aerobic bacteria, total psychrophile aerobic bacteria, *Enterobactericeae* bacteria and yeast-mould) of the samples were determined in every three days period. The count of TMAB was determined exceeded the acceptable limit value (7 log cfu/g) on the 9th day in A group samples. Groups with essential oil were below this value during storage. The highest count of TPAB count at the end of the storage, 8.03±0.02 log cfu/g in group control (A), the lowest 3.05±0.09 log cfu/g in D group. It was concluded that black cumin oil added in different concentrations had positive microbiological effects on trout fillets.

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Introduction

Today, the increasing number of female employees, workload, advancing technology, and different habits of taste necessitate the catering industry. Aquaculture products can be made to ready for consumption by processing in different ways, as in other foodstuffs (Ovayolu, 1997; Metin, 2001; Kılınççeker et al., 2009; Can, 2011; Kılınççeker, 2014).

By consuming processed and consumed seafood, the product is protected, benefit more from the products, job opportunities are increased, the residues are brought to the economy, the consumer is provided with ease, the product is given a different flavor and these products are also economically utilized (Oğuzhan et al., 2006; Kılıç, 2016).

Synthetic and natural additives have been used good alternatives to ensure food safety many years. Consumers prefer natural additives due to the detrimental effects of synthetic additives health (Pizzale et al., 2002; Duman et al., 2012; Kuş, 2012; Mutlu and Bilgin, 2016; Emir Çoban et al., 2018; Oğuzhan Yıldız, 2019). For this reason, the use of natural plant extracts/oils that have antimicrobial and antioxidant effects has become widespread (Akarsu, 2016).

Black cumin (*Nigella sativa* L.) is a plant from *Ranunculaceae* family (buttercup family) which can be found in many countries, notably as East Mediterranean countries. Black cumin oil is widely used in several fields such as cosmetics, food and drug industry. It is one of the most frequently used oils in the fields of health and food. In our country, the oil is widely produced in Afyon, Burdur, Konya and

* Corresponding author
E-mail address: gokhan.arslan@atauni.edu.tr

Isparta provinces. 13 different species of black cumin is produced in our country and it is mostly consumed as a spice (Bourgou et al., 2012; Rooney and Ryan, 2005; Kar et al., 2007; Bulca, 2014; Kılıç, 2016).

The nutrient content of the black seed is composed of 20.8% crude protein, 3.7% ash, 7.0% moisture, 34.8% lipids, and 33.7% carbohydrate (Öz et al., 2017). Black cumin has been reported to posses natural antioxidant, antibacterial and antifungal effects and has been used as a food preservative because of its antioxidant and antimicrobial effect (Yimer et al., 2019). Black cumin oil is highly effective in some pathogenic Gram-positive and Gram-negative bacteria (Öz et al., 2017).

Arıcı et al., (2005) investigated the antimicrobial activity of black cumin oil. They found that black cumin oil addition improved the microbiological quality. Özpolat and Duman (2016) studied the antimicrobial properties of black cumin oil during storage at 2± 1°C and they found that black cumin oil reduced the growth of bacteria. This study aims to examine the effects of black cumin oil in different concentrations for prevention or controlling microbial growth in rainbow trout (*O. mykiss)* fillets.

Materials and Methods

Sample Preparation

The black cumin oil used in the study was procured from a private firm in Ardahan province. Rainbow trouts, 250-300 g on average weight, were obtained from a trout farming business in the Şavşat district of Artvin province and were transferred to the laboratory in cold chain conditions within the ice. Their head was cut, internal organs were removed and made into fillets. Two fillets were obtained from each fish. Then, the fillets were separated in four as control group (without added cumin black oil-A), added 1% black cumin oil (B), added 1.5% black cumin oil (C) and added 2% black cumin oil (D). A total of 8 fish samples were in each of treatment groups. Black cumin oil was applied to both sides of the fillets with a brush in appropriate volumes. It was first applied to one surface of the fillet and then to the other surface after it dried slightly (5 minutes). The fillets were then placed in foam plates and covered with stretch film and stored under refrigerator conditions (4° C ± 1). The microbiological analyses of the fillets were examined on the 0, 3, 6 and 9 days of storage. The study was conducted with two replications.

Microbiological Analyses

25 g samples were taken from the fish for microbiological analyses, transferred into stomacher bags and 225 ml of sterile peptone water was added. Then the bags were homogenized in a stomacher device. Plate Count Agar (PCA) medium was prepared in order to count the total aerobic mesophilic bacteria (TAMB) and total psychrophilic bacteria (TPAB). Two parallel plantings were performed with the prepared dilutions in accordance with the spread plate method. TAMB was left for incubation at 30°C for 2 days and TPAB was left for incubation at 7°C for 7 days. Man Rogosa Sharpe Agar medium was used for the enumeration of lactic acid bacteria. The petri dishes were incubated at 30°C for 2 days for LAB count. Violet Red Bile Dextrose (VRBD) Agar was prepared for the enumeration of *Enterobacteriaceae* and was incubated at 30°C for 2 days in anaerobic conditions. Rose Bengal Chloramphenicol Agar was used for the enumeration of yeast-mould and sonar microbial enumeration was conducted after the petri dishes were left for incubation at 25°C for 5 days (Gökalp et al., 2001).

Statistical Analysis

Data were evaluated using analysis of variance (ANOVA) using the statistical package for social scientists- SPSS 22 software at significance level of 95%. The variance of significance was verified using Duncan test.

Results and Discussion

The microbiological analysis results of rainbow trout which were added black cumin oil extract in different concentrations (%1, 1.5% and 2%) were demonstrated in Table 1.

Significant increases were determined in the amount of TAMB during the preservation process ($p<0.05$). While the highest increase was observed in the control group, the lowest amount of TAMB was determined in the D sample. On the first day of storage, TAMB bacteria values were respectively determined as 4.07, 3.09. 2.87 and 2.71 log cfu/g in group A, B, C and D. The acceptable amount of TAMB and TAPB in fresh fish were determined as 7 log cfu/g by the ICMSF (1986). Samples in group A reached limit value (7 log cfu/g) on the 9th day. Groups with essential oil were below this value during storage period. Akarsu (2016) examined the amount of TAMB in thyme extracts which were obtained by applying different processing techniques (hot infusion, cold infusion, distillation and boiling) on trout fillets. As a result of the 21-days of storage, it was reported that the amount of TMAB exceeded the limit value on the 17th day in the control group and the 21st day in other groups. In the study of Andevari and Rezai (2011) in which the effects of cinnamon oil extract in different concentrations (1%, 1.5% and 2%) were examined on the quality of rainbow trout, it was stated that the amount of TAMB bacteria in cinnamon oil additive samples were lower than the control group. In the study of Uçak (2019) which was conducted with green tiger shrimp and japanese shrimp that were treated with onion peel extract, it was reported that the onion peel extract significantly slowed the bacterial growth and total viable count on the shrimps which were treated with onion skin extract was significantly lower than the control groups ($p<0.05$). Similar results were found by previous studies (Özpolat and Duman 2016; Öz et al., 2017).

An increase was observed in the amount of TPAB depending on the storage duration in all of the groups and the amount of TPAB were respectively determined as 4.36, 3.45, 3.22 and 3.05 log cfu/g in the group A, B, C and D. The determined value in the control group was higher than the other groups ($p<0.05$). Erkan et al. (2011) emphasized that the laurel and cinnamon ail applications on the bluefish were effective on the amount of TPAB. In the study of Duman et al. (2012), rosemary and thyme oil were applied on marinated crayfish and it was reported that the amount of TPAB was higher in the control

group than the essential oil added groups. Our study is similar to the findings of this research. When the TMAB and TPAB values of this study are examined, it can be stated that the essential oils have an antimicrobial effect. This study showed black cumin oil addition improved the microbiological quality.

Table 1. The results of microbiological analyzes of rainbow trout samples applied with black cumin oil (log cfu/g)

Microbiological Analyzes	Group	0. day	3. day	6. day	9. day
Total Aerobic Mesophilic Bacteria	A	4.07±0.03^{a}	5.51±0.05^{b}	6.91±0.08^{c}	7.92±0.09^{d}
	B	3.09±0.14^{a}	3.80±0.14^{b}	4.75±0.12^{c}	5.21±0.02^{d}
	C	2.87±0.12^{a}	3.48±0.24^{b}	4.32±0.16^{c}	5.02±0.18^{d}
	D	2.71±0.04^{a}	3.24±0.08^{b}	4.13±0.07^{c}	4.66±0.12^{d}
Total Psychrotrophic Bacteria	A	4.36±0.06^{a}	5.86±0.05^{b}	7.02±0.02^{c}	8.03±0.02^{d}
	B	3.45±0.14^{a}	4.25±0.10^{b}	4.63±0.21^{b}	5.82±0.07^{c}
	C	3.22±0.07^{a}	3.94±0.09^{b}	4.30±0.11^{c}	5.56±0.14^{d}
	D	3.05±0.09^{a}	3.65±0.17^{b}	3.96±0.05^{b}	5.11±0.09^{c}
Lactic Acid Bacteria	A	2.16±0.06^{a}	2.92±0.09^{b}	3.31±0.14^{c}	5.03±0.12^{d}
	B	2.02±0.03^{a}	2.50±0.24^{b}	3.09±0.12^{c}	4.36±0.10^{d}
	C	2.00±0.00^{a}	2.18±0.04^{a}	2.86±0.11^{b}	3.82±0.23^{c}
	D	2.00±0.00^{a}	2.07±0.06^{a}	2.60±0.07^{b}	3.53±0.07^{c}
Enterobacteriaceae	A	2.00±0.00^{a}	2.97±0.04^{b}	3.55±0.13^{c}	4.09±0.14^{d}
	B	2.00±0.00^{a}	2.31±0.19^{a}	2.95±0.09^{b}	3.30±0.12^{b}
	C	2.00±0.00^{a}	2.08±0.07^{a}	2.78±0.12^{b}	3.12±0.07^{b}
	D	2.00±0.00^{a}	2.04±0.06^{a}	2.52±0.07^{b}	2.95±0.06^{c}
Yeast-Mould	A	2.00±0.00^{a}	2.25±0.11^{a}	2.67±0.12^{b}	3,07±0.11^{c}
	B	2.00±0.00^{a}	2.11±0.10^{a}	2.42±0.03^{b}	2.65±0.08^{c}
	C	2.00±0.00^{a}	2.00±0.00^{a}	2.33±0.07^{b}	2.51±0.05^{c}
	D	2.00±0.00^{a}	2.00±0.00^{a}	2.09±0.06^{a}	2.37±0.05^{b}

A= control; B= sample applied with 1% black cumin oil; C= sample applied with %1,5 black cumin oil; D= sample applied with 2% black cumin oil.

The highest amount of LAB was determined in the samples of the control group (A), and the lowest amount of bacteria was determined in the samples of group D of which 2% black cumin oil was added. An increase was observed in the amount of LAB in all of the groups throughout the storage duration. Duman et al. (2012) emphasized that the amount of LAB in the rosemary and thyme oil added groups were lower than the control group. Patır et al. (2015) reported that the amount of LAB in marinated trout (*O. mykiss*) with eugenol-added samples were lower than the control group. Compared to other studies, our current research results showed similar results both in terms of storage and positive effects of plant oils.

At the end of the storage process, the amount of *Enterobacteriaceae* were determined as 4.09 log cfu/g in the samples of group A, 3.30 log cfu/g in the samples of group B, 3.12 log cfu/g in the samples of group C, and 2.95 log cfu/g in the samples of group D and the difference between the groups were significant. In the study of İnanlı et al. (2018) in which the effect of chitosan coating enriched with blueberry and goji berry extracts was examined on the microbial growth of rainbow trout fillets, it was reported that the highest amount of *Enterobacteriaceae* was determined in the control group (İnanlı et al., 2018). Özpolat and Duman (2016) reported higher Enterobacteriaceae count control group and lower the treatment groups black cumin oil.

While the amount of yeast-mould increased in all of the groups depending on the storage duration, the highest value was determined in the samples of group A (3.07±0.11 log cfu/g) at the last day of the storage. Significant differences were determined between the groups throughout the storage duration ($p<0.05$). İnanlı et al. (2018) determined the highest amount of yeast-mould in the rainbow trout fillets as 5.14±0.12 log cfu/g in the samples of the control group and the lowest amount of yeast-mould in the samples which were coated with goji berry added chitosan as 3.74±0.19 log cfu/g. Our study is similar to the findings of this research.

Conclusion

When the overrall microbiological analysis results were evaluated, it can be stated that black cumin oils added with different concentrations have an antibacterial effect on the rainbow trout fillets.

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Alınteri

Yazışma Adresi / Correspondence address

Kastamonu Üniversitesi Su Ürünleri Fakültesi KASTAMONU

Tlf: 0366 280 23 07 **Fax:** 0366 280 23 13

email: alinteridergisi@hotmail.com

www.alinteridergisi.com