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The Leaf Properties, Stomatal Index and Chlorophyll Content of Turkish Hazelnut (*Corylus avellana L.*) Cultivars

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ABSTRACT
This study examined the leaf micromorphological properties, stomatal indexes, and chlorophyll contents of 20 Turkish hazelnuts (*Corylus avellana*) cultivars. The cultivars examined included the "Acı, Allahverdi, Cavcava, Çakıldak, Foşa, Giresun melezi, Incekara, Kalınkara, Kan, Karafındık, Kargalak, Kuş, Mincane, Okay 28, Palaz, Sivri, Uzunmusa, Tombul, Yassı Badem, and Yuvarlak Badem". The chlorophyll content was measured by a portable chlorophyll meter and the surface sections of leaves were excised by hand and all measurements were obtained by using imaging software (NIS - Elements, Version 3.00 SP5). The stomatal index per unit area ($1x10^4$ μm^2) was calculated. For scanning electron microscope (SEM) imaging, the dried leaves were mounted on stubs using double-sided adhesive tape. The leaf samples were coated with 12.5-15.0 nm of gold and the coated leaves were photographed using a Hitachi SU 1510 SEM. Three wax ornamentation types were found in the leaf samples (e.g., crust, smooth, and granules). The epidermal features, stomatal index, and chlorophyll quantities showed some differences among the *C. avellana* cultivars. The importance of stoma width and stoma length were determined for the "Palaz", "Kuş", "Yuvarlak Badem", and "Yassı Badem". The stomatal index and width and length of upper epidermis and lower epidermis were identified as distinctive properties for the "Allahverdi", "Kargalak", "Kara", and "Mincane". The chlorophyll density was identified as a distinctive feature of the "Sivri", "Çakıldak", "Incekara", and "Acı" cultivars. The highest correlation was found at a rate of 0.98 between "Okay 28" and "Tombul" while the lowest correlation was found at a rate of 0.87 between "Sivri - Karafındık", "Sivri -Foşa", and "Sivri - Kargalak".

Keywords: *Corylus avellana*, Leaf micromorphology, Scanning electron microscopy, SPAD, Stoma

1. Introduction

The Betulaceae family consists of six genera and 120 species around the world (Hardin & Bell 1986) and includes 5 genera and 12 species in Turkey (Güner et al. 2012). The *Corylus* L. genus belongs to the family Betulaceae. According to Davis (1982) and Güner et al. (2012), the genus is represented by three species in Turkey; *C. avellana* L., *C. maxima* Mill. and *C. colurna* L. Today, however, many researchers agree that the genus should be represented in Turkey by 2 species as *C. colurna* and *C. avellana*. According to these researchers, *C. maxima* species should be included in *C. avellana* species due to its continuous variation in morphology, hybridizes easily, and overlaps geographical distribution. In addition, DNA fingerprint dataset analysis supports a common origin for the *C. maxima* and *C. avellana* species (Mehlenbacher 1991; Rovira 1997; Botta et al. 2019; Erdogan & Mehlenbacher 2000; 2002). The common hazelnut (*C. avellana*) is an important horticultural crop and is grown for consumption worldwide. There are 20 hazelnut cultivars in Turkey, of which 18 are registered and 2 are unregistered. The registered cultivars include "Allahverdi, Cavcava, Çakıldak, Foşa, Giresun Melezi, Incekara, Kalınkara, Kan, Karafındık, Kargalak, Mincane, Okay 28, Palaz, Sivri, Uzunmusa, Tombul, Yassı Badem, Yuvarlak Badem". "Acı" and "Kuş" are unregistered cultivars (Balık et al. 2016). "Kargalak" has the biggest nut and kernel size among the other Turkish hazelnut cultivars, while the "Tombul" is reported to be the highest quality and the most productive hazelnut in Turkey (Akçin & Bostan 2018).

The leaf characteristics such as chlorophyll quantities, stomata and epidermal structures are effective on hazelnut yields, fruit quality and resistance to ecological conditions (Rong-hua et al. 2006). For this reason, it is important to determine the characteristics of cultivars such as stomatal characteristics and chlorophyll quantities. The ability of plants to adapt to an ecological environment is related to the processes of transpiration and photosynthesis that occur in the leaves. In addition, the number of stomata and stomatal properties affect gas exchange, photosynthesis production, drought resistance, and vegetative development (Çağlar & Tekin 1999; Çağlar et al. 2004; Drake et al. 2013). The number of stomata per unit area, stomata, and epidermis properties varies according to species and cultivars (Çağlar et al. 2004; Akçin et al. 2013; Avcı & Aygün 2014; Hurt & Doğan 2020). Although leaf micromorphological features such as cuticular wax types, and epidermal and stomatal properties have been used in the identification of plants, the literature survey has shown that no comprehensive study has yet been conducted.

The quantity of chlorophyll in leaves is typically expressed in terms of either concentration or content and can vary significantly in value among different plant taxa and growing stages (Taiz et al. 2014).

There are some data on leaf epidermis micromorphologies of the *Corylus* species. Uzunova (1999) investigated the leaf epidermis in European Corylaceae while Avcı & Aygün (2014) determined the stomata density and distribution in the leaves of 18 varieties of Turkish hazelnuts. There is, however, no data on the micromorphological properties of Turkish hazelnut cultivars.

This study aims to determine the differences between the stomatal index and chlorophyll content (SPAD value) of 20 Turkish hazelnut cultivars and determine the similarities and differences between them.

2. Material and Methods

The specimens of 20 hazelnut cultivars were collected from the Hazelnut Research Station (Giresun -Turkey- coordinate: 40°54'35.2"N, 38°21'09.7"E), which sits at an altitude of 14 m, in 2021. The studied cultivars were ''Acı, Allahverdi, Cavcava, Çakıldak, Foşa, Giresun melezi, Incekara, Kalınkara, Kan, Karafındık, Kargalak, Kuş, Mincane, Okay 28, Palaz, Sivri, Tombul, Uzunmusa, Yassı Badem, and Yuvarlak Badem''. The experimental design was planned in a randomized manner with five replications (5 bushes with multi stems), and a plant represented by 5 leaves in each replication. A total of 10 measurements were obtained for each leaf. Leaves of the same size at the tips of south-facing branches were used for measurements. Chlorophyll measurements were conducted at 13:00-14:00 on 7 July. The SPAD value of each leaf was obtained by an average of 250 measurements. Chlorophyll content was measured through a portable chlorophyll meter (Minolta SPAD-502, Osaka, Japan). In each cultivar, the quantity of chlorophyll in the leaves was measured, after which the leaves were placed in a 70% alcohol solution to determine the stomatal index of the cultivars. The surface sections of leaves were excised by hand and they covered with glycerin-gelatin (Vardar 1987). All measurements were obtained using imaging software (NIS - Elements, Version 3.00 SP5). The stomatal index per unit area ($1x10^4$ μm^2) was calculated according to Meidner and Mansfield (1968). For scanning electron microscope (SEM) imaging, dried leaves were mounted on stubs using double-sided adhesive tape. The samples were coated with 12.5-15.0 nm of gold and the coated leaves were examined and photographed using a Hitachi SU 1510 SEM (Figures 1, 2).

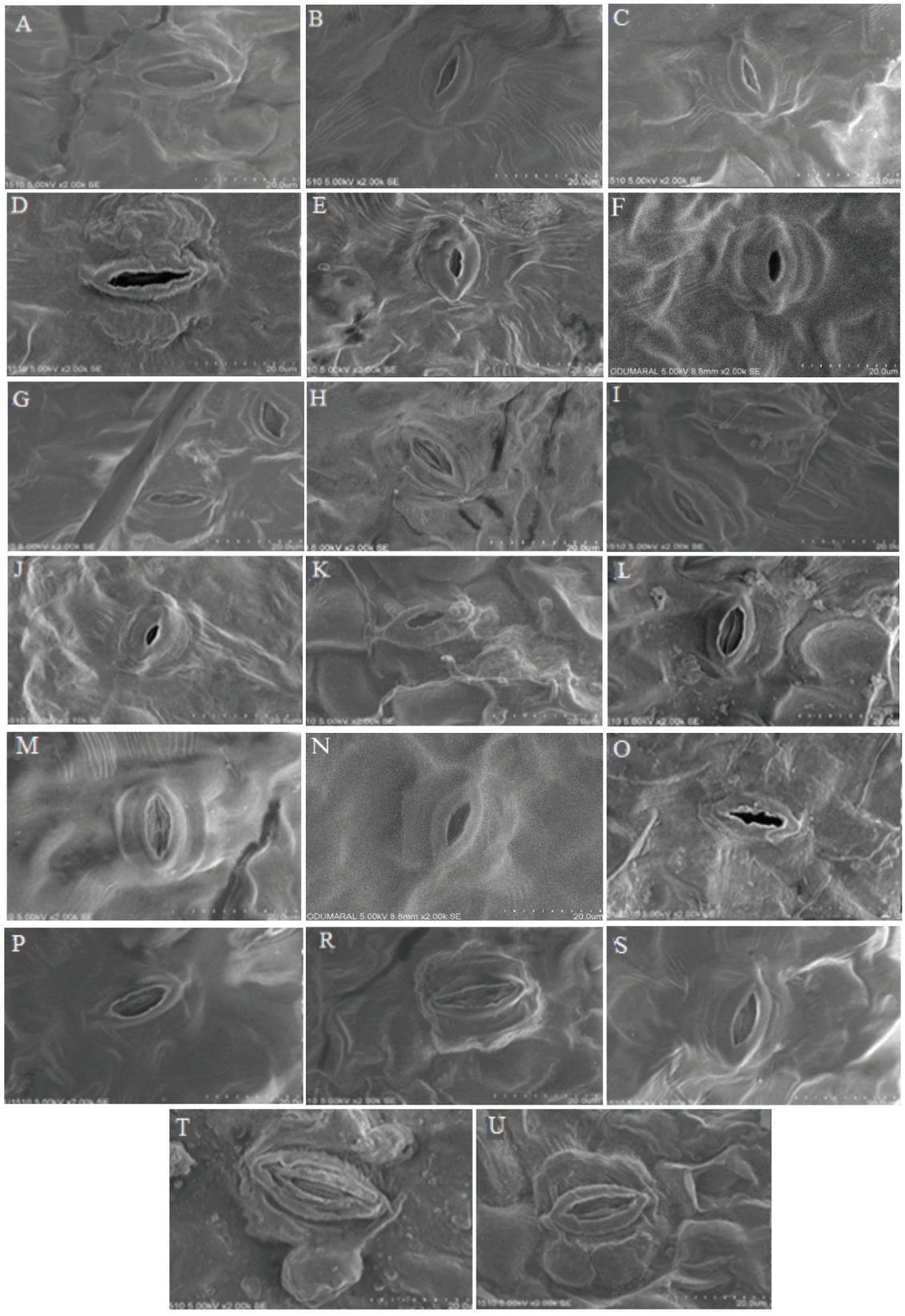

Figure 1- Scanning electron micrographs of upper leaf surface of *C. avellana* cultivars. A: Acı, B: Allahverdi, C: Cavcava, D: Çakıldak, E: Foşa, F: Giresun Melezi, G: Incekara, H: Kalınkara, I: Kan, J: Kara, K: Kargalak, L: Kuş, M: Mincane, N: Okay 28, O: Palaz, P: Sivri, R: Tombul, S: Uzunmusa, T: Yassı Badem, U: Yuvarlak Badem

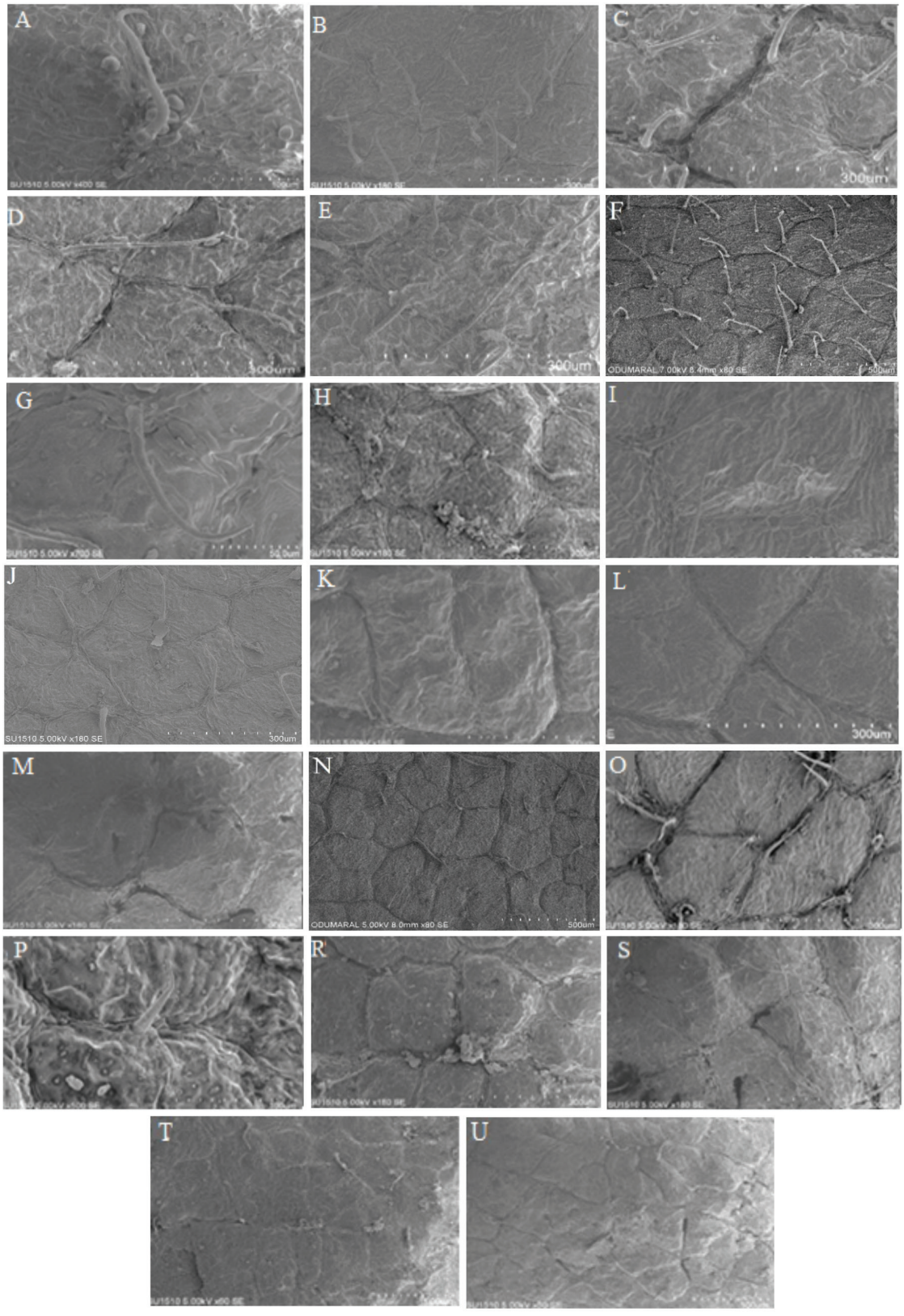

Figure 2- Scanning electron micrographs of leaf lower surface of *C. avellana* cultivars. A: Acı, B: Allahverdi, C: Cavcava, D: Çakıldak, E: Foşa, F: Giresun Melezi, G: Incekara, H: Kalınkara, I: Kan, J: Kara, K: Kargalak, L: Kuş, M: Mincane, N: Okay 28, O: Palaz, P: Sivri, R: Tombul, S: Uzunmusa, T: Yassı Badem, U: Yuvarlak Badem

Table 1- Some morphological properties of leaf epidermis, stomata and chlorophyll density in 20 cultivars of *C. avellana* species

Cultivar	*Epidermis cell (mean)*						*Stomata cell (mean)*				
	Length (µm)		*Width (µm)*		*Number of epidermis cells (1x10⁴ µm²)*		*Length (µm)*	*Width (µm)*	*Number of stomata (1x 10⁴ µm²)*	*Stomatal index*	*Chloropyll content*
	Ue	*Le*	*Ue*	*Le*	*Ue*	*Le*					
Acı	17.99BCDEFG	20.77ABCDEF	20.50EFG	34.83ABCD	31.22EF	24.77EFGH	24.46BCDEFG	20.81ABCDE	2.33AB	8.60ABCD	47.24AB
Allahverdi	14.74EFG	26.04AB	20.83EFG	38.92AB	36.55BCD	22.33GHI	26.06ABCDE	18.59CDEF	2.11AB	8.66ABCD	46.32ABC
Cavcava	17.21CDEFG	22.91ABCD	26.67BCDE	37.64ABC	31.55EF	19.55IJK	27.75AB	23.49A	1.77B	8.26ABCD	46.13ABCD
Çakıldak	18.69BCDEF	16.16F	24.09CDEFG	28.4D	27G	24.77EFGH	23.53DEFG	21.67ABC	2.55AB	9.33ABCD	47.18AB
Foşa	20.66ABC	24.87ABC	31.43B	39.45A	16.77K	16.44K	27.21ABC	22.19AB	2.33AB	12.44A	41.18FG
Giresun Melezi	15.90DEFG	19.52CDEF	22.50DEFG	33.18ABCD	40.55AB	38A	21.69G	17.58EF	2.66AB	6.56CD	39.58G
İncekara	14.25FG	18.62DEF	20.33EFG	29.87CD	40.44AB	26.66CDEF	26.39ABCDE	22.47AB	2.44AB	8.39ABCD	46.44ABC
Kalınkara	16.56DEFG	20.93ABCDEF	23.58CDEFG	30.85BCD	31.55EF	29.77BC	24.62BCDEFG	19.28BCDEF	2.88AB	6.15D	45.91ABCD
Kan	13.62G	18.04DEF	21.80EFG	30.02CD	35CDE	30.77B	23.11EFG	18.63CDEF	3.22A	9.46ABCD	41.97EFG
Kara	22.10AB	22.01ABCDEF	30.06BC	33.70ABCD	21.11IJ	24.88DEFG	26.35ABCDE	20.04BCDE	2.77AB	10.04ABCD	42.68DEFG
Kargalak	20.95ABC	22.65ABCDE	26.95BCDE	36.63ABCD	22.88HI	28.33BCD	26.82ABCD	21.62ABC	2.55AB	8.25ABCD	30.39H
Kuş	17.38CDEFG	21.80ABCDEF	18.65G	37.32ABC	37BCD	17.66JK	26.83ABCD	20.51ABCDE	2.11AB	10.60ABC	47.69A
Mincane	23.42A	26.40A	25.63BCDEF	35.79ABCD	26.11GH	20.22IJ	23.55DEFG	16.70F	2.22 AB	9.86ABCD	44.02 BCDEF
Okay 28	14.69EFG	21.87ABCDEF	24.55CDEFG	31.90ABCD	42.22A	28.77BC	22.32FG	17.87DEF	3.33A	10.42ABC	45.60ABCD
Palaz	20.42ABCD	20.15BCDEF	24.88BCDEFG	30.16CD	25.88GH	25.88HI	25.36ABCDEF	19.91BCDEF	2.77 AB	11.34AB	47.03AB
Sivri	14.89EFG	16.97EF	18.90FG	28.61D	37.44BC	29.88BC	25.22ABCDEFG	19.43BCDEF	2.11AB	6.50CD	45.51ABCD
Tombul	15.27EFG	19.73CDEF	24.13CDEFG	31.39ABCD	33.11DE	28.22BCDE	22.08G	17.9EF	2.33AB	7.65BCD	46.37ABC
Uzunmusa.	17.86BCDEFG	20.54ABCDEF	26.72BCDE	32.01ABCD	27.55FG	24.55FGH	23.80CDEFG	18.66CDEF	2.44AB	8.95ABCD	44.89ABCDE
Yassı Badem	18.89ABCDE	20.94ABCDEF	38.76A	38.82AB	18.77JK	20.11IJ	28.37A	20.87ABCDE	2.11AB	9.31ABCD	43.29CDEF
Yuvarlak Badem	18.26BCDEFG	22.43ABCDE	29.12BCD	33.78ABCD	21.66IJ	22.66GHI	25.90ABCDEF	21.17±ABCD	3AB	11.56AB	43.36CDEF
P value	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Ue: Upper epidermis surface, Le: Lower epidermis

Table 2- Some micromorphological and morphological properties of leaf epidermis and stomata in 20 cultivars of *C. avellana* species

Cultivars	*Epidermis cell*				*Stomata cell*			
	Shape		*Anticlinal walls*		*Outer stomatal rim*	*Peristomatal rim*	*Aperture*	*Membrane - wax ornamentation*
	Ue	*Le*	*Ue*	*Le*				
Acı	Rec.	Irr.	Undulate	Sinuous	Raised	Evident	Long and wide	Striated-crust
Allahverdi	Irr.	Irr.	Sinuous	Sinuous	Raised	Stout and raised	Long and narrow	Striated -crust
Cavcava	Rec.-pol.	Irr.	Undulate	Sinuous	Raised	Evident	Long and narrow	Striated-smooth
Çakıldak	Rec.-pol.	Irr.	Str-cur	Undulate	Raised	Raised, wide and amorphous	Long and narrow	Smooth- granules
Foşa	Rec.-irr.	Rec.	Undulate	Undulate	Raised and wide	Barely perceptible	Short and wide	Striated-smooth or crust
Giresun Melezi	Rec.-pol.	Rec.-pol.	Str-cur	Str-cur	Raised	Raised and double ring	Short and wide	Striated-smooth
Incekara	Rec.-irr.	Irr.	Str-cur	Sinuous	Raised	Raised	Long and wide	Smooth or striated-granules
Kalınkara	Rec.-pol.	Rec.-pol.	Undulate	Sinuous	Raised	Evident	Long and wide	Striated-smooth or granules
Kan	Rec.-irr.	Rec.-irr.	Str-cur	Undulate	Raised	Overlapping	Long and narrow	Striated- crust
Kara	Rec.	Rec.	Str-cur	Undulate	Raised and wide	Raised	Short and narrow	Striated-smooth
Kargalak	Rec.	Irr.	Str-cur	Sinuous	Raised	Barely perceptible	Short and narrow	Smooth-crust
Kuş	Rec.-pol.	Rec.-pol.	Str-cur	Undulate	Raised	Evident and raised	Short and narrow	Smooth or striated- crust
Mincane	Rec.-pol.	Irr.	Undulate	Sinuous	Raised	Evident and raised	Long and wide	Striated-smooth
Okay 28	Rec.-pol.	Rec.-pol.	Str-cur	Str-cur	Raised	Raised	Long and narrow	Smooth-striated
Palaz	Pol.-rec.	Irr.	Str-cur	Undulate	Raised	Barely perceptible	Short and narrow	Granular-crust
Sivri	Rec.-pol.	Irr.	Undulate	Undulate	Raised	Raised	Long and wide	Striated-granules
Tombul	Rec.-pol.	Irr.	Str-cur	Sinuous	Raised	Overlapping and raised	Long and narrow	Smooth- granules or crust
Uzunmusa.	Pol.	Irr.	Str-cur	Sinuous	Raised	Evident and not raised	Long and wide	Striated-smooth
Yassı Badem	Pol.	Irr.	Str-cur	Sinuous	Raised	Very evident, stout, raised and amorphous	Long and narrow	Granular-smooth
Yuvarlak Badem	Rec.-pol.	Irr.	Str-cur	Sinuous	Raised	Stout, wide, raised and amorphous	Long and narrow	Granular-smooth

Ue: Upper epidermis surface, Le: Lower epidermis, Rec.: Rectangular, Irr.: Irregular, Pol.: Polygonal, Str-cur: Straight to curved

Analysis of variance, Tukey multiple comparison tests and the principal component analysis (PCA) methods were used for statistical analysis of the obtained data. The significance level (α) was determined as 0.05 in calculations and interpretations. The Minitab 17 statistical package program was used for statistical analysis.

3. Results and Discussion

Table 1 shows some morphological properties of leaf epidermis and stomata, stomatal index, and chlorophyll contents in 20 cultivars of *C. avellana* species. The micromorphological characteristics of leaf epidermal cells such as shape, the structure of the anticlinal walls, outer stomatal rims, peristomal rims, apertures, wax ornamentation, and membrane ornamentation are summarized in Table 2. Some significant differences were found among cultivars in terms of the epidermal properties, stomatal index, and chlorophyll contents.

3.1. Epidermis cells

Statistically significant differences were found in the width, length, and number of epidermis cells on the upper and lower surfaces of the leaves in the 20 hazelnut cultivars examined ($p<0.000$) (Table 1). The highest values of upper and lower epidermis lengths were determined in "Mincane" with 23.42 and 26.40, respectively. The smallest epidermis length value was found in "Kan" with 13.62 for the upper epidermis and in "Çakıldak" with 16.16 for the lower epidermis. The largest upper epidermis width was measured in "Yassı Badem" (38.76), and the smallest width was measured in "Kuş". It was determined that "Foşa" has the highest value in the lower epidermis (39.45). The number of epidermis cells in the leaves varies between 16.77-42.22 on the upper surface and 16.44-38 on the lower surface of the examined hazelnut cultivars. The lowest number of the epidermis was found in the "Foşa" on both surfaces.

Leaf anatomy, leaf epidermis morphology, and micromorphology and stomata properties provide relative taxonomic data (Uzunova 1999; Nabin et al. 2000; Chen 2008; Akçin et al. 2013; Razaz et al. 2015) Uzunova (1999) stated that there are differences in the epidermal structures of taxa belonging to the Corylaceae family. Various studies have been conducted on the determination of the leaf anatomical and morphological structures of the cultivars and thus a better recognition of the cultivars was defined (Sagaram & Lambardini 2007; Nur Fatıhah et al. 2014; Najmaddin & Saeed 2020). The anatomical and palynological structures of *Bougainvillea glabra* cultivars were examined and it was determined that there were differences among leaf characteristics. (Najmaddin & Saeed 2020). In our study, statistically significant differences were found among the sizes of epidermis cells, the sizes of stomatal cells, the stomatal index, and the number of stomata and epidermis cells in hazelnut cultivars.

The micromorphological features of epidermis cells are shown in Table 2. The epidermal cell shapes on both surfaces of the hazelnut cultivars are rectangular, polygonal, rectangular-polygonal, or irregular. The irregular epidermis is the most common shape on the lower surface. There are usually rectangular-polygonal cells on the upper surface. The "Allahverdi" has an irregular epidermis shape on the upper surface while the "Foşa", "Incekara", and "Kan" cultivars have rectangular-irregular shapes. The cells on the upper surface of "Acı", "Karafındık" and "Kargalak" are rectangular in shape. The anticlinal walls of the epidermis cells show some differences in the examined specimens. The anticlinal walls of epidermis cells are sinuous and undulate on the lower surface. Eleven cultivars have sinuous anticlinal walls. Undulate, sinuous and straight to curved anticlinal walls are present on the upper surfaces of leaves of the cultivars examined. Straight to curved walls are the most common type on the upper surface of leaves. "Allahverdi" has a sinuous type, and "Foşa" and "Sivri" have undulate type anticlinal walls on both surfaces of a leaf (Figures 1, 2).

There are different opinions about the systematic importance of the shapes of epidermis cells. Chen et al. (2008) stated that the shapes of epidermal cells were not useful in the systematic of the *Salix* genus or Salicaceae family. Cheng (2006) noted that some epidermal characteristics such as the shape of epidermal cells, type of stomata, and cuticular ornamentation in the Schisandraceae family are usually constant within species and this factor is useful in defining the relationship between species. According to present study, anticlinal walls of leaf epidermal cells show differences among the studied cultivars; three cultivars (Allahverdi, Foşa, and Sivri) have the same anticlinal walls on both upper and lower surfaces. In other specimens differences are apparent between the surfaces. These properties can help determine the boundaries of the cultivars "Allahverdi", "Foşa", and "Sivri". Yang and Lin (2005) and Zamani et al. (2015) reported that the properties of an anticlinal wall can be regarded as a diagnostic feature at the species level.

3.2. Stomata

All hazelnut cultivars have stoma only on the lower surfaces of the leaves. Leaves are hypostomatic. The stoma sizes, the number of stomata, and stomatal index were statistically significant in hazelnut cultivars ($p<0.000$) (Table 1). Uzunova (1999) reported

that *C. avellana* and *C. colurna* L. have stomata only on a lower surface of a leaf. The widest stomata were determined in the "Cavcava" cultivar (23.49) while the longest stomata were found in "Yassı Badem" (28.37). Avcı and Aygün (2014) stated that the stomatal characteristics of Turkish hazelnut cultivars are unique and can be used for cultivar identification. Their study results from 18 hazelnut cultivars showed that the average stomatal width was 20.02 µm among the cultivars and varied between 17.00 µm (Sivri) and 22.61 µm (Yassı Badem). It was found that "Yassı Badem" has the widest stoma both in the present study and in Avcı and Aygün's (2014) study. Avcı and Aygün (2014) stated that the number of stomata varied between 83.08-117.73 in 1 mm^2 and the highest number of stomata were determined in "Sivri". In our study, it was determined that the number of stomata varied between 1.77-3.33 per area ($1x10^4$ μm^2). While the highest number of stomata was found in the "Okay 28", the lowest number of stomata was found in "Cavcava". In a study performed on 11 hazelnut cultivars and genotypes, it was determined that hazelnut cultivars and genotypes had different stomatal characteristics such as stomatal number and stomatal size (Hurt & Doğan 2020). In previous studies, it was observed that as the stomatal width in leaves increased, the stomatal density decreased (Mert et al. 2009; Avcı & Aygün 2014; Hurt & Doğan 2020). Our results generally support this statement. While "Cavcava" had the widest stomata with 23.49, it was also found to be the lowest cultivar in terms of stomatal density.

The highest stomatal index was found in "Foşa" with 12.44 and the lowest in "Kalınkara" with 6.15. Avcı and Aygün (2014) reported that the stomatal index values in hazelnut cultivars varied between 10.55 and 17.15. Their study found that "Sivri" had the highest stomatal index and "Kalınkara" had the lowest stomatal index. The lowest stomatal index in "Kalınkara" is in line with our findings. The difference in the stomatal index in cultivars can be explained by differences in the water uptake capacity, light requirement level, and plant growth rate (Warrit et al. 1980; Mert et al. 2009; Avcı & Aygün 2014). Metcalfe and Chalk (1979) stated that changes in the stomatal index may be caused by factors such as humidity and nutritional conditions.

According to the micromorphological features of stomata given in Table 2, the outer stomatal rims are raised in all examined specimens. Wide outer stomatal rims are found in "Foşa" and "Karafındık". The peristomal rims are stout, raised, overlapping, and amorphous in all hazelnut cultivars. In "Foşa", "Kargalak", and "Palaz", the peristomal rim is barely perceptible while "Çakıldak", "Yassı Badem" and "Yuvarlak Badem" have amorphous peristomal rims. "Giresun Melezi" has a raised and double ring rim. Wilkinson (1979) reported that peristomatal rims may vary in different plants.

In present study the stomata aperture is usually long. While "Karafındık", "Kargalak", "Kuş", and "Palaz" have short and narrow apertures, "Foşa" and "Giresun Melezi" have short and wide apertures (Figures 1, 2).

3.3. Cell membrane and wax ornamentation

Three wax ornamentation types are recognized: crust, smooth, and granules in the present study. All hazelnut cultivars. The crust type is the most common wax ornamentation type on both surfaces of hazelnut cultivars. The cell membrane ornamentation types are striated or smooth. Most cultivars have roughly striated cuticles around their stomata which is evident in the "Allahverdi", "Foşa" and "Mincane" cultivars (Table 2, Figures 1, 2). Previous studies have emphasized that wax ornamentations are important in epidermal micromorphological characters (Sonibare et al. 2005; Akçin et al. 2013; Zamani et al. 2015).

3.4. Chlorophyll content (SPAD values)

The chlorophyll content of the 20 hazelnut cultivars of *C. avellana* species is shown in Table 1. The chlorophyll contents were statistically significant in the hazelnut cultivars ($p<0.000$) in which the chlorophyll content of the investigated cultivars varies between 47.69-30.39 values. While the highest chlorophyll content was detected in "Kuş", the lowest value was found in "Kargalak".

Recent studies have shown that the use of physiological characteristics such as chlorophyll content as selection criteria affect yield. Statistically significant correlations were found between the chlorophyll contents and main yield components in wheat where an increase in the amount of chlorophyll affected the yield positively. The photosynthetic pigment concentration in the leaf is related to the amount of sunlight absorbed by the leaf. Therefore, low chlorophyll concentration directly limits photosynthetic potential and primary production (Fillella et al. 1995; Bahar 2015). The most important factor in differentiating the chlorophyll levels of plants is the genetic structure. (Taner & Sade 2005). The amount of chlorophyll varies between species as well as within species according to subspecies, varieties, and forms (Canova et al. 2008; Cetin 2017). It is known that one of the important factors determining the amount of chlorophyll is the leaf structure (Taner & Sade 2005; Atar et al. 2020). In this study, chlorophyll contents were statistically very significant in hazelnut cultivars ($p<0.000$). Chlorophyll SPAD >30 in hazelnut plants was indicated as having a high chlorophyll

Table 3- Similarity rates of cultivars according to Bray-Curtis similarity index

	TMBL	*KRFK*	*ÇKDK*	*FŞ*	*KŞ*	*CVCV*	*UNMS*	*YSBM*	*PLZ*	*KLKR*	*KGLK*	*MNCN*	*YVKB*	*INKR*	*KN*	*SVR*	*AC*	*ALVD*	*OK28*	*GMLZ*
TMBL	1.00																			
KRFK	0.89	1.00																		
ÇKDK	0.95	0.89	1.00																	
FŞ	0.89	0.93	0.90	1.00																
KŞ	0.93	0.91	0.93	0.92	1.00															
CVCV	0.93	0.92	0.93	0.95	0.96	1.00														
UNMS	0.97	0.92	0.95	0.92	0.94	0.95	1.00													
YSBM	0.90	0.96	0.90	0.95	0.92	0.94	0.93	1.00												
PLZ	0.96	0.92	0.96	0.92	0.95	0.94	0.96	0.92	1.00											
KLKR	0.97	0.90	0.95	0.90	0.94	0.94	0.97	0.91	0.96	1.00										
KGLK	0.89	0.91	0.89	0.94	0.91	0.94	0.92	0.92	0.91	0.90	1.00									
MNCN	0.93	0.92	0.92	0.93	0.92	0.93	0.95	0.91	0.94	0.93	0.92	1.00								
YVKB	0.93	0.95	0.93	0.96	0.95	0.96	0.96	0.95	0.95	0.94	0.94	0.94	1.00							
INKR	0.95	0.89	0.95	0.89	0.95	0.94	0.94	0.90	0.95	0.96	0.89	0.90	0.93	1.00						
KN	0.96	0.89	0.95	0.89	0.92	0.91	0.95	0.89	0.94	0.95	0.88	0.91	0.93	0.96	1.00					
SVR	0.95	0.87	0.95	0.87	0.93	0.91	0.94	0.88	0.94	0.96	0.87	0.89	0.91	0.97	0.95	1.00				
AC	0.95	0.91	0.95	0.91	0.97	0.95	0.96	0.92	0.96	0.96	0.91	0.93	0.95	0.96	0.94	0.94	1.00			
ALVD	0.94	0.89	0.91	0.92	0.96	0.95	0.94	0.92	0.93	0.94	0.90	0.94	0.93	0.94	0.93	0.93	0.96	1.00		
OK28	0.98	0.91	0.94	0.90	0.94	0.94	0.97	0.91	0.96	0.97	0.90	0.94	0.95	0.94	0.96	0.94	0.95	0.94	1.00	
GMLZ	0.96	0.89	0.92	0.89	0.92	0.91	0.95	0.89	0.92	0.95	0.90	0.92	0.92	0.93	0.96	0.93	0.94	0.92	0.95	1.00

TMBL: Tombul, KRFK: Kara, ÇKDK: Çakıldak, FŞ: Foşa, KŞ: Kuş, CVCV: Cavcava, UNMS: Uzunmusa, YSBM: Yassı Badem, PLZ: Palaz, KLKR: Kalınkara, KGLK: Kargalak, MNCN: Mincane, YVKB: Yuvarlak Badem, INKR: Incekara, KN: Kan, SVR: Sivri, AC: Acı, ALVD: Allahverdi, OK28: Okay 28, GMLZ: Giresun Melezi

content (Hand & Reed 2014). In our study, the chlorophyll content of the examined cultivars was high, and the chlorophyll SPAD values varied 30.39 and 47.69. The highest chlorophyll content was detected in "Kuş", the lowest value was found in "Kargalak". Atar et al. (2020) reported that *C. avellana* has 30.6-48.9 SPAD values.

According to the Bray-Curtis similarity index (Table 3), the highest correlation was found between "Okay 28" and "Tombul" cultivars with a ratio of 0.98 in terms of the traits examined. The lowest correlation was found between "Sivri - Karafındık", "Sivri - Foşa", and "Sivri - Kargalak" with a 0.87 ratio. The correlation ratio between "Giresun Melezi and Tombul" was 0.96, and the correlations between "Giresun Melezi and Kargalak" and "Okay 28 and Kargalak" were 0.90.

It was determined that the examined epidermal features, stomatal index, and chlorophyll quantities according to the PCA showed some differences among hazelnut cultivars. Stoma width and stoma length were determined to be significant for "Palaz, Kuş, and Yuvarlak Badem" and "Yassı Badem" (Figure 3). However, no statistical correlation was found between the amount of chlorophyll and the stomatal characteristics.

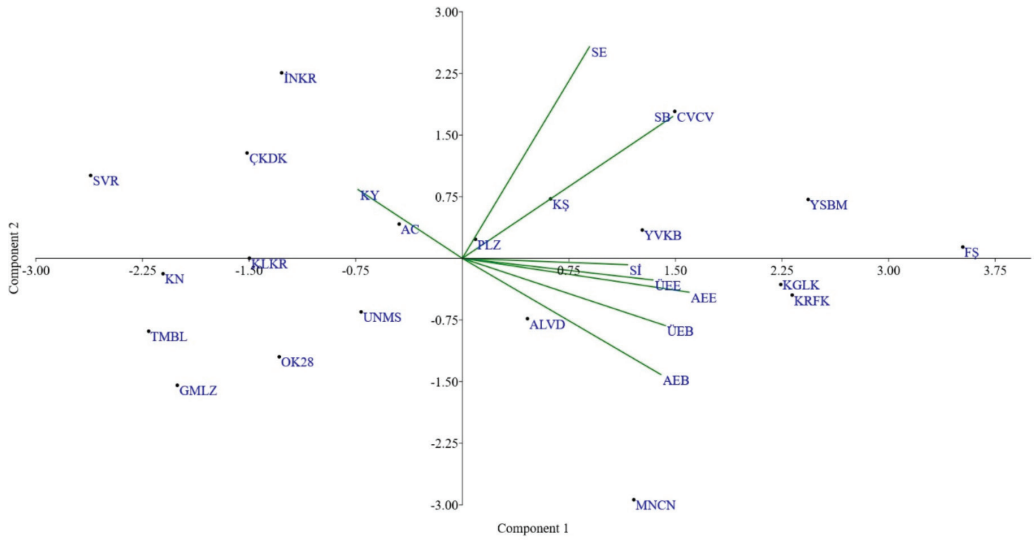

Figure 3- Principal component analysis of investigated traits in hazelnut cultivars. TMBL: Tombul, KRFK: Kara, ÇKDK: Çakıldak, FŞ: Foşa, KŞ: Kuş, CVCV: Cavcava, UNMS: Uzunmusa, YSBM: Yassı Badem, PLZ: Palaz, KLKR: Kalınkara, KGLK: Kargalak, MNCN: Mincane, YVKB: Yuvarlak Badem, INKR: Incekara, KN: Kan, SVR: Sivri, AC: Acı, ALVD: Allahverdi, OK28: Okay 28, GMLZ: Giresun Melezi, SE: Stoma width, SB: Stoma lenght, SI: Stomatal index, UEE: Upper epidermis width, UEB: Upper epidermis lenght, AEE: Lower epidermis width, AEB: Lower epidermis lenght, KY: Chlorophyll content

4. Conclusions

There are 20 hazelnut cultivars in Turkey, 18 of them are registered and 2 of them are unregistered. The determination of hazelnut cultivars is typically performed according to their pomological characteristics. Recently, it has been used in some molecular studies to determine hazelnut varieties. It is crucial to know the anatomical and micromorphological characteristics of the plants to recognize the cultivars better and increase the yield. For this reason, studies have been carried out to better understand the anatomical and micromorphological structures of cultivars in many agricultural products. In our study, the leaf epidermis and stomata characteristics and chlorophyll quantities of 20 hazelnut cultivars were determined in comparatively and in detail. Our study's findings show that the epidermal features, stomatal index, and chlorophyll quantities can be used as distinguishing features in the identification of cultivars.

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References

Akçin Ö E, Şenel G & Akçin Y (2013). Leaf epidermis morphology of some Onosma (Boraginaceae) species from Turkey. *Turkish Journal of Botany* 37(1): 55-64. doi.org/10.3906/bot-1202-33

Akçin Y & Bostan S Z (2018). Variations in some traits affecting yield in different hazelnut cultivars. *Acta Hortic* 1226: 149-152. doi.org/10.17660/actahortic.2018.1226.21

Atar F, Güney D, Bayraktar A, Yıldırım N & Turna I (2020). Seasonal Change Of Chlorophyll Content (Spad Value) In Some Tree And Shrub Species. *Turkish Journal of Forest Science* 4(2): 245-256. doi.org/10.32328/turkjforsci.711389

Avcı N & Aygün A (2014). Determination of Stomatal Density and Distribution on Leaves of Turkish Hazelnut (*Corylus avellana* L.) Cultivars. *Journal of Agricultural Sciences* 20(4): 454-459. doi.org/10.1501/tarimbil_0000001304

Bahar B (2015). Relationships among flag leaf chlorophyll content, agronomical traits, and some physiological traits of winter wheat genotypes. *Dicle University Institute of Natural and Applied Science Journal* 4(1): 1-5.

Balık H I, Kayalak Balık S, Beyhan N & Erdoğan V (2016). Hazelnut Cultivars. Klasmat Press, Trabzon, Turkey.

Bartholtt W, Neinhuis C, Cutler D, Ditsch F, Meusel I, Theisen I & Wilhelmi H (1998). Classification and terminology of plant epicuticular waxes. *Botanical Journal of Linnean Society* 126: 237-260. doi.org/10.1111/j.1095-8339.1998.tb02529.x

Botta R, Molnar T J, Erdogan V, Valentini N, Marinoni D T & Mehlenbacher S A (2019). Hazelnut (*Corylus* spp.) Breeding. *Advances in Plant Breeding Strategies: Nut and Beverage Crops* 4: 157-219. doi.org/10.1007/978-3-030-23112-5_6

Canova I, Durkovic J & Hladka D (2008). Stomatal and chlorophyll fluorescence characteristics in European beech cultivars during leaf development. *Biologia Plantarum* 52(3): 577-581. doi.org/10.1007/s10535-008-0115-3

Chen J, Sun H & Yang Y (2008). Comparative morphology of leaf epidermis of *Salix* (Salicaceae) with special emphasis on sections Lindleyanae and Retusae. *Botanical Journal of Linnean Society* 157: 311-322. doi.org/10.1111/j.1095-8339.2008.00809.x

Cheng Q A (2006). Morphological characters of leaf epidermis in Schisandraceae and their systematic significance. *Journal of Plant Biology* 49(1): 80-87. doi.org/10.1007/bf03030791

Cetin İ Z, Cesur A, Saleh E A A & Mossi M M M (2017). Variation of Chlorophyll Amount in Some Landscape Plants: a case study of Rize, *Journal of Chemical, Biological and Physical Sciences* 7(3): 807-817. doi.org/10.24011/barofd.33134

Çağlar S & Tekin H (1999). Farklı Pistacia anaçlarına aşılı Antepfıstığı çeşitlerinin stoma yoğunlukları. *Turkish Journal of Agriculture and Forestry* 23(5): 1029-1032

Çağlar S, Sütyemez M & Beyazıt S (2004). Stomatal density in some selected walnut (Juglans regia) types. *Mediterranean Agricultural Sciences* 17: 169-174

Davis P H (1982). Flora of Turkey and the East Aegean Islands. Edinburgh University Press 7: 947.

Drake P L, Froend R H & Franks P J (2013). Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. *Journal of Experimental Botany* 64: 495-505. doi.org/10.1093/jxb/ers347

Erdogan V & Mehlenbacher S A (2000). Phylogenetic relationships of *Corylus* species (Betulaceae) based on nuclear ribosomal DNA ITS region and chloroplast matK gene sequences. *Systematic Botany* 25(4): 727-737. doi.org/10.2307/2666730

Erdogan V & Mehlenbacher S A (2002). Phylogenetic analysis of hazelnut species Corylus Corylaceae based on morphology and phenology. *Herb J Syst Bot* 9(1): 83-100

Fillela I, Serrano L, Serra J & Peñuelas J (1995). Evaluating wheat nitrogen status with canopy reflectance indices and discriminant analysis. In: Richardson A D, Duigan S P & Berlyn G P An evaluation of noninvasive methods to estimate foliar chlorophyll content. *New Phytologist* 153(1): 185-194. doi.org/10.1046/j.0028-646x.2001.00289.x

Güner A, Aslan S, Ekim, T, Vural M & Babaç M T (Eds) (2012). List of Turkey Plants (Vascular Plants). Nezahat Gökyiğit Botanik Garden and Flora Researchers Association Press, İstanbul.

Hand C & Reed B M (2014). Minor nutrients are critical for the improved growth of *Corylus avellana* shoot cultures. *Plant Cell Tissue and Organ Culture* 119(2): 427-439. doi.org/10.1007/s11240-014-0545-x

Hardin J W & Bell J M (1986). Atlas of Foliar Surface Features in Woody Plants, IX Betulaceae of Eastern United States. *Brittonia* 38(2): 133-144. doi.org/10.2307/2807266

Hurt H & Doğan A (2020). Determination of Stoma Density and Distribution of Hazelnut (*Corylus avellana* L.) Cultivars and Genotypes Grown in Different Ecologies. *YYU Journal of Agricultural Science* 30(3): 544-552. doi.org/10.29133/yyutbd.705671

Mehlenbacher S A (1991). Hazelnuts (*Corylus*). Genetic resources of temperate fruit and nut crops. *Acta Horticulturae* 290: 791-836. doi.org/10.17660/actahortic.1991.290.18

Meidner H & Mansfield T A (1968). Physiology of Stomata.

Mert C, Barut E & Uysal T (2009). Farklı anaçlar üzerine aşılı elma çeşitlerinin stoma morfolojilerinin araştırılması. *Tarım Bilimleri Araştırma Dergisi* 2(2): 61-64. doi.org/10.29050/harranziraat.259104

Metcalfe C R & Chalk L (1979). Anatomy of Dicotyledons II. Oxford University Press.

Nabin S, Nath S C & Simanta D (2000). Foliar micromorphological characters of few taxa of the genus *Aquilaria* Lamk growing Lamk growing North East India. *Advanced Plant Science* 13(2): 551-558

Najmaddin C & Saeed M M (2020). Anatomical and Palynological Studies of Some Bougainvillea Glabra Cultivars. *Systematic Reviews in Pharmacy* 11(11): 1177-1191

Nur Fatıhah H, Nashrıyah M, Nor Zaımah A, Khaırıl M & Alı A M (2014). Leaf morphology and anatomy of 7 varieties of Ficus deltoidea (Moraceae). *Turkish Journal of Botany* 38(4): 677-685. doi.org/10.3906/bot-1301-7

Razaz M, Naqinezhad A, Mahmoodi A, Hosseinzadeh A & Azimi R A (2015). Comparative Anatomy of *Carpinus orientalis* Mill. (Betulaceae) Populations in Iran. *Journal of Genetic Resources* 1(1): 45-54. doi.org/10.22080/JGR.2015.1123

Rong-hua L, Guo P, Michael B, Stefania G & Salvatore C (2006). Evaluation of Chlorophyll Content and Fluorescence Parameters as Indicators of Drought Tolerance in Barley. *Agricultural Sciences in China* 5(10): 751-757. doi.org/10.1016/s1671-2927(06)60120-x

Rovira M (1997). Genetic variability among hazelnut (*C. avellana* L.) cultivars. *Acta Horticulturae* 445:45-50. doi.org/10.17660/actahortic.1997.445.6

Sagaram M & Lombardini L (2007). Variation in Leaf Anatomy of Pecan Cultivars from Three Ecogeographic Locations. *Journal of the American Society for Horticultural Science* 132(5): 592-596. doi.org/10.21273/jashs.132.5.592

Sonibare M A, Jayeola A A, Egunyomi A & Murata J (2005). A survey of epidermal morphology in *Ficus* Linn. (Moraceae) of Nigeria. *Botanical* Bulletin *of Academia Sinica* 46: 231-238. doi.org/10.7016/BBAS.200507.0231

Taiz L, Zeiger E, Moller I M & Murphy A (2014). Plant Physiology and development.

Taner S & Sade B (2005). Low temperature effect of cereal. *Journal of Crop Research* 2(2): 19-28.

Uzunova K R (1999). A comparative study of leaf epidermis in European Corylaceae. *Feddes Repert* 110(3): 209-218. doi.org/10.1002/fedr.4921100307

Vardar Y (1987). Preparation Techniques in Botany. Ege University Press.

Warrit B, Landsberg J J & Thorpe M R (1980). Responses of apple leaf stomata to environmental factors. *Plant, Cell and Environment.* 3(1): 13-22. doi.org/10.1111/1365-3040.ep11580397

Wilkinson H P (1979). The plant surface (mainly leaf). *Anatomy of the Dicotyledons.* 1: 97-165.

Yang Z R & Lin Q (2005). Comparative morphology of the leaf epidermis in Schisandra (Schisandraceae). *Botanical Journal of Linnean Society* 148(1): 39-56. doi.org/10.1111/j.1095-8339.2005.00396.x

Zamani A, Attar F & Civeyrel L (2015). Leaf epidermis characters of Iranian Pyrus L. (Rosaceaea) and their taxonomic implications. Genetic Resources *and* Crop Evolution 64: 159-176. doi.org/10.1007/s10722-015-0341-4

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Bacterial Dynamics of Hardaliye, a Fermented Grape Beverage, Determined by High-throughput Sequencing

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ABSTRACT

Hardaliye is a traditional beverage produced by fermenting red grapes with mustard seeds and sour cherry leaves in the Thrace region of Turkey. Few studies have been conducted that have determined the microorganisms responsible for hardaliye fermentation, and those that have are limited to lactic acid bacteria (LAB) using culture-dependent techniques. This study aims to determine the bacterial dynamics of hardaliye fermentation using a culture-independent approach, high-throughput sequencing of 16S rRNA amplicons. Hardaliye was produced using the traditional method, and samples were taken and analyzed on days 0, 2, 4, 6, and 10 of fermentation. During the fermentation period, the pH decreased from 3.65 to 3.23. Amplicon sequencing showed that bacterial diversity was highest at 2 d, and lowest at 10 d, the final day. Although *Enterobacteriaceae* was the most dominant family at 0 and 2 d, *Acetobacteriaceae*, specifically *Gluconobacter frateurii*, became dominant with ~50% relative abundance at 4 d, and increased its abundance to >98% at 6 and 10 d. Although a slight increase in the relative abundance of ~1% (0 d) to ~5% (4 d) was observed in LAB, their presence was limited. This study showed that acetic acid bacteria should not be overlooked in hardaliye fermentation.

Keywords: 16S rRNA targeted metagenomics, Acetic acid bacteria, Amplicon sequencing, Fermented foods, *Gluconobacter frateurii*

1. Introduction

Hardaliye is a traditional grape-based beverage from the Thracian region of Turkey (Arici & Coskun 2001). It is produced by fermenting aromatic red grapes together with crushed raw mustard seeds, sour cherry leaves, sorbic and/or benzoic acid as a preservative for 7 to 10 d (Coşkun et al. 2012; Aydogdu et al. 2014). Yeast growth and ethanol fermentation in hardaliye are limited due to the protective effect of active compounds in mustard seeds together with the action of the preservative (Coşkun et al. 2012). After production, hardaliye is filtered and stored at 4 °C (Arici & Coskun 2001; Coskun 2017). Even if it is stored in the cold, there may be changes in its properties after one year of storage (Coşkun et al. 2012).

Fruits and fruit products are important components of a healthy diet due to their bioactive compounds, poor fat content, and low sodium and potassium (Rodríguez et al. 2021). Fermentation is an alternative preservation technique to extend the short shelf life of fruits; moreover, it improves the functional properties, including nutritional value and sensory attributes (Prado et al. 2008). Because the fermentation takes place with the seeds and skins of grapes, hardaliye is rich in antioxidant polyphenols, such as resveratrol, gallic acid, and flavonoids, such as quercetin and anthocyanidins (Amoutzopoulos et al. 2013). The 2013 study of Amoutzopoulos et al. (2013) showed that hardaliye consumption significantly decreased oxidative stress markers compared to the control group suggesting an antioxidative effect (Amoutzopoulos et al. 2013). Hardaliye is also shown to reduce the formation of the lipid oxidation

product, malondialdehyde, in meat products during *in vitro*-digestion, indicating a potential health effect when consumed together with meat (Aksoy et al. 2022). Since it is non-alcoholic and plant-based, hardaliye is a beverage suitable for the consumption of a wide variety of consumer groups, including children and vegetarians (Prado et al. 2008).

Hardaliye has been described as a lactic acid fermented product (Arici & Coskun 2001; Bayram et al. 2015; Pehlivanoglu et al. 2015; Arici et al. 2017; Coskun 2017). Studies on the microbiology of hardaliye have predominantly focused on culture-dependent methods using plate counts, including total mesophilic aerobic bacteria, lactic acid bacteria (LAB), coliforms, and yeasts (Arici & Coskun 2001; Coşkun et al. 2012; Aydogdu et al. 2014; Bayram et al. 2015). In addition, two studies have described the isolation of LAB species from hardaliye (Arici & Coskun 2001; Arici et al. 2017).

Culture-independent microbial profiling techniques, which involve extracting DNA directly from samples, allow for the analysis of microorganisms without isolating and culturing them. Owing to the decrease in sequencing costs and the increasing availability of bioinformatics analysis tools, high-throughput sequencing (HTS) techniques have been ubiquitously used in fermented food community analysis for the past two decades (Chen et al. 2017; Ferrocino & Cocolin 2017; Rizo et al. 2020). In HTS, shotgun sequencing of whole genomes can be used for a comprehensive analysis involving identifying microbial communities and their functional potential (Rizo et al. 2020). Meanwhile, amplicon-based "targeted" approaches, which involve sequencing only an informative region of the genome, are more accessible and cheaper when the aim is to identify the community members and their relative abundance (De Filippis et al. 2017; Chen et al. 2017; Gołębiewski & Tretyn 2020). For the identification of bacteria, the most widely used taxonomically informative region is the *16S rRNA* gene (Ferrocino & Cocolin 2017; Gołębiewski & Tretyn 2020). Amplicon sequencing is performed after amplification of 16S rRNA with universal primers. The resulting operational taxonomic unit (OTU) abundance is proportional to the number of reads, allowing the method to be quantitative (De Filippis et al. 2018).

Considering the scarcity of studies on hardaliye microbiology, this study's aim is to determine the bacterial community of hardaliye during the 10-d fermentation period using a culture-independent method, namely, 16S rRNA-targeted amplicon sequencing, and to correlate this with the pH changes.

2. Material and Methods

2.1. Hardaliye production

Hardaliye was produced using traditional methods (Arici & Coskun 2001; Aydogdu et al. 2014). A traditional French red grape variety Alphonse Lavallée (*Vitis vinifera* L.) (Aubert & Chalot 2018) cultivated in Tekirdag was used in production. Black mustard (*Brassica nigra* L.) seeds were obtained from local stores in Istanbul, and sour cherry (*Prunus cerasus* L.) leaves were picked from a tree at the Istanbul Sabahattin Zaim University campus the same day that fermentation began. Two parallel samples (fermentation samples 1 and 2) were used for the fermentation process. Five-L plastic barrels with a tap at the bottom were filled in three layers with 5 kg crashed grapes, 2 g/kg crushed raw black mustard seeds, 2.5 g/kg sour cherry leaves, and 0.5 g/kg each sodium benzoate and potassium sorbate as preservatives. The contents were fermented at room temperature (~25 °C) for 10 d and mixed with a sterile ladle every other day.

2.2. Chemical analyses

The pH of both samples on days 0, 2, 4, 6, 8, and 10 of fermentation was measured using an HI 2211 pH meter (Hanna Instruments Inc., Woonsocket, RI, USA).

2.3. DNA isolation

For bacterial diversity analysis, genomic DNA was extracted directly from hardaliye samples without microorganism cultivation using samples obtained at 0, 2, 4, 6, and 10 d of the fermentation of sample 1 and at 10 d (the final day) of the fermentation of sample 2. The Meta-G-Nome™ DNA isolation kit (Epicentre Biotechnologies, Madison, WI, USA) was used for extraction. To prepare samples for DNA isolation, 50 mL hardaliye sample was first filtered through four layers of Miracloth (Merck KGaA, Darmstadt, Germany) and then through a 1.2-µm filter supplied with the kit to remove impurities. The bacteria were then captured using a 0.45-µm filter and the kit protocol was followed. Isolated DNA in 50 µL TE buffer [10 mM Tris-HCl (pH 7.5) and 1 mM ethylenediaminetetraacetic acid] was stored at -20 °C. The quality of the metagenomic DNA was analyzed using the BioSpec Nano spectrophotometer (Shimadzu, Kyoto, Japan).

2.4. 16S rRNA amplicon sequencing

Amplicons were sequenced using Macrogen (Seoul, Korea). The sequencing library was first prepared using the Illumina (San Diego, CA, USA) 16S metagenomic sequencing library preparation protocol. This protocol involves amplification of the ~460 bp hypervariable V3-V4 region of the 16S rRNA gene using Kapa HiFi HotStart polymerase chain reaction (PCR) kit (Kapa Biosystems, Cape Town, South Africa) with adapter-added primers (Klindworth et al. 2012). Following the purification of the PCR products using AMPure XP beads (Beckman Coulter Inc, Indianapolis, IN, USA), a second index PCR was conducted to add dual indices and sequencing adapters using the Nextera XT Index kit (Illumina). PCR clean-up was conducted, and the library size and quantity were determined using the 2100 DNA 1000 reagent kit and 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany). Library sequencing was conducted using the Miseq system (Illumina) with its own software, MiSeq Control v 2.2. Base-calling was performed using Real Time Analysis v 1.18 (Illumina), and FASTQ files were generated using the package bcl2fastq v 1.8.4 (Illumina). The adapter sequences were removed using Scythe v 0.991 Beta (https://github.com/vsbuffalo/scythe) and Sickle (https://github.com/najoshi/sickle). FLASH was used for assembly (Magoč & Salzberg 2011). Denoising, OTU clustering (97% cutoff), and taxonomic and diversity analyses were conducted using CD-HIT-OTU, rDnaTools, and QIIME, respectively (Schloss et al. 2009; Caporaso et al. 2010; Li et al. 2012). Sequences were submitted to GenBank (accession numbers: OK217199-OK217226).

3. Results and Discussion

3.1. Change of pH during hardaliye fermentation

The pH of hardaliye decreased from 3.65 (±0.10) to 3.23 (±0.04) (average values of two samples) during fermentation (Figure 1). A study examining the hardaliye samples (n=26) obtained from the market indicated that the pH varies between 3.21 and 3.97 (Arici & Coskun 2001). The final pH of the hardaliye produced in the present study was close to the lower end of the pH range in the study by Arici & Coskun (2001). The differences in the final pH values may be the result of a variety of factors, such as the raw materials, microorganism load on the raw materials, fermentation temperature, fermentation duration, and storage period.

Laboratory-produced hardaliye samples in many of the previous studies showed a similar decrease in pH during the fermentation period (Arici & Coskun 2001; Coskun & Arici 2006; Aydogdu et al. 2014), while some studies indicated an increase in pH but not pH >4 (Çoşkun & Arici 2011) or a relatively constant value remaining close to pH 4.0 (Bayram et al. 2015). Coskun & Arici (2011) have shown that different grape varieties result in different pH trends and that the pH trend of the same grape variety is similar even though different mustard seeds are used. This indicated that grape variety was more effective than the type of mustard seeds in determining the pH trend. The Alphonse Lavallée grape variety was used in the present study, which might have been responsible for our relatively lower pH values compared to that of other studies. In a previous study using the same grape variety for hardaliye production, the pH decreased from 3.86 to 3.39 in 7 days of fermentation (Arici & Coskun 2001) with low pH values similar to that of our study. Aydoğdu et al. (2014) also observed a pH decrease from 4.24 to 3.82 with the Alphonse Lavallée grape variety; however, those pH values were higher than that in the current study.

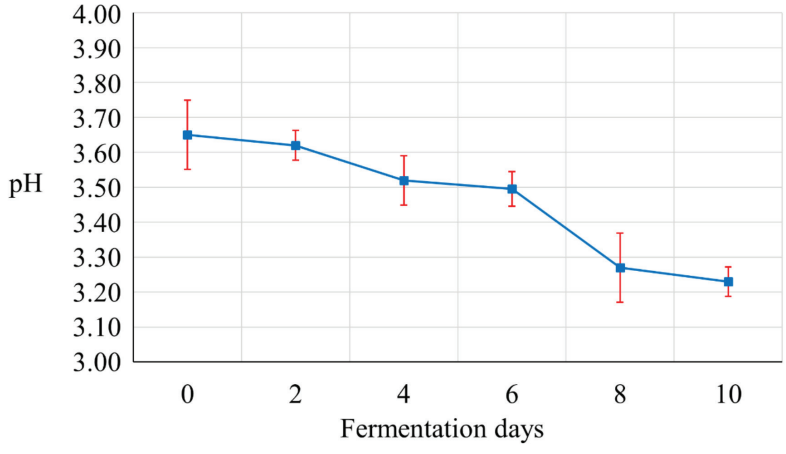

Figure 1- Change in pH during hardaliye fermentation

3.2. Bacterial dynamics of hardaliye

Amplicon sequencing was conducted at 0, 2, 4, 6, and 10 d of fermentation for sample 1 and at 10 d of fermentation for sample 2. The number of reads obtained by sequencing the six samples were between 1,327,260 and 1,548,056, which were reduced between 459,864 and 614,148 after adapter removal and assembly (Table S1). Quality scores, Q20 and Q30, for the assembled data were between 97.19 and 97.47 and between 90.10 and 90.97, respectively (Table S1). As a result of the analysis, 140 OTUs were obtained. After cleaning chloroplast and mitochondrial sequences, 135 OTUs were determined (Table S2).

The Good's coverage index showing how well the data represented the larger set was near 1 for all samples (Table 1). The microbial diversity during the course of fermentation was estimated using the number of OTUs, Chao1 richness, and Shannon and Simpson diversity indices (Table 1). The number of OTUs first increased from 23 at 0 d to 95 at 2 d and then decreased to 15 at 10 d. Similarly, the second fermentation sample contained 14 OTUs at 10 d. All diversity estimates indicated that although the highest diversity was observed at 2 d, the lowest was observed at the end of the fermentation period at 10 d (Table 1). Starter culture is not used in hardaliye production; therefore, fermentation is spontaneous and driven by the microorganisms either in the raw materials or the environment in which the fermentation takes place. Diversity analyses indicated that in the nutrient-rich fermentation medium, the bacterial diversity first increased up to day 2 of fermentation, after which it began to decrease and reached its lowest values by the end of the process, parallel to the decrease in the pH. Towards the end of the fermentation period, dominance by the main microorganism eliminated the other species.

Table 1- Diversity analyses of hardaliye samples: Community richness and alpha diversity indices

Fermentation sample no	***Fermentation day***	***Number of OTUs***	***Chao1 richness***	***Shannon diversity index***	***Simpson diversity index***	***Good's coverage index***
1	0	23	63.5	1.9440	4.1383	0.9695
	2	95	95.5	3.0674	8.8777	0.9999
	4	32	32.1	1.6842	3.2071	0.9997
	6	38	38.2	0.0913	1.0237	0.9999
	10	15	17.0	0.0572	1.0191	0.9999
2	10	14	26.5	0.0582	1.0164	0.9999

OTU: Operational taxonomic unit

Taxonomical analyses showed that at 0 d, the bacteria in the fermentation medium was composed mainly of *Acetobacteriaceae* and *Enterobacteriaceae* (Figure 2A). At the species level, *Gluconobacter frateurii* and *Tatumella pytseos* had the greatest relative abundances (Figure 2B). At 2 d, the bacterial diversity increased and *Escherichia*/*Shigella* sp. within *Enterobacteriaceae* became dominant. At 4 d, when pH was slightly decreased, *Acetobacteriaceae*, specifically *G. frateurii* at the species level, became dominant with ~50% relative abundance (Figure 2, Table S2). Beginning at 6 d until the end of the fermentation period at 10 d, fermentation was nearly entirely (>98%) dominated by *G. frateurii* (Figure 2, Table S2). The presence of *G. frateurii* with a similar relative abundance of ~99% at the end of the fermentation period was confirmed using the second fermentation sample (Figure 2, Table S2). Although LAB species, such as *Levilactobacillus brevis*, *Companilactobacillus musae/farciminis* clade, *Streptococcus thermophilus*, *Lactococcus lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Limosilactobacillus fermentum*, and *Weissella confusa/cibaria* clade were detected, their numbers were limited and greatly decreased after 4 d (Figure 2, Table S2).

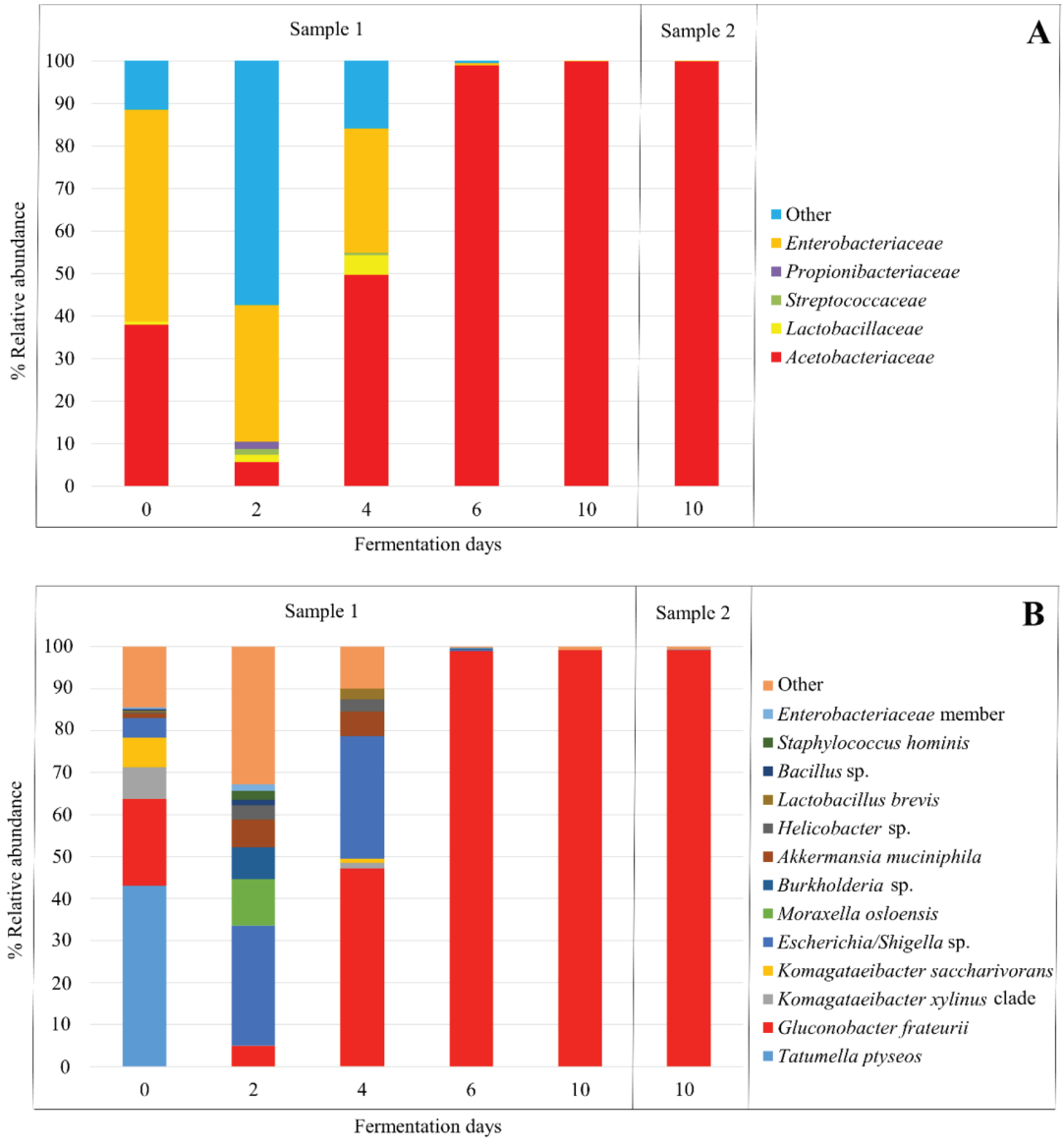

Figure 2-Bacterial diversity of hardaliye during the fermentation period. A) Diversity at the family level. B) Diversity at the species level determined using the five most abundant species on each fermentation day. The remaining species are represented by "other".

Previous studies identifying bacteria species in hardaliye were focused on LAB. One such study identified *Lacticaseibacillus paracasei, Lacticaseibacillus casei, Limosilactobacillus pontis, L. brevis, Lactobacillus acetotolerans, Fructilactobacillus sanfranciscensis,* and *Paucilactobacillus vaccinostercus* (Arici & Coskun 2001). In another study, *Lactiplantibacillus plantarum* and *Lactiplantibacillus pentosus* were primarily isolated in addition to *L. brevis* and *Secundilactobacillus collinoides* (Arici et al. 2017). The common LAB species we identified in these two studies was *L. brevis*.

In the present study, we detected acetic acid bacteria (AAB) as the leading microorganism group in hardaliye fermentation. AAB have not been enumerated or isolated in previous hardaliye studies because, in general, AAB have not been studied as widely as other food-related bacteria (Pothakos et al. 2016), most likely because cultivation and isolation are difficult in spontaneously fermented food ecosystems harboring a variety of different bacteria and yeasts (De Roos & De Vuyst 2018). In addition, AAB are known to have a viable-but-not-culturable state, especially under low oxygen conditions, which causes an underestimation of the population (Bartowsky & Henschke 2008; Pothakos et al. 2016). The process of determining AAB in various fermented foods has increased, especially after the use of culture-independent high-throughput techniques (Pothakos et al. 2016; De Roos & De Vuyst 2018). For example, the unexpected presence of AAB, specifically *Gluconobacter* species, has been demonstrated in the spontaneous fermentation of the Grenache grape

variety using HTS (Portillo & Mas 2016). Similar to that study, HTS has also shown that low sulfited wine fermentations involve AAB, specifically *Gluconobacter* species, more often than LAB (Bokulich et al. 2015). AAB has also been reported to represent an important group of microbiota in various fermented products, such as vinegar (Buyukduman et al. 2022; Lynch et al. 2019), Lambic beer and other sour beers (Bouchez & De Vuyst 2022; De Roos et al. 2018), water kefir (Martínez-Torres et al. 2017), Kombucha (Villarreal-Soto et al. 2020), and cocoa (De Vuyst & Leroy 2020).

AAB are able to oxidize ethanol, carbohydrates, and sugar alcohols to their corresponding oxidation products (Lynch et al. 2019). For example, the conversion of ethanol to acetic acid is the key reaction taking place in vinegar production. Gluconic acid is another metabolite of AAB converted from glucose prominently by *Gluconobacter* species (García-García et al. 2017). While gluconic acid is a mild organic acid providing a refreshing sour taste, acetic acid provides an astringent and strong acidic flavor (Li et al. 2022; Sainz et al. 2016). Kombucha contains gluconic acid and acetic acid as the primary organic acids, and the presence of the former one in higher proportion was reported to be correlated with higher sensory scores (Li et al. 2022). Because ethanol production in hardaliye is limited, the main organic acid produced by AAB may be expected to be gluconic acid, which could be analyzed in future studies.

The finding that AAB rather than LAB is dominant in hardaliye fermentation might reorient the studies on starter culture for hardaliye that previously involved only LAB (Coşkun et al. 2012; Coskun & Arici 2006); however, we admit that the information obtained from this culture-independent study should be confirmed by culture-dependent analyses in addition to metabolomics, which will give a more complete view of hardaliye fermentation in terms of the microorganisms involved and the metabolites produced.

4. Conclusions

In the present study, the bacterial dynamics of hardaliye, a traditional grape-based beverage in the Thracian region of Turkey, was determined for the first time using a culture-independent method. The pH decreased from 3.65 to 3.23 during the 10 d fermentation period. Bacterial diversity increased at 2 d, then decreased until the end of the fermentation period along with pH decrease. HTS of 16S rRNA amplicons revealed the dominance of AAB, specifically *G. frateurii*, especially at 4 d. To our knowledge, this is the first report of AAB in hardaliye fermentation and has important implications for the development of starter culture. In future studies, comprehensive analyses involving both culture-dependent and culture-independent techniques for determining microbial dynamics in addition to a metabolomics approach will present a more comprehensive picture of hardaliye fermentation.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: B.M., H.P., Design: B.M., H.P., Data Collection or Processing: B.M., H.P., E.Y.S., Analysis or Interpretation: B.M., H.P., E.Y.S., M.A., Literature Search: B.M., H.P., E.Y.S., M.A.,Writing: B.M., H.P., M.A.

Conflict of Interest: No conflict of interest was declared by the authors.

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References

Aksoy AS, Arici M & Yaman M (2022). The effect of hardaliye on reducing the formation of malondialdehyde during in vitro gastrointestinal digestion of meat products. *Food Bioscience* 47(4): 101747. doi.org/10.1016/j.fbio.2022.101747

Amoutzopoulos B, Löker GB, Samur G, Çevikkalp SA, Yaman M, Köse T & Pelvan E (2013). Effects of a traditional fermented grape-based drink 'hardaliye'on antioxidant status of healthy adults: a randomized controlled clinical trial. *Journal of the Science of Food and Agriculture* 93(14): 3604-3610. doi.org/10.1002/jsfa.6158

Arici M & Coskun F (2001). Hardaliye: Fermented grape juice as a traditional Turkish beverage. *Food Microbiology* 18(4): 417-421. doi.org/10.1006/fmic.2001.0413

Arici M, Coşkun F, Çelikyurt G, Mirik M, Gülcü M & Tokatli N (2017). Some technological and functional properties of lactic acid bacteria isolated from hardaliye. *Journal of Agricultural Sciences* 23(4): 428-437. doi.org/10.15832/ankutbd.385870

Aubert C & Chalot G (2018). Chemical composition, bioactive compounds, and volatiles of six table grape varieties (Vitis vinifera L.). *Food Chemistry* 240: 524-533. doi.org/10.1016/j.foodchem.2017.07.152

Aydogdu H, Yildirim S, Halkman A K & Durgun T (2014). A study on production and quality criteria of hardaliye; a traditional drink from Thrace region of Turkey. *Gida (The Journal of Food)* 39(3): 193-145. doi.org/10.5505/gida.36844

Bartowsky EJ & Henschke P A (2008). Acetic acid bacteria spoilage of bottled red wine—A review. *International Journal of Food Microbiology* 125(1): 60-70. doi.org/10.1016/j.ijfoodmicro.2007.10.016

Bayram M, Esin Y, Kaya C, İlhan M, Akin G & Etdöğer R (2015). Geleneksel yöntemle müşküle üzümünden üretilen hardaliyenin bazı özelliklerinin belirlenmesi. *Akademik Gıda* 13(2): 119-126.

Bokulich N A, Swadener M, Sakamoto K, Mills D A & Bisson L F (2015). Sulfur dioxide treatment alters wine microbial diversity and fermentation progression in a dose-dependent fashion. *American Journal of Enology and Viticulture* 66(1): 73-79. doi.org/10.5344/ajev.2014.14096

Bouchez A & De Vuyst L (2022) Acetic acid bacteria in sour beer production: Friend or foe? *Frontiers in Microbiology* 13: 957167. doi.org/10.3389/fmicb.2022.957167

Buyukduman E, Kirtil H E, & Metin B (2022). Molecular identification and technological properties of acetic acid bacteria isolated from Malatya apricot and home-made fruit vinegars. *Microbiology and Biotechnology Letters* 50(1):81-88. doi.org/10.48022/mbl.2109.09017

Caporaso J G, Kuczynski J, Stombaugh J, Bittinger K, Bushman F D, Costello E K, Fierer N, Peña A G, Goodrich, J K, Gordon, J I, Huttley G A, Kelley S T, Knights D, Koenig J E, Ley R E, Lozupone C A, McDonald D, Muegge B D, Pirrung M, & Knight R (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* 7(5): 335-336. doi.org/10.1038/nmeth.f.30

Chen G, Chen C & Lei Z (2017). Meta-omics insights in the microbial community profiling and functional characterization of fermented foods. *Trends in Food Science & Technology* 65: 23-31. doi.org/10.1016/j.tifs.2017.05.002

Coskun F (2017). A traditional Turkish fermented non-alcoholic grape-based beverage, "hardaliye." *Beverages* 3(1): 2. doi.org/10.3390/beverages3010002

Coskun F & Arici M (2006). The effects of using different mustard seeds and starter cultures on some properties of hardaliye. *Annals of Microbiology* 56(4): 335-337. doi.org/10.1007/BF03175027

Çoşkun F & Arici M (2011). Hardaliyenin bazi ozellikleri üzerine farkli hardal tohumlari ve üzüm cesitleri kullaniminin etkisi. *Akademik Gida (Academic Food Journal)* 9(3): 6-11.

Coşkun F, Arıcı M, Çelikyurt G & Gülcü M (2012). Farklı yöntemler kullanılarak üretilen hardaliyelerin bazı özelliklerinde depolama sonunda meydana gelen değişmeler. *Tekirdağ Ziraat Fakültesi Dergisi* 9(3): 62-67.

De Filippis F, Parente E & Ercolini D (2017). Metagenomics insights into food fermentations. *Microbial Biotechnology* 10(1): 91-102. doi.org/10.1111/1751-7915.12421

De Roos J & De Vuyst L (2018). Acetic acid bacteria in fermented foods and beverages. *Current Opinion in Biotechnology* 49: 115-119. doi.org/10.1016/j.cop bio.2017.08.007

De Roos J, Verce M, Aerts M, Vandamme P, De Vuyst L & Schaffner Donald W (2018). Temporal and spatial distribution of the acetic acid bacterium communities throughout the wooden casks used for the fermentation and maturation of Lambic beer underlines their functional role. *Applied and Environmental Microbiology* 84(7): e02846-17. doi.org/10.1128/AEM.02846-17

De Vuyst L & Leroy F (2020). Functional role of yeasts, lactic acid bacteria and acetic acid bacteria in cocoa fermentation processes. *FEMS Microbiology Reviews* 44(4): 432-453. doi.org/10.1093/femsre/fuaa014

Ferrocino I, & Cocolin L (2017). Current perspectives in food-based studies exploiting multi-omics approaches. *Current Opinion in Food Science*, 13; 10-15. doi.org/10.1016/j.cofs.2017.01.002

García-García I, Cañete-Rodríguez A M, Santos-Dueñas I M, Jiménez-Hornero J E, Ehrenreich A, Liebl W, García-Martínez T & Mauricio J C (2017). Biotechnologically relevant features of gluconic acid production by acetic acid bacteria. *Acetic Acid Bacteria*, 6(1): 6458. doi.org/10.4081/aab.2017.6458

Gołębiewski M, & Tretyn A (2020). Generating amplicon reads for microbial community assessment with next-generation sequencing. *Journal of Applied Microbiology* 128(2): 330-354. doi.org/10.1111/jam.14380

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M & Glöckner F O (2012). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research* 41(1): e1. doi.org/10.1093/nar/gks808

Li R, Xu Y, Chen J, Wang F, Zou C, & Yin J (2022) Enhancing the proportion of gluconic acid with a microbial community reconstruction method to improve the taste quality of Kombucha. *LWT* 155: 112937. doi.org/10.1016/j.lwt.2021.112937

Li W, Fu L, Niu B, Wu S & Wooley J (2012). Ultrafast clustering algorithms for metagenomic sequence analysis. *Briefings in Bioinformatics* 13(6): 656-668. doi.org/10.1093/bib/bbs035

Lynch K M, Zannini E, Wilkinson S, Daenen L & Arendt E K (2019). Physiology of acetic acid bacteria and their role in vinegar and fermented beverages. *Comprehensive Reviews in Food Science and Food Safety* 18(3): 587-625. doi.org/10.1111/1541-4337.12440

Magoč T & Salzberg S L (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27(21): 2957-2963. doi.org/10.1093/bioinformatics/btr507

Martínez-Torres A, Gutiérrez-Ambrocio S, Heredia-del-Orbe P, Villa-Tanaca L & Hernández-Rodríguez C (2017). Inferring the role of microorganisms in water kefir fermentations. *International Journal of Food Science & Technology* 52(2): 559-571. doi.org/10.1111/ijfs.13312

Pehlivanoglu H, Gunduz H H, Ozulku G, Demirci M (2015). An investigation of antimicrobial activity of wheat grass juice,barley grass juice, hardaliye and boza. I*nternational Interdisciplinary Journal of Scientific Research* 2(1): 8-14.

Portillo M del C & Mas A (2016). Analysis of microbial diversity and dynamics during wine fermentation of Grenache grape variety by high-throughput barcoding sequencing. *LWT - Food Science and Technology* 72: 317-321. doi.org/10.1016/j.lwt.2016.05.009

Pothakos V, Illeghems K, Laureys D, Spitaels F, Vandamme P & De Vuyst L (2016). Acetic acid bacteria in fermented food and beverage ecosystems. In K. Matsushita H Toyama, N Tonouchi & A Okamoto-Kainuma (Eds.), *Acetic Acid Bacteria: Ecology and Physiology* pp. 73-99. doi. org/10.1007/978-4-431-55933-7_3

Prado FC, Parada JL, Pandey A & Soccol CR (2008). Trends in non-dairy probiotic beverages. *Food Research International* 41(2): 111-123. doi.10.1016/j.foodres.2007.10.010

Rizo J, Guillén D, Farrés A, Díaz-Ruiz G, Sánchez S, Wacher C & Rodríguez-Sanoja R (2020). Omics in traditional vegetable fermented foods and beverages. *Critical Reviews in Food Science and Nutrition* 60(5): 791-809. doi.org/10.1080/10408398.2018.1551189

Rodríguez LGR, Gasga VMZ, Pescuma M, Van Nieuwenhove C, Mozzi F, & Burgos J (2021). Fruits and fruit by-products as sources of bioactive compounds. Benefits and trends of lactic acid fermentation in the development of novel fruit-based functional beverages. *Food Research International* 140: 109854. doi.org/10.1016/j.foodres.2020.109854

Sainz F, Navarro D, Mateo E, Torija M J & Mas A (2016). Comparison of D-gluconic acid production in selected strains of acetic acid bacteria. *International Journal of Food Microbiology* 222: 40-47. doi.org/10.1016/j.ijfoodmicro.2016.01.015

Schloss P D, Westcott S L, Ryabin T, Hall J R, Hartmann M, Hollister E B, Lesniewski R A, Oakley B B, Parks D H, Robinson C J, Sahl J W, Stres B, Thallinger G G, Van Horn D J & Weber C F (2009). Introducing mothur: Open-Source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* 75(23): 7537-7541. doi.org/10.1128/AEM.01541-09

Villarreal-Soto S A, Bouajila J, Pace M, Leech J, Cotter P D, Souchard J P, Taillandier P & Beaufort S (2020). Metabolome-microbiome signatures in the fermented beverage, Kombucha. *International Journal of Food Microbiology* 333: 108778. doi.org/10.1016/j.ijfoodmicro.2020.108778

Table S1. High-throughput sequencing statistics

Table S2. Operational taxonomic units (OTUs) defined in hardaliye fermentation

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An Unmanned Aerial Vehicle Based Artificial Pollination in a Frost-affected Walnut (*Juglans regia L.*) Orchard

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ABSTRACT

The winter and spring frosts pose a significant problem in many walnut cultivation areas as frost damage to catkins and female flowers results in low fruit set and yield. In January 2021, the temperature dropped to -17.2 °C in Yenişehir, Bursa, an important walnut production area in North-Western Turkey. The present study was carried out to determine the natural frost damage on catkins of some walnut cultivars and the effectiveness of an unmanned aerial vehicle (UAV) pollination, which has been developed and used in artificial pollination studies recently, on fruit set and fruit characteristics. For this purpose, two pollen concentrations (T1: 5% pollen two times; T2: 5% pollen in the first, 20% pollen in the second time) and the open pollination (control) were tested. Observations showed that frost damage on catkins varied from 57.31% (Ronde de Montignac) to 99.33% (Franquette). The initial fruit set was significantly higher in the T1 (87.74%) followed by T2 (83.89%). The final fruit set in T2 (75.16%) was higher than the T1 (74.11%), but the difference was found to be insignificant. The box and whisker plot shows that UAV pollination treatments (T1, T2) increased the fruit set, although this not uniform on the tree compared to open pollination. The highest nut weight, thickness, and length were obtained from control, followed by T1. The results showed that the fruit set was higher in pollination with UAV, and using less pollen (T1) was sufficient for fruit set. The research results support the use of UAV treatment on supplementary pollination for walnut.

Keywords: Agriculture drones, Winter frost, UAV, Pollen, Supplemental pollination, Fruit set, Catkins

1. Introduction

Juglans regia L., a species of nut, has a high degree of plasticity and can withstand cold winter temperatures in various regions without suffering winter injuries. However, its cultivation in the Mediterranean region is more limited due to its sensitivity to intense winter cold (Gandev 2013). Some cultivars may not show much damage during the cold winter between -9 °C and -11.5 °C. However, at lower temperatures (mainly when temperatures fall below -20 ºC), they are damaged and begin to dry from the tips of the branches (Şen 2011). Low-temperature damage differs between walnut species and cultivars, as well as between different organs and tissues (Lapins 1961; Malone & Ashworth 1991; Takeda et al. 1993; Aslamarz et al. 2010). Previous studies have determined some data belonging to frost-damaged walnut tree organs with artificial tests (Rochette et al. 2004; Poirier et al. 2010; Charrier et al. 2013; Aleta et al. 2014). Charrier et al. (2013) reported that buds were resistant but remained more sensitive than other organs in winter. However, frost damage observed in catkin buds was only found in Gandev's (2013) research on natural frost events. Gandev (2013) noted that in some cultivar's the male buds were more susceptible to winter frost than female buds in Bulgaria.

During active growth, the frost tolerance of tissues is relatively low, but when growth stops, cold-hardiness develops from fall to winter (Aslamarz et al. 2010). Recent developments in climate simulations indicate that climate change negatively affects winter chilling hours, and dramatic declines in cold-hardiness are predicted over the following several decades (Luedeling et al. 2011; Charrier et al. 2013; Vahdati et al. 2019, Özcan et al. 2019), which affects plants' cold acclimation and frost tolerance (Shepherd 2016; Liu et al.

2019). Warm spells may cause early dehardening, which could increase the risk of further freezing injuries in the late winter and early spring (Pagter & William 2011 and lead to damage in walnut shoots and buds, particularly male catkins. The lack of pollenizer resulting from damage to catkins causes a decrease in the natural pollination rate and fruit set. For this reason, supplemental pollination measures should be taken during the walnut blooming period. For that purpose, producers whose catkins have been damaged by winter frost collect healthy catkins from other orchards during the pollination period, putting them inside the net and hanging them on the branches to ensure wind pollination. Even though it is simple to utilize in small orchards, it wastes time and labor in commercial and industrial orchards. With this in mind, there is a need to consider artificial pollination as a supplemental pollination measure to increase the fruit set of walnut. Equipment used in pollination is significant since artificial pollination effectiveness depends on it. (Gianni & Michelotti 2018). There are two main approaches applied for artificial pollination equipment: aerial and ground platforms (Mazinani et al. 2021). A ground platform pollination system with electrostatic sprayers has previously been used to pollinate kiwifruit (Barnett et al. 2017; Williams et al. 2019), cherry (Whiting 2015), and date palm trees (Mostan 2012; Soliman et al. 2017).

Another approach, aerial platforms, including agriculture drones, have attracted attention because of their small size, convenient handling, and the way they reduce working hours (Lan et al. 2017; Kim et al. 2019). Agricultural robots include various unmanned aerial vehicle (UAVs) designs that spray pollen onto the crop canopies above. Pollination by wind power generated from the UAVs has been tested on rice (Wang et al. 2013; Li et al. 2015; Li et al. 2017) and sugar cane (Zhang et al. 2021). In addition, simulation flights were performed on walnut (Mazinani et al. 2021), and a pollen dump drone pollinating fruits (apples, cherries, and almonds) was recently developed by a New York-based startup (Matt Koball 2019). When considering UAVs pollination research, Matt Koball (2019) stated that controlling UAV flight height due to the uneven height of almond trees creates more challenges. Li et al. (2017) reported that the wind force generated by UAVs had been observed to disperse the rice pollen asymmetrically. In addition, simulation flights with UAVs in walnuts have shown that pollen spread may not be uniform in some cases due to wind effect. (Mazinani et al. 2021). However, no study has been found on the effect of pollination by UAVs and the amount of pollen used in pollination treatment on walnut fruit set and fruit characteristics. For these reasons, the present study seeks to determine the effectiveness of pollination by UAV as a supplemental pollination treatment on walnut orchards due to catkin damage in cultivars after a natural frost event.

The objectives were to 1) evaluate natural frost damage on male catkins of cultivars; 2) determine the effectiveness of UAV pollination on fruit set and fruit characteristics depending on the pollen dose used.

2. Materials and Methods

2.1. Observation of frost damage

The present study was carried out at Yenişehir, Bursa, (north-west Turkey; 40° 13′ 16″ N, 29° 31′ 04″ E latitude 40°11’ and longitude 29°3’) in 2021. The experiment plantation is characterized by 350 mm average annual rainfall and six months of frost danger (late November to April). The orchard was established in 2008 with a planting distance of 7x7 m and trained as a modified leader system. Catkins of seven (Chandler, Fernor, Ronde de Montignac, Cisco, Franquette, Meylanise, Kaman 1) cultivars grafted on *Juglans regia* were evaluated for natural frost damage. Temperature data were obtained from the automated climatic monitoring equipment during the frost period (Table 1).

Table 1- Minimum and maximum temperatures at the experimental orchard in the frost period

Frost period	*Minimum (°C)*	*Maximum (°C)*
January 17	-2.9	0.5
January 18	-16.0	-2.1
January 19	-17.2	-7.9
January 20	-16.7	-2.7
January 21	-10.8	1.8
January 22	-5.9	7.9

Seventy five catkins per tree were collected from 2.0-2.5 m above the ground, on four sides of the tree one month after the frost incident. The experiment was designed as randomized blocks with three replicates and two trees per replicate. Catkin buds were left for slow acclimatization at a low temperature (4 °C) for 24 hours, after which the temperature was gradually (16 hour) increased to 20 °C. To determine the frost damage percentage (FDP) of catkins, the buds were cut with a scalpel.

The observation was scored for visible symptoms of frost damage on a 1-4 scale: 1- completely green, living bud; 2- largely green bud; 3- partially green, browning bud; 4- dead bud (Figure 1). Following this, the frost damage index (FDI) was calculated according to the following formula: FDI= $\sum (n_i \times i)/N$, where "n_i" is the number of buds receiving the mark "I" (from 1 to 4) and "N" is the total number of buds in each cultivar. In addition, the FDP was recorded according to the presence or absence of buds damage.

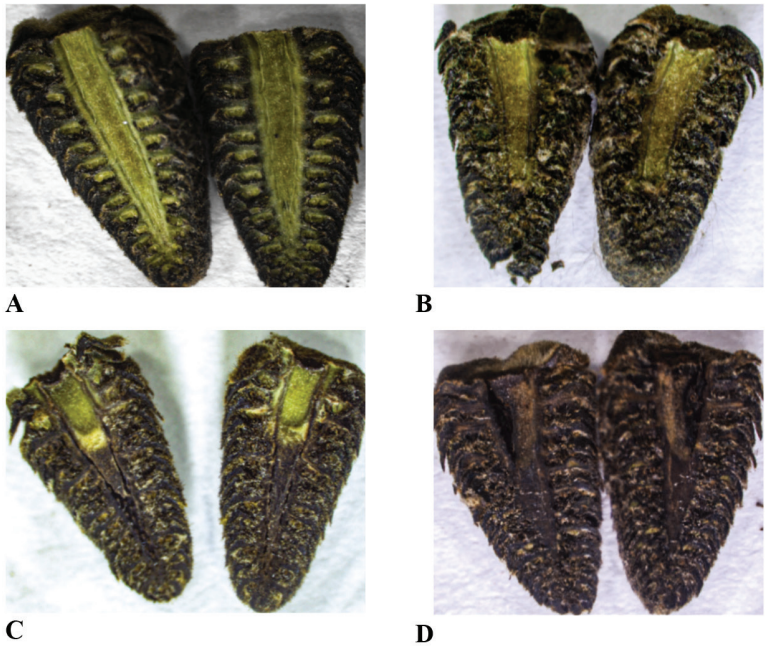


Figure 1. Visible symptoms of frost damage on young male catkins: (A) 1- scale, completely green bud; (B) 2- scale, largely green bud; (C) 3- scale, partially green; (D) 4- scale, dead bud

2.2. Artificial pollination with UAV

Chandler trees, the main cultivar in the orchard, were used in artificial pollination experiments. In the artificial pollination experiments, Chandler catkins were collected from the Agricultural Application and Research Center of Bursa Uludağ University's walnut orchard. Catkins were collected plump and yellow before they shed their pollen at the phenologic period termed the Fm stage (UPOV 1994). They were kept in a sieve in a warehouse at room temperature to gain pollen. Every day, the sieves were checked, and some calcium chloride was placed inside the containers to absorb moisture. The pollen that passed through the sieve was gradually kept at -20 °C until the female flowers were in the receptive state. The pollen was diluted with flour (w/w) (Kuru 1995) to obtain 5% and 20% (weight/weight) concentration (Figure 2). To estimate the viability of applied pollen, the 1% 2,3,5-triphenyl tetrazolium chloride method was used (Mert 2009; Özcan et al. 2017). The viability of pollen was determined by counting approximately 150 pollen using four replicates under the light microscope (×40; Leica DC 500, Wetzlar, Germany). A red color was considered viable, light red was semi-viable, and white pollen was non-viable (Mert 2009).

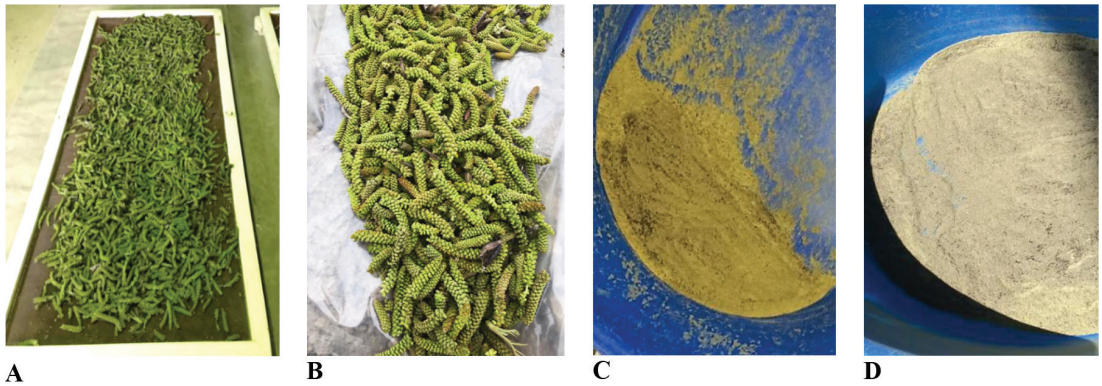


Figure 2. The pollen procedure: (A), (B) catkins in the sieve; (C) pollen; (D) pollen and flour mixture

Artificial pollination was repeated twice due to the gradual stigma receptivity. The treatments were composed of 5% in the first and second pollination (T1), 5% in the first, 20% pollen in the second pollination (T2), and open pollination (control). Trees belonging to the treatments were selected in different areas (Figure 3). The distance of the control area to the T1 and T2 treatment areas was 503 m and 731 m, respectively, and the T1 to T2 was 420 m. Trees for each treatment were selected according to the randomized blocks trial design with three replications and two trees per replication before the pollination. Three branches were marked from three sides and the middle layers of each tree. The 25 female flowers labeled per branch and 150 female flowers per replication were counted during female receptivity. The female flower and fruit marked branches were counted 25, 55, and 95 days (at harvest) after the pollination treatments, and the final fruit set rates were determined.

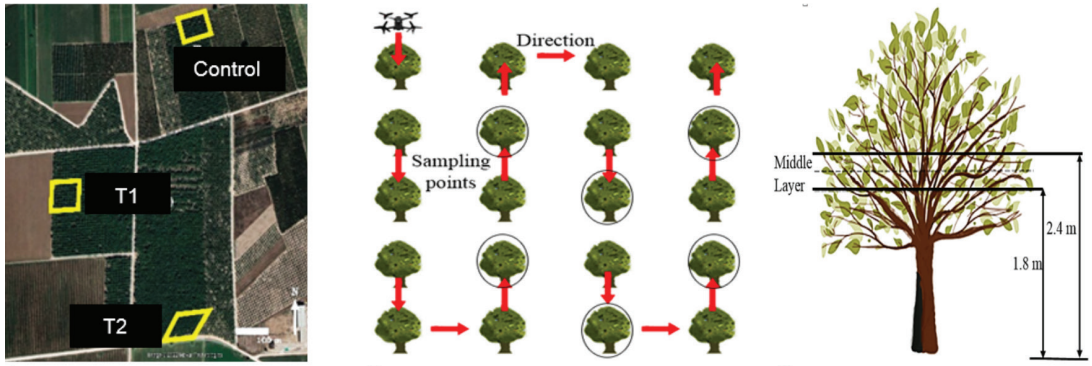


Figure 3. Artificial pollination procedure: (A) Experiment areas; (B) flight information for the walnut tree; (C) tree's middle layer where female flowers are marked. Note: This image is only for illustration

The quadrotor UAV (DJI Matrice 200 produced by Dà-Jiāng Innovations Science and Technology Co. Ltd Shenzhen, China) was used in the experiments. The quadrotor UAV has a 20 MP camera; it works with GPS navigation systems. The maximum ascent speed can reach up to 5 m/s and has a simple structure, autonomous obstacle avoidance, and program control. The specific technical parameters are shown in Table 2.

Table 2- Technical parameters of DJI Matrice 200 quadrotor UAV

Item	***Parameter***
Size (mm)	716×220×236 mm
Number of rotors (number)	4
Motor model	DJI 3515
Battery (mAh)	7660 mAh
Battery (number)	2
Maximum flying time (min)	13
Maximum ascent and descent speed (m/s)	5;3
Maximum wind resistance (m/s)	12
Maximum load (kg)	1.61
Operating temperature (°C)	-20 °C to 45 °C

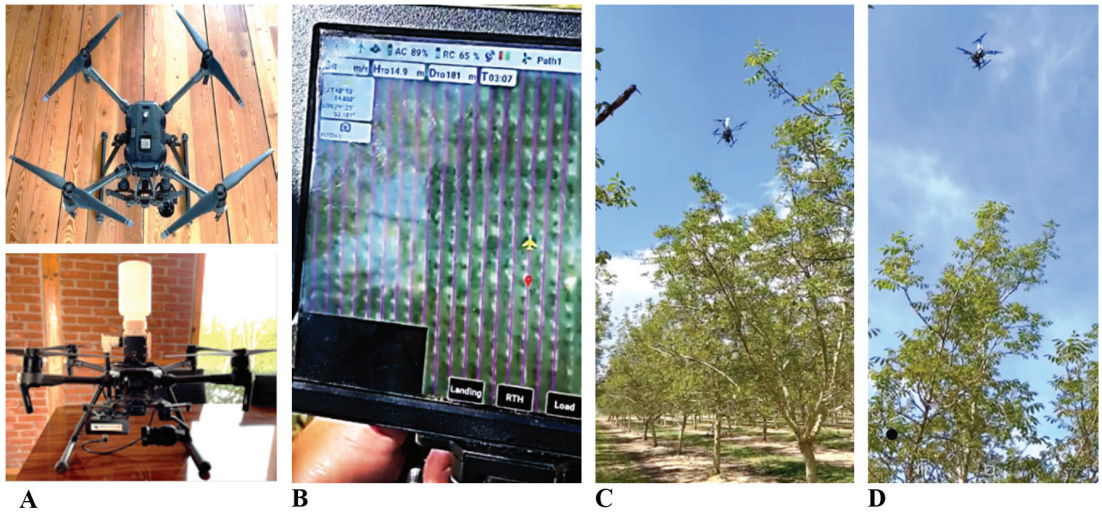
The UAV, equipped with a 1-liter distribution kit, was produced by BLY-A matica and can pollinate 20 acres in a single flight (Figure 4). DJI APP software was used to calculate general parameters such as altitude, speed, battery information, and distance to the take-off site. All data specified with this software range 20 km, and an Android-based tablet was used as a mobile station.

The characteristics of the walnut trees, the weather conditions, and the UAV operation parameters during the artificial pollination experiment are shown in Table 3. The UAV was operated at 2 m above the canopy with an optimum flight speed of about 4 m/s (Meng et al. 2019).

Table 3- The walnut characteristics, weather conditions, and UAV operation parameters

Experiment time	*Phenological period*	*Tree height (m)*	*Wind speed (m/s)*	*Mean temperature (°C)*	*Drone flight height (m)*	*Drone flight speed (m/s)*
May 03 May 06	Stigma receptivity	12±0.60	0.78±0.40	18±1.50	3	4

The GPS coordinates were entered autonomously in the mobile station, the experiment orchard's planting frequency (7x7 m) was recorded, and the UAV dispersed the pollen on the rows via the kit. After beginning from the start of the row and finishing that row, the drone spread pollen from the end of the other row to the beginning, as shown in Figures 3 and 4.



A B C D

Figure 4- Pollination by UAV: (A) DJI Matrice 200 quadrotor UAV; (B) GPS coordinates entering on the tablet used as a mobile station; (C), (D) Polen disperse via kit on rows

2.3. Pistil squash method

Ten days after pollination, 8 female flowers were collected per replicate and fixed in FAA solution. Pistils fixed in FAA were washed three times with distilled water. The stigmas and the styles were separated from the ovary for examination. They were softened in 7% sodium sulfite by boiled for 50 min and then washed in distilled water three times. Afterwards, they were stained with lacmoide 0.2%, and kept for one night. The stained stigma and styles were placed on the slide and covered with a coverslip by dripping one drop of glycerin and lightly squashed. Pollen tube formations were observed under a light microscope (Ayfer 1967).

2.4. Fruit traits

After the harvest, the walnut fruits were dried and stored at 25 °C until the analysis. Fruit traits were analyzed based on the nut's weight, length, thickness, width, kernel weight, shell thickness, and kernel percentage in 3 replications of 30 fruits per replication.

2.5. Statistical analysis

An analysis of variance (ANOVA) was used for statistical analysis performed by Minitab version 16.1. The Tukey test was used to make pairwise comparisons between pollination treatments. The significance level used was $p \leq 0.05$. The boxplots were comprised using Minitab version 16.1 to identify the dispersion of the data set.

3. Results and Discussion

3.1. Observation of frost damage

A severe midwinter frost caused damage to the walnut cultivars' male catkins (Table 4). The percentage of frost damage ranged from 57.31% (Ronde de Montignac) to 99.33% (Franquette). All cultivars with the exception of Ronde de Montignac and Kaman 1 showed a high rate of catkin damage. The catkin of Kaman 1 and Ronde de Montignac cultivar were generally greener with 1.94 and 2.25 FDI values, respectively.

Table 4- Evaluation of frost damage to catkins of walnut selection and cultivars

Cultivars	*FDP (%)*	*FDI*
Ronde de Montignac	57.31±4.64 b	2,25 b
Chandler	99.00±0.57 a	3,94 a
Cisco	98.33±1.52 a	3,96 a
Fernor	98.92±1.86 a	3,80 a
Franquette	99.33±0.60 a	3,96 a
Meylanise	96.00±2.65 a	3,78 a
Kaman 1	57.45±7.80 b	1,94 b

Each data value is represented as means ± standard deviation. Means within columns followed by different letters differ significantly ($p \leq 0.05$).
FDP: Frost damage percent, FDI: Frost damage index

Winter frost damage was observed in walnut cultivars catkins in varying degrees. The damage percent of cultivars with the exception of Ronde de Montignac and Kaman were found to be similar. Gandev (2013) observed 40% to 98% damage on catkins of walnut cultivars at temperatures ranging from -13 °C to -24 °C. Similarly, Aslamarz et al. (2010) reported that a midwinter frost (-17 °C) at the experimental orchard caused moderate tissue browning in the buds and stems of cultivars and genotypes. Aleta et al. (2014) and Poirier et al. (2005) reported that 50% of the bud sticks and stem sections of Chandler, Fernor, and Franquette cultivars were damaged at -24.5 °C, -21.3 °C, -20.9 °C, and -22 °C, -24 °C, -26 °C respectively, in their artificial frost test. In the present study, we observed that catkins were damaged at -18 °C depending on the cultivars. This case may be explained when considering, the longevity of the frost, the rapidity of frost, the stage of dormancy of the trees, and that frost resistance varies by organs (Webster & Looney 1996). Furthermore, the results may differ depending on whether the frost tests are performed in the field or in the laboratory (Lapins 1961; Malone & Ashworth 1991; Takeda et al. 1993).

3.2. Artificial Pollination with UAV

The results showed that pollination treatments affected the flower abscission and fruit set (Table 5). It was determined that 30.37% of flower abscission occurred 25 days after pollination in control, followed by T2 (15.15%). 55 days after pollination, the fruit abscission ratio ranged between 8.75% (control) and 12.76% (T1), but there were no significant differences between pollination treatments. According to the flower abscission result of control treatment, it is assumed that a lack of pollen caused abscission due to the low number of viable catkins that provide pollination close to the orchard. For regular fruit set, 10-18 pollen grains should penetrate the stigmas. (Kaveckaja & Tokar 1963). Following the control treatment, the highest flower abscission was obtained from T2 (Figure 5). The pistillate flower abscission (PFA) at T2 can be explained by a high dose of pollen on the stigma. Previous studies recorded that the pollen load and level of abortion of pistillate flowers have a positive relationship (Por & Por 1990; Polito et al. 1996).

Table 5- Flower, fruit abscission and initial, final fruit set of pollination treatments (%)

Treatment	*Flower abscission*	*Fruit abscission*	*Initial fruit set*	*Final fruit set*
Control	30.37±0.59 a	8.75±4.56 ns	69.05±0.59 b	59.82±2.92 b
T1	11.64±3.29 b	12.76±5.95 ns	87.74±4.29 a	74.60±5.97 a
T2	15.15±8.00 ab	9.06±5.78 ns	83.89±8.00 ab	75.16±5.72 a

Each data value is represented as means ± standard deviation. Means within columns followed by different letters differ significantly ($p \leq 0.05$)

McGranahan et al. (1994) cited that lower flower abscission has been reported with few pollen grains on the stigma, which may be the result of less competition between pollen tubes in the pollen tube pathway. Similarly, Gonzalez et al. (2018) found that 1% pollen application with 5 pollen grains per mm^2 on the stigma surface provided maximum fruit set, and flower abscission increased with the increasing pollen concentration. Gün et al. (2010) reported that the PFA was 48.6%, 54%, and 77% when they controlled the pollination of female flowers with 5%, 50%, and 100% pollen.

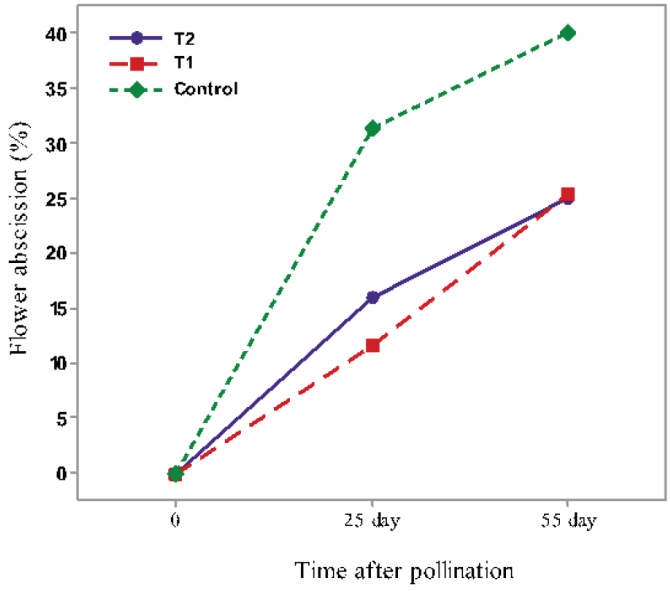


Figure 5- Flower and fruit abscission 25 and 55 days after pollination

41% of the pollen from the Chandler cultivar used in UAV pollination was found viable and 40% semi-viable. According to the pistil squash method, in open pollination (control) treatment, germination was not uniform across the stigmatic surface. In some areas, germination frequency was high, while another part of the stigma lobes had little germination. In the stigma lobes of the T2 treatment, numerous pollens were observed, and many of them were germinated. Pollen germination frequency was high in styles belonging to T1 and T2 treatment, and it was observed that some pollen tubes were very close to the ovary. The squashed pistil verified that the UAV airflow field could help the pollen spread and pollen germination (Figure 6).

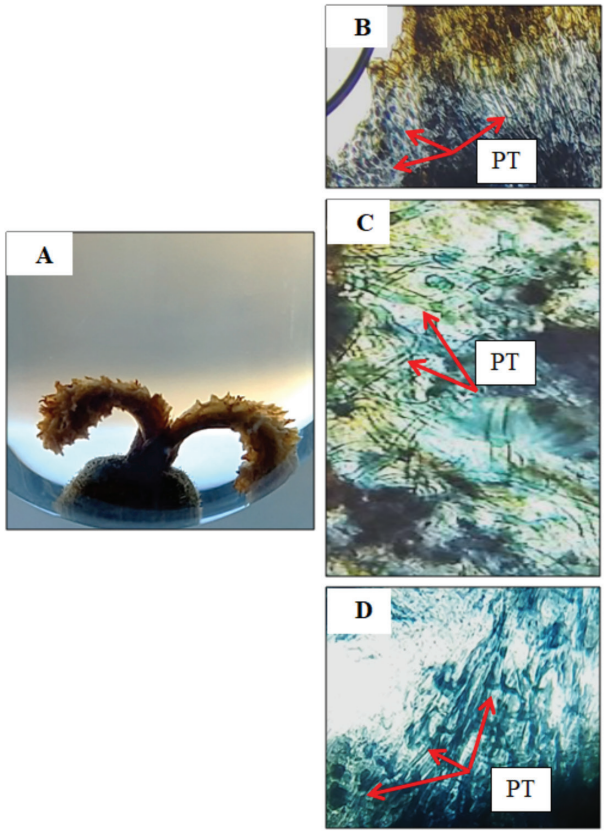


Figure 6- Pollen germination and pollen tube growth: (A) T1; (B) T2; (C) open pollination; (D) pollen tube formation (4x10)

The initial fruit set was found to be significantly high in the T1 (87.74%) and T2 (83.89%). The mean values of T1 and T2 were 21.30% and 17.68% higher than control, respectively. Similarly, the final fruit set was significantly higher in T2 (75.16%) and T1 (74.60%) compared with control (59.82%).

UAV pollination treatments increased the initial and final fruit sets compared to open pollination. The initial fruit set was greater than 80% in pollination with UAV, consistent with Lemus's (2010) findings that the best walnut yield is obtained when the fruit set reaches 80%. An initial and final fruit set in open pollination was found to be 69.05% and 59.82%, respectively. The distance of the control trees from pollination treatment areas (T1=503; T2=731 m) with the UAV suggests that the control trees did not receive pollen from these areas because the catkins of the Fernette and Franquette, which generally pollinate the Chandler cultivar, were damaged. However, the less damaged catkins of the Ronde de Montinag trees, about 420 meters from the control area, were able toollinate the last receptive female flowers of the Chandler cultivar. It has been reported that the pollen of walnut species can maintain its vitality when traveling distances of 1 km (Robichaud 2007), but the effective pollenizer distances rarely exceed 457 meters for Persian walnut orchards in California (Polito et al. 2005).

The pollination results with UAV are compatible with Mazinani et al. (2021), who reported the UAV could be used in walnut pollination successfully as a result of the simulation test. Similarly, Whiting (2015) studied artificial pollination in cherry trees using ground robots and revealed that it increased pollen density on flower stigma three-fold compared to pollination through bees. Liu et al. (2017) stated that UAVs could be used in supplemental pollination for hybrid rice seed production. In contrast, Wang et al. (2020) reported that UAV with low volume pollen spraying has varying particle size and coverage of the droplets deposited in the canopy of pear trees and that the fruit set rates were low. Also, Al-Wusaibai et al. (2012) reported that a higher date palm fruit set was obtained through manual treatment than mechanical pollination.

The box and whisker plot shows that 75% of the initial fruit set data of control treatment was less than 72.29%, and the values were distributed in a short range compared to T1 and T2. UAV pollination treatments increased the initial and final fruit set, but its variability was greater than open pollination (Figure 7). This may suggest that pollen might not be distributed evenly in the tree canopy during drone pollination. Liu et al. (2017) reported that the wind field produced by the rotor-wing UAV had an asymmetrical impact on pollen dispersal. Similarly, Mazinani et al. (2021) reported that pollen could not steer to walnut trees if the wind flow changes the path off the pollen. In quadrotors, the wind greatly influences their motion, making them unstable. The wind parallels to the flight direction are more useful for supplementary pollination (Mazinani et al. 2021; Zhang et al. 2021).

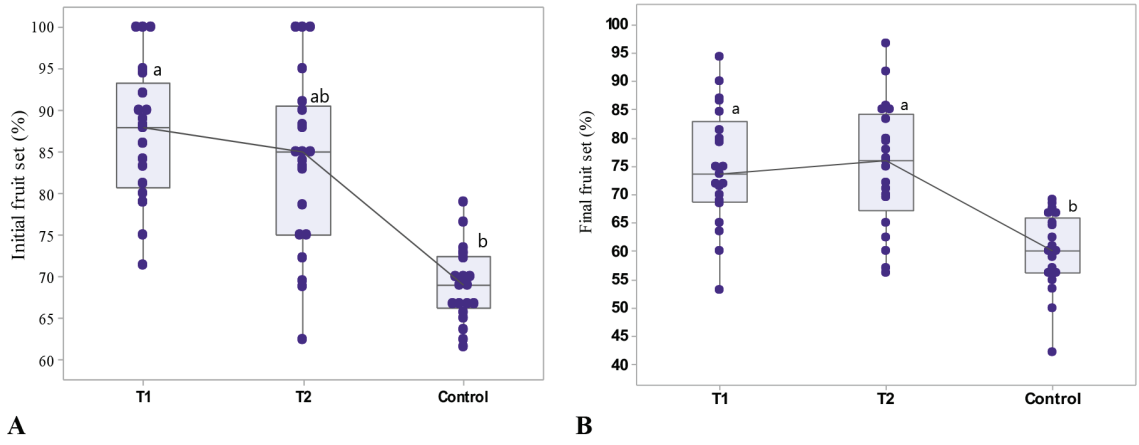


Figure 7- Boxplot graphs: (A) representing initial fruit set; (B) final fruit set value and its distribution in treatment. The central line displays the median, the bottom and the top of the box are the first and third quartiles, and the dots represent sample values. Lowercase letters are used to show statistical results of multiple comparisons treatments

Many previous studies reported that flight speed and flight height significantly impacted the pollen dispersal characteristics. Matt Koball (2019) noted that controlling UAV flight height due to the uneven height of almond trees creates more challenges. In the present study, it was tried to choose equal heights of trees to be pollinated by drone, the drone flight speed was set to be 4.0 m/s and the flight height to be 2 m above the canopy, as suggested in previous studies (Meng et al. 2019). In addition, attention was paid to selecting branches from the middle part of the tree. However, one of the reasons for the variation in fruit set could be that pollen distribution is not uniform since the height of the trees is not exactly uniform.

Furthermore, it has been reported in UAV spraying research that the tree canopy and the lower, middle, or upper parts of the tree affect the operation of agricultural UAVs. Zhang et al. (2016) reported that UAVs performed better when working on open center-shaped plants than round-shaped trees at a 1.0 m working height compared to other heights. Meng et al. (2019) also stated that with UAV spraying research comparing Y-shaped and Central leader peach, the droplet coverage in the upper layer was significantly higher than in the lower layers and the distribution was not uniform in the CL-shaped peach tree. Tang et al. (2018) reported that the operational height of 1.2 m and citrus trees with inverted triangle shapes yield optimal droplet deposition performance based on UAV spraying. According to the above mentioned research, in the present study, the high variability between the fruit sets obtained as a result of pollination with UAV may be related to the fact that the pollen on the layer from which the branches were selected may not be dispersed uniformly in relation to the flight speed, height and wind direction due to the height of the walnut trees.

The results showed that the highest initial fruit set was obtained from T1. Similarly, the highest final fruit set was obtained from T2, although there was no significant difference between treatments. Jameela & Alagirisamy (2021) reported that the date palm fruit set was significantly higher in liquid mechanical pollination, and there was no significant difference between the concentrations. Similarly, Akhavan et al. (2021) found no difference between pollen doses in fruit set in the date palm cultivar applied to different pollen doses through UAV material. According to the result of UAV pollination using 50 gr pollen in a 1-liter kit, 2 ha (120 trees) area was pollinated at once, and increasing the pollen dose did not affect the fruit set. Thus, it has been concluded that 5% of pollen treatment is sufficient for UAV pollination of the walnut trees. In the present study, approximately 0.5 mg of pollen was obtained from 1 catkin, and obtaining the pollen (50 g/L) required to pollinate 2 ha area simultaneously requires the collection of 50,000 catkins. Since it is known that a walnut tree can produce approximately 5000 catkins (Şen 1986), catkins must be collected from approximately 20 trees to pollinate 2 hectares of area with the drone. Considering that it is easy to harvest the catkins by shaking them mechanically and subsequently obtaining pollen, it would be reasonable to use this pollen dose for pollination.

The results showed that nut traits were affected by the pollination treatment (Table 6). The highest nut weight, thickness, and length were obtained from the control treatment. The kernel weight and shell thickness were unaffected by the pollination treatments. The kernel percent of fruits gave similar results in all treatments. Moreover, the nut length/width ratio (fruit shape) ranged from 1.20 to 1.24 and was unaffected by pollination treatment.

Table 6. Nut traits of the pollination treatments

Treatment	*Nut weight (g)*	*Nut width (mm)*	*Nut thickness (mm)*	*Nut length (mm)*	*Kernel weight (mm)*	*Kernel percent (%)*	*Shell thickness (mm)*	*Nut length/ width*
Control	12.47±0.26 a	33.30±0.32 ns	34.25±0.56 a	40.18±0.46 a	5.99±0.24 ns	47.26±0.92 ns	1.86±1.84 ns	1.20±0.20 ns
T1	11.53±0.43 ab	32.23±0.67	32.73±0.20 b	38.77±0.41 b	5.73±0.39	49.25±2.26	1.85±1.76	1.20±0.20
T2	11.29±0.53 b	32.24±0.41	32.55±0.29 b	40.06±0.12 a	5.31±0.38	46.40±1.94	1.92±1.91	1.24±0.20

ns: Not significant. Each data value is represented as means ± standard deviations of three replications. Means within columns followed by different letters differ significantly ($p \leq 0.05$)

The results of nut traits of the walnut cultivars are shown in Table 6. The nut weight, nut thickness, and nut length values were significantly higher in the control than in other treatments. Although the fruit setting of the drone pollination treatment (T1, T2) was about 20 % higher than the control, the control treatment fruits were heavier than others. Akhavan et al. (2021) observed almost similar findings in date palm pollination; the treatments with higher fruit set ratios had a lower fruit weight. Likewise, Awad (2010) reported that fruit diameter and length were significantly higher in traditional pollination than in spray pollination treatments. El-Mardi et al. (2007) reported no significant difference between hand pollination and mechanical pollination methods in fruit diameter in date palm cultivars, but fruit weight and length values were lower in mechanical pollination. In contrast, Razeto et al. (2005) reported that the fruit shape of kiwifruit was affected by the pollination treatment and the fruit size increased with machine pollination.

4. Conclusions

The present study determined that winter temperatures of around -17.2 °C damaged the catkins of walnut cultivars at different rates. Due to the effects of the frost, with the catkins being damaged, there was a lack of pollenizers in the orchard. The pollination of walnuts was tested using the UAV, which was attempting to be adapted to agriculture in the recent years, in order to find a solution to the problem which is experienced in the present study and may be experienced in the future due to climate changes. The findings of this study reveal that using UAVs on walnut trees could increase fruit sets by about 20%. Furthermore, increasing the pollen doses used in UAVs pollination did not increase the fruit set. Thus, a single operator using this machine could pollinate with 5% pollen doses more

than 120 walnut trees per hour. UAV pollination did not affect the fruit quality but did affect fruit weight, thickness, and length. The fruits of open-pollinated trees were found to be heavier and larger than the others. These results are significant in showing the effect of UAVs pollination on walnut fruit sets and fruit characteristics. UAVs may require a significant initial investment, but fruit sets can be increased with the UAV pollination when catkins are damaged in commercial walnut orchards. Although pollination with UAVs increased fruit set, it was not uniform on trees compared to open pollination. For this purpose, to improve pollination efficiency, further experiments will be needed to determine the effects of the operation height and speed on pollen flow, including both the distance and intensity of pollen dispersal on different layers of walnut trees using UAV. In addition, considering that different pollen doses in the present study did not cause a difference in fruit set, pollination tests can be performed on walnuts by using less pollen in future UAV studies.

Data availability: Data are available on request due to privacy or other restrictions.

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References

Akhavan F, Kamgar S, Nematollahi M A, Golneshan A A, Nassiri S M & Khaneghah A M (2021). Design, development, and performance evaluation of a ducted fan date palm (Phoenix dactylifera L.) pollinator. *Scientia Horticulturae* 277: 1-6. doi.org/10.1016/j.scienta.2020.109808

Al-Wusaibai N A, Ben Abdallah A, Al-Husainai M S, Al-Salman H & Elballaj M A (2012). Comparative study between mechanical and manual pollination in two premier Saudi Arabian date palm cultivars. *Indian Journal of Science and Technology* 5(4): 2487-2490. doi.org/10.17485/ijst/2012/v5i4.4

Aleta N, Vilanova A, Tomas E & Guardia M (2014). Frost resistance in seven commercial walnut cultivars. *Acta Horticulturae* 1050: 389-393. doi.org/10.17660/actahortic.2014.1050.54

Aslamarz A A, Vahdati K, Rahemi M, Hassani D & Leslie C (2010). Supercooling and cold-hardiness of acclimated and deacclimated buds and stems of Persian walnut cultivars and selections. *HortScience* 45(11): 1662-1667. doi.org/10.21273/hortsci.45.11.1662

Awad M A (2010). Pollination of date palm (Phoenix dactylifera L.) cv. Khenazy by pollen grain-water suspension spray. *Journal of Food Agriculture and Environment* 88(3): 313-317. doi.org/10.4197/met.22-1.7

Ayfer M (1967). Antepfıstığında Megasporognesis, Megagametogenesis, Embriyogenesis ve Bunlarla Meyve Dökümleri Arasındaki Münasebetler. Tarım Bakanlığı Teknik Kitap, İstanbul.

Barnett J, Seabright M, Williams H, Nejati M, Scarfe A, Bell J & Duke M (2017). Robotic Pollination - Targeting kiwifruit flowers for commercial application. International Tri-Conference for Precision Agriculture.

Charrier G, Poirier M, Bonhomme M, Lacointe A & Améglio T (2013). Frost hardiness in walnut trees (*Juglans regia* L.): How to link physiology and modelling? *Tree Physiology* 33(11): 1229-1241. doi.org/10.1093/treephys/tpt090

El-Mardi M O, Al Said F A J, Sakit C B, Al Kharusi L M, Al Rahbi I N & Al Mahrazi K (2007). Effect of pollination method, fertilizer and mulch treatments on the physical and chemical characteristics of date palm (Phoenix dactylifera) fruit. I: Physical characteristics. *Acta Horticulturae* 736: 317-328. doi.org/10.17660/actahortic.2007.736.30

Gandev S (2013). Winter hardiness of reproductive organs of the walnut cultivars Izvor 10, Lara and Fernor at extreme low temperatures in South Bulgaria. *Bulgarian Journal of Agricultural Science* 19(5): 1068-1071.

Gianni T & Michelotti V (2018). Artificial Pollination in Kiwifruit and Olive Trees. *Pollination in Plants*. 59-66. doi.org/10.5772/intechopen.74831

Gonzalez R, Lemus G & Reginato G (2018). Pistillate Flower abscission symptoms of Serr walnut (*Juglans regia* L.). *Chilean Journal of Agricultural Research* 68: 183-191. doi.org/10.4067/s0718-58392008000200008

Gün A, Erdogan V, Akçay M E, Fidanci A & Tosun İ (2010). Pistillate Flower Abscission in Turkish Walnut Cultivars and Its Reduction. *Science and Technology* 3: 29-34.

Jameela R V & Alagirisamy M (2021). Effect of Mechanical (Dry and Wet) Pollination of Date Palms on Cultivar Naghal in the Sultanate of Oman. *International Conference on Information and Communication Technology Convergence (ICTC)* 967-972. doi.org/10.1109/ictc52510.2021.9621169

Kaveckaja A A & Tokar K O (1963). The unfavorable effect of large amount of pollen in the pollination of walnuts. *Botanichnyi Zhurnal* 48: 580-585.

Kim J, Kim S, Ju C & Son H (2019). Unmanned Aerial Vehicles in Agriculture: A Review of Perspective of Platform, Control, and Applications. *IEEE Access* 99: 1. doi.org/10.1109/access.2019.2932119

Kuru C (1995). Artificial pollination of pistachio trees under unsufficient pollination conditions. *Acta Horticulturae* 419(18): 121-124. doi.org/10.17660/actahortic.1995.419.18

Lan Y, Shengde C & Fritz B K (2017). Current status and future trends of precision agricultural aviation technologies. *International Journal of Agricultural and Biological Engineering* 10(3): 1-17.

Lapins K (1961). Artifical freezing of 1-year old shoots of apple varieties. *Canadian Journal of Plant Science* 41(2): 381-393. doi.org/10.4141/cjps61-051

Lemus G (2010). PFA Control in 'Serr' in Chilie. *Acta Horticulturae* 861(34): 263-266. doi.org/10.17660/actahortic.2010.861.34

Li J Y, Lan Y B, Wang J W, Chen S D, Huang C, Liu Q & Liang Q (2017). Distribution law of rice pollen in the wind field of small UAV. *International Journal of Agricultural and Biological Engineering* 10(4): 32-40..

Li J, Zhou Z, Lan Y, Hu L, Zang Y, Liu A & Zhang T (2015). Distribution of canopy wind field produced by rotor unmanned aerial vehicle pollination operation. *Transactions of the Chinese Society of Agricultural Engineering* 31(3): 77-86.

Liu A, Zhang H, Liao C, Zhang Q, Xiao C, He J, Zhang J, He Y, Li J & Luo X (2017). Effects of supplementary pollination by single-rotor agricultural unmanned aerial vehicle in hybrid rice seed production. *Agricultural Science and Technology* 18: 543-547.

Liu Y, Dang P, Liu L & He C (2019). Cold acclimation by the CBF–COR pathway in a changing climate: Lessons from Arabidopsis thaliana. *Plant Cell Reports* 38(5): 511-519. doi.org/10.1007/s00299-019-02376-3

Luedeling E, Girvetz E H, Semenov M A & Brown P H (2011). Climate change affects winter chill for temperate fruit and nut trees. *PLoS ONE* 6(5): e20155. doi.org/10.1371/journal.pone.0020155

Malone S R & Ashworth E N (1991). Freezing Stress Response in Woody Tissues Observed Using Low-Temperature Scanning Electron Microscopy and Freeze Substitution Techniques. *Plant Physiology* 95(3): 871-81. doi.org/10.1104/pp.95.3.871

Matt Koball W M (2019). Dropcopter.

Mazinani M, Dehghani M, Zarafshan P, Etezadi H, Vahdati K & Chegini G (2021). Design and Manufacture of an Aerial Pollinator Robot for Walnut Trees. Proceedings of the 9th *RSI International Conference on Robotics and Mechatronics* (*ICRoM)* 445-450. doi.org/10.1109/icrom54204.2021.9663500

McGranahan G H, Voyatzis D G, Catlin P B & Polito V S (1994). High pollen loads can cause pistillate flower abscission in walnut. *Journal of the American Society for Horticultural Science* 119(3): 505-509. doi.org/10.21273/jashs.119.3.505

Meng X, He Y & Han J (2019). Survey on Aerial Manipulator: System, Modeling, and Control. *Robotica* 38(7): 1288-1317. doi.org/10.1017/s0263574719001450

Mert C (2009). Temperature responses of pollen germination in Walnut (Juglans regia L.). *Journal of Biological and Environmental Science* 3(8): 37-43.

Mostan A (2012). Mechanization in Date Palm Pollination. *An overview of date palm production* 129-139. doi.org/10.1201/b11874-13

Özcan A, Bükücü Ş B & Sütyemez M (2017). Determination of pollen quality and production in new walnut cultivars. *Asian Journal of Agriculture Research* 11(3): 93-97.

Özcan A, Sütyemez M, Bükücü Ş B & Ergun M (2019). Pollen Viability and Germinability of Walnut A Comparison Between Storage at Cold and Room Temperatures. *Fresenius Environmental Bulletin* 28(1): 111-115.

Pagter M & Williams M (2011). Frost dehardening and rehardening of hydrangea macrophylla stems and buds. *HortScience* 46: 1121-1126. doi.org/10.21273/hortsci.46.8.1121

Poirier M, Bodet C, Ploquin S, Saint-Joanis B, Lacointe A & Ameglio T (2005). Walnut Cultivar Performance of Cold Resistance in South Central France. *Acta Horticulturae* 705: 281-285. doi.org/10.17660/actahortic.2005.705.35

Poirier M, Lacointe A & Améglio T (2010). A semi-physiological model of cold hardening and dehardening in walnut stem. *Tree Physiology* 30(12): 1555-1569. doi.org/10.17660/actahortic.2005.705.35

Polito V S, Pinney K, Weinbaum S, Aradhya M K, Dangl J, Yanknin Y & Grant J A (2005). Walnut Pollination Dynamics: Pollen Flow in Walnut Orchards. *Acta Horticulturae* 705: 465-472. doi.org/10.17660/actahortic.2005.705.68

Polito V, Coates B, Grant J, Hasey J, Micke W, Olson B & Pinney K (1996). Pollen, Pistillate flower abortion/ abscission. *Walnut research reports* 77-87.

Por A & Por J (1990). The effect of the excess pollen on the fruit set of walnuts in Balatonboglar. *Acta Horticulturae* 284: 253-256.

Razeto B, Reginato G & Larraín A (2005). Hand and Machine Pollination of Kiwifruit. *International Journal of Fruit Science* 5(2): 37-44. doi.org/10.1300/j492v05n02_05

Robichaud R L (2007). Pollen Gene Dispersal in Black Walnut across a Heterogeneous Landscape in Central Indiana. *PhD Thesis* doi.org/10.2737/rds-2017-0002

Rochette P, Angers D A, Bélanger G, Chantigny M H, Prévost D & Lévesque G (2004). Emissions of N_2O from alfalfa and soybean crops in Eastern Canada. *Soil Science Society of America Journal* 68(2): 493-506. doi.org/10.2136/sssaj2004.4930

Şen S M (1986). Ceviz Yetiştiriciliği. Eser Matbaası. Samsun.

Şen S M (2011). Ceviz yetiştiriciliği ve besin değeri. ÜÇM Yayıncılık. Ankara.

Shepherd T G (2016). Effects of a warming Arctic. *Science* 353(6303): 989-990. doi.org/10.1126/science.aag2349

Soliman S S, Alebidi A I, Al-Saif A M, Al-Obeed R S, Al-Bahelly A N (2017). Impact of pollination by pollen-grain-water suspension spray on yield and fruit quality of segae date palm cultivar (Phoenix dactylifera l.). *Pakistan Journal of Botany* 49(1): 119-123. doi.org/10.37855/jah.2011.v13i01.10

Takeda F, Arora R, Wisniewski M E Davis G A & Warmund M R (1993). Assessment of freeze injury in 'Boskoop Giant' black currant buds. *HortScience* 28: 652-654. doi.org/10.21273/hortsci.28.6.652

Tang Y, Hou C J, Luo S M, Lin J T, Yang Z & Huang W F (2018). Effects of operation height and tree shape on droplet deposition in citrus trees using an unmanned aerial vehicle. *Computers and Electronics in Agriculture* 148: 1-7. doi.org/10.1016/j.compag.2018.02.026

UPOV (1994). Walnut (*Juglans regia* L.) Guidelines for the Conduct of Test for Distinctness, Uniformity and Stability. Union Production New Varietes Plants.

Vahdati K, Arab MM, Sarikhani S, Sadat Hosseini M, Leslie C A & Brown P J (2019). Advances in walnut breeding strategies. *Advances in Plant Breeding Strategies: Nut and Beverage Crops* 401-172. doi.org/10.1007/978-3-030-23112-5_11

Wang P, Hu L, Zhou Z, Yang W, Liu A, Luo X, Xue X, He J & Yan Y (2013). Wind field measurement for supplementary pollination in hybrid rice breeding using unmanned gasoline engine single-rotor helicopter. *Transactions of the Chinese Society of Agricultural Engineering* 29(3): 54-61. doi.org/10.13031/aim.20131592053

Wang S, Lei X, Tang Y, Chang Y& Lv X (2020). Pear tree spray pollination technology based on multi-rotor UAV. *Jiangsu Agricultural Sciences* 48: 210-214.

Webster A D & Looney N E (1996). World distribution of sweet and sour cherry production: national statistics. In: Webster, AD, Looney NE (Eds.), *Cherries: crop physiology, production and uses* 25-69. doi.org/10.1079/9780851989365.0000

Whiting M (2015). Mechanical pollination for yield security. WSU.

Williams H, Nejati M, Hussein S, Penball N, Lim J Y, Jones M H, Ahn H S, Bradley S, Schaare P, Martinsen P, Alomar M, Patel P, Seabright M, Duke M, Scarfe A & MacDonald B (2019). Autonomous pollination of individual kiwifruit flowers: Toward a robotic kiwifruit pollinator. *Journal of Field Robotics* 37(2): 246-262. doi.org/10.1002/rob.21861

Zhang P, Deng L, Lyu Q, He S, Yi S, Liu Y, Yu Y & Pan H (2016). Effects of citrus tree-shape and spraying height of small unmanned aerial vehicle on droplet distribution. *International Journal of Agricultural and Biological Engineering* 9(4): 45-52.

Zhang S, Cai C, Li J, Sun T, Liu X, Tian Y & Xue X (2021). The Airflow Field Characteristics of the Unmanned Agricultural Aerial System on Oilseed Rape (*Brassica napus*) Canopy for Supplementary Pollination. *Agronomy* 11(10): 1-13. doi.org/10.3390/agronomy11102035

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Impact of Bio-fertilizers under Supplementary Irrigation and Rain-fed Conditions on Some Physiological Responses and Forage Quality of Smooth Vetch (*Vicia dasycarpa L.*)

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ABSTRACT

In arid and semi-arid regions, water scarcity and declining soil fertility limit the supply of livestock forage. This study evaluates the use of bio-fertilizers to adjustment water shortage stress and improved smooth vetch (*Vicia dasycarpa* L.) yield under water deficit stress conditions. A 2-year experiment was performed in an agrisilviculture system of plum orchard in 2016 and 2017. In this study, the single, double and triple combined of Arbuscular mycorrhizal fungi (AMF)- *Rhizophagus intraradices*, *Azotobacter chroococcum* (*Az*) and *Thiobacillus* spp. (*Th*) on smooth vetch plants were evaluated under rain-fed and supplemental irrigation. The results indicated that irrigated plants had more Fe and Zn nutrients than rain-fed plants. In comparison with single inoculation, the combined use of AMF + *A. chroococcum* facilitated the forage dry matter digestibility, total digestible nutrient, net energy for lactation, dry matter intake and relative feed value. In irrigated, double and triple combination of AMF with *A. chroococcum* and/or *Th*. improved chlorophyll-a, chlorophyll-b, total chlorophyll, relative water content, total soluble sugar, ascorbic acid, and glutathione while lowering proline and malondialdehyde. The results show that synthesis non-enzymatic antioxidant because of the combined use of bio-fertilizers (AMF, *Az* and *Th*) can reduce reactive oxygen species damage and improve water deficit resistance and yield in smooth vetch rain-fed plants.

Keywords: Chlorophyll, Irrigation, Non-enzymatic antioxidants, Osmolytes adjustment, Yield

1. Introduction

Smooth vetch (*Vicia dasycarpa* L.) is an important annual legume forage plant due to its multiple uses (seed, forage, silage, and green manure), its high nutritional value, and its potential to grow in a wide variety of climatic and soil conditions. Furthermore, vetch plants by fixing atmospheric nitrogen increase soil fertility (Haffani et al. 2017). Smooth vetch can be used in integrated systems such as agroforestry. Agroforestry systems are a sustainable and integrated system that, via some processes such as erosion control, improving water cycle, soil organic matter and nutrient, will lead to soil fertility and conservation improvements that help improve the inhabitant's livelihood (Heydarzadeh et al. 2022). So, in correct management of land-use, it is possible to achieve sustainable development in agriculture (Heydarzadeh et al. 2022).

In arid and semi-arid environments, water is the most limiting factor in reducing agricultural forage production (Saadat et al. 2019). In rain-fed farming, a plant's response to water deficit stress is complicated because of the varying frequency of dry and wet periods, the patterns of soil and atmospheric water deficits, and the degree and timing of drought (Balazadeh et al. 2021). Indeed, global warming, drought, and climate change require new strategies to increase forage cultivation (Tan & Yolcu 2021). Smooth vetch is a rain-fed crop and is moderately drought tolerant, but its yield is radically reduced with increasing drought conditions (Haffani et al. 2017). Consequently, supplemental irrigation is an effective measure that complements crop production and improves the living conditions of a region (Saadat et al. 2021). Under water deficit stress, plants usually respond by osmotic adjustment, stomata regulation, and antioxidant defense to reduce stress-related damages (Habibzadeh et al. 2015). Osmotic adjustment, is defined as a procedure of solute

accumulation in dividing cells when the water potential is decreased, and thereby enables the preservation of the cells turgor. This is considered a kind of adaptation to water scarcity in order to limit the damage of water deficit stress (Rahimzadeh & Pirzad 2017).

Sustainable agricultural systems involve the knowledge of interactions between crops and microorganisms, particularly those that have a direct effect on crop development and stress tolerance (Rahimzadeh & Pirzad 2017). Thus, in agricultural systems, the application of microorganism inoculants is of strategic interest in order to reduce chemical fertilizer usage, and improve environmental sustainability (Saadat et al. 2021). Microorganisms that assist plant growth often include arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria such as *Azotobacter* and *Thiobacillus* spp. (*Th*) that enhance growth by increasing the access to mineral nutrition, drought tolerance, and disease resistance (Heydari & Pirzad 2020; Heydarzadeh et al. 2022). For mycorrhiza and *Azotobacter*, several mechanisms have been proposed to improve water deficit tolerance in plants, the accumulation of osmolytes and antioxidant defense (Mohammadi et al. 2019). Produce various siderophores, plant growth hormones, antifungal, antibiotics and amino acids are the other beneficial of biofertilizers (Heydarzadeh et al. 2022). In fact, mycorrhizal fungi and their coexistence with plants alter water absorption and so improve drought resistance in the host plant (Habibzadeh et al. 2015). It clear that biofertilizers protects plants against the damaging impacts of reactive oxygen species (ROS) produced by water deficit stress. Therefore, biofertilizers improved non-enzymatic antioxidants (mainly proline, soluble carbohydrates, GSH, and AsA) and reduced water stress damage (Sohrabi et al. 2012a; Sohrabi et al. 2012b). The effect of combined inoculation of *Th* and AMF has been stated on dragon's head (Heydari & Pirzad 2020), as well as, the combined use of *Azotobacter* and mycorrhiza on lentils (Amirnia et al. 2019). These effects may be varied in terms of soil water deficit stress. Moreover, the use combined of bio-fertilizers has been proposed as an effective way to increase plant growth, development and production under rain-fed conditions. For this reason, this study assesses the impact of bacteria (*Az* and *Th*) and fungi (AMF) under supplementary irrigation and rain-fed conditions on some physiological responses and the forage quality of smooth vetch in an agrisilviculture system.

2. Material and Methods

In the plum [*Prunus domestica* (L.), Opal] orchard in Urmia, west Azerbaijan, Iran (37°39′24.82″N latitude, 44°58′12.42″ E longitude, 1338 m elevation), these two-years (2016 and 2017) field experiment were conducted based on a randomized complete block design with three replications. The single, double and triple combined of *Rhizophagus intraradices* (AMF), *Azotobacter chroococcum* (*Az*) and *Th*, AMF + *Az*, AMF + *Th*, *Az* + *Th*, AMF + *Az* + *Th* were used as inoculation for smooth vetch seeds, under rain-fed and supplemental irrigation.

Monthly rainfall and air temperature as an average are shown in Table 1.

Table 1- The average monthly temperature and rainfall in the growing seasons of 2016 and 2017 compared to the average of the period from 1987 to 2017 in Urmia

Month	*Jan.*	*Feb.*	*Mar.*	*Apr.*	*May*	*June*	*Jul.*	*Aug.*	*Sep.*	*Oct.*	*Nov.*	*Des.*	*Average/ total*
2015-2016													
Temperature (°C)	-0.4	3.8	7.4	12.2	15.4	20.9	24	22.8	19	11.2	7.3	-5.9	11.47
Rainfall (mm)	44	9.2	19.3	32.9	45.1	6	0	0.1	0	9.2	62	41.8	269.5
2016-2017													
Temperature (°C)	-5.2	-4.3	2.73	9.31	15.9	21.21	25	26	22.87	13.59	7.55	-1.23	11.15
Rainfall (mm)	10.6	43	9.3	69.8	22.3	0.8	0	0.7	0	0	28.2	55.33	240.4
Long term 1987-2017													
Temperature (°C)	-2.1	0.2	5.22	11.26	15.81	20.84	24	23.32	18.9	6.12	5.77	0.35	11.33
Rainfall (mm)	25.4	28.	46.7	54.7	37.1	10	5.6	2.8	4.3	30.5	39.4	28.1	312.8

The mycorrhiza inoculum was composed of sterile sand, mycorrhizal hyphae and spores (10 spores' g^{-1} inoculum) and colonized root fragments of corn crops (*Zea mays* L.) below the seeds. During the sowing time only in the rows below the seeds, 12 g m^{-2} of mycorrhiza inoculum was added to the plots. Prior to sowing, the seeds were inoculated with 10^8 CFU mL^{-1} (Colony-Forming Unit per mL) bacterial population of *A. chroococcum* and *Th*. at a rate of 2 L ha^{-1} in shadow (Wani & Gopalakrishnan 2019).

The following soil physicochemical characteristics were measured: the hydrometer method was used to determine the soil texture (clay, silt 23%, clay 42%, sand 35%) (Day 1965). Soil reaction (pH) was 7.95 that measured by using a pH meter described by Carter and Gregorich (2007), and electrical conductivity (EC) was 0.52 dS m^{-1} that determined in 1:2.5 soil-water suspension (Okalebo et al. 2002). Field capacity (FC=30%) and permanent wilting point (PWP=16%) were determined at soil water potentials of -0.33 bar and -15 bar, respectively, by pressure plates (Richards 1965). Organic matter was 1.35% (multiplying the percent organic carbon value by 1.724) that determined by method as described by Walkley and Black (1934), Soil nitrogen was 1.35 % that analyzed by the Kjeldahl method (Bremner 1996). The available P and K were 10.52 and 488 mg kg^{-1} that determined by the standard Olsen method (Olsen 1954) and flame photometer as described by Rowell (2014), respectively.

The Maragheh Dryland Agricultural Research Institute (MDARI) provided the smooth vetch seeds. On 8th March 2016 and 10th March 2017, the seeds were planted at a depth of 8 cm in plots of 150×200 cm in size with plant spacing between the row and plant (20×2 cm).

To determine the photosynthetic pigments, relative water content (RWC), osmolyte content, ROS, and antioxidants fresh leaves were sampled at the end of the flowering stage (87 and 89 days after sowing in the first and second year, respectively). Fresh leaf samples were held with aluminum foil, frozen in liquid nitrogen, and placed in plastic packets at -80 °C before being stored. Supplemental irrigation was applied at the 10% of flowering stage in both years. The plants were harvested on the 25th June 2016 and 27th June 2017. The water that supply by irrigation were 580 m^3 ha^{-1} in 2016 and 620 m^3 ha^{-1} in 2017 (Richards 1965).

To determine forage quality indices, a Near Infra-Red Spectroscopy (NIR, Inframatic 8600 Perten instruments- with wavelengths ranging from 500 to 2400 nm) was used and calibrated based on Jafari et al. (2003) method. At the end of flowering stage, 100 grams of dried forage (samples were dried in an oven at 68 °C for 72 h) from each plot was ground in order to determine the forage quality. Dry matter digestibility (DMD), acid detergent fiber (ADF), neutral detergent fiber (NDF), total digestible nutrients (TDN), dry matter intake (DMI), net energy for lactation (NEL) and relative feed value (RFV) were the forage quality indices that were measured using NIR (Horrocks & Valentine 1999; Jafari et al. 2003).

To determine the Fe and Zn content, the dried grains of smooth vetch, were milled, and underwent combustion (4 h at 500 °C), after which the grain ashes (5 mg) were digested in 1 mL of 2 N HCl, and the extracts acquired filtered (Whatman filter paper: grade 42).

The Fe and Zn was determined in dry digestion extract using an atomic absorption (2380 Perkin Elmer); they were measured in mg L^{-1}, and expressed as mg kg^{-1} (Houba et al. 1988). The content of chlorophyll-a and b were determined by extracting the fresh leaves using 80% acetone using a spectrophotometer at wavelengths of 646.8 and 663.2 nm, respectively (Lichtenthaler & Wellburn 1983). In addition, the relative content of leaves water (RWC) was measured according to the following equation (Saadat et al. 2021).

$$\% \text{ RWC} = [(\text{fresh weight-dry weight})/(\text{turgid weight-dry weight})] \times 100 \quad \text{(Eq. 1)}$$

For measuring turgid, the leaves were soaking for 16 to 18 h in distilled water, after which the leaves were carefully and quickly blotted dry with tissue paper. The leaf dry weight was determined after drying the leaf sample for 72 h at 70 °C.

The fresh leaf (0.5 *g*) was used to evaluate the concentration of proline by the ninhydrin method (Bates et al. 1973). The absorbance was evaluated at 520 nm using a Spectronic 20 colorimeter (SP 6-200 Unican). A calibration curve was used to determine the concentration of proline. Leaf total soluble sugars (TSS) were determined based on the phenol sulfuric acid method (Dubois et al. 1956). In this method, 0.5 g of fresh leaves were homogenized with ethanol. The extract was filtered and treated with 5% phenol and 98% sulfuric acid. This mixture was left for 1 h and its absorption was measured by spectrophotometer at 485 nm. Malondialdehyde (MDA) was evaluated with minor changes according to the thiobarbituric acid (TBA) reaction (Zhou et al. 2004). One mL of extract was mixed with 0.5% TBA in 2.5 mL and then heated for 20 min at 100 °C. The spectrophotometric absorbance of the supernatant was calculated at 532, 600 and 450 nm (A532, A600 and A450) and the concentration of MDA was calculated using the following formula (Eq. 2):

$$6.45 \times (A532 - A600) - 0.56 \times A450 \quad \text{(Eq. 2)}$$

The ascorbic acid (AsA) measurement was based on creating a purple complex between ferrous ion and bipyridyl generated at 525 nm (reduction of ferric to ferrous ion with ascorbate in acid solution). The AsA content was determined using 0.2 *g* of fresh leaves (Li et al. 2015). The AsA standards were prepared in the range of 0-15 mg L^{-1} in 1 mL of 5% (w/v) trichloroacetic acid (TCA), 1 mL of alcohol, 0.5 mL of 0.4% H_3PO_4 alcohol, 1 mL of 0.5% bathophenanthroline alcohol and 0.5 mL of 0.03% $FeCl_3$ alcohol for 90 min at 30 °C and determined at 534 nm.

The fresh leaves (0.2 *g*) were mixed with 4 mL of 5 mM EDTA-TCA to determine glutathione (GSH) concentration, which was estimated using 5,5′-dithio-bis (2 nitrobenzoic acids) (Khan et al. 2014). Changes in reaction mixture absorbance were reported at 420 nm and GSH concentration was determined from a standard GSH curve. After the flowering stage, 10 plants per plot were randomly harvested from a depth of 10-30 cm. After washing fresh root samples, about 1 *g* of roots were transmitted to the laboratory and cleaned with 10% KOH, marked with 0.05% trypan blue in lactic acid (Phillips & Hayman 1970), and the proportion of root colonization was calculated using the gridline intercept method (McGonigle et al. 1990).

An analysis of variance for the two-year results was performed using the generalized linear model (SAS 9.1.3) combined over the two years. The effects of rain-fed and supplementary irrigation, the application of biofertilizers and the interactions between these two variables were evaluated by ANOVA and distinctions between means were compared using Duncan's multiple range test at $p \leq 0.05$.

3. Results and Discussion

The combined analysis of the 2-year data for the ADF, NDF, DMD, TDN, DMI, RFV and NEL of smooth vetch indicated that the simple effects of year, irrigation conditions and biofertilizers were significant (Table 2). The interaction effect of "irrigation conditions × biofertilizer" was significant on the Fe and Zn of smooth vetch grains (Table 2). According to the combined ANOVA of 2-years, the simple effects of irrigation conditions and biofertilizers on chlorophyll-a, chlorophyll-b and total chlorophyll were significant (Table 2). The interaction effect of "irrigation conditions × biofertilizers" and year had a significant effect on proline, MDA, RWC, GSH and seed yield (Table 2). In addition, the TSS, AsA and root colonization were affected by the interaction of "irrigation conditions × biofertilizer" (Table 2).

Table 2. Variance analysis of *V. dasycarpa* forage quality traits and some physiological traits as affected by irrigation and biofertilizers

Source of variation	***df***	***ADF***	***NDF***	***DMD***	***TDN***	***DMI***	***RFV***	***NEL***	***Fe***	***Zn***	***Chl-a***
Year (Y)	1	124.32**	108.16**	75.38**	207.15**	0.61**	3569.59**	0.08**	3.51^{ns}	4.13^{ns}	0.0008ns
Repeat /Y	4	1.04	0.08	0.63	1.74	0.0009	12.92	0.0007	63.91	3.64	0.001
Irrigation conditions (IC)	1	245.15**	284.48**	148.72**	408.37**	1.62**	8542.26**	0.16**	41293.34**	2990.76**	0.85**
Y × IC	1	0.05^{ns}	0.36^{ns}	0.03^{ns}	0.08^{ns}	0.0006^{ns}	9.03^{ns}	0.00008^{ns}	18.69^{ns}	9.33^{ns}	0.001ns
Biofertilizer (Biof)	7	36.69**	55.33**	22.29**	61.17**	0.29**	1418.25**	0.02**	6924.18**	244.10**	0.51**
Y × Biof	7	0.001^{ns}	0.01^{ns}	0.001^{ns}	0.003^{ns}	0.001^{ns}	6.28^{ns}	0.000003^{ns}	21.76^{ns}	0.62^{ns}	0.01ns
IC × Biof	7	0.08^{ns}	0.01^{ns}	0.05^{ns}	0.14^{ns}	0.002^{ns}	16.69^{ns}	0.00005^{ns}	488.38**	11.43*	0.001ns
Y × IC × Biof	7	0.09^{ns}	0.01^{ns}	0.05^{ns}	0.15^{ns}	0.00009^{ns}	0.79^{ns}	0.00006^{ns}	5.13^{ns}	0.22^{ns}	0.001ns
Error	60	0.11	0.28	0.07	0.19	0.002	7.89	0.00008	20.14	4.73	0.007
Coefficient of variation (%)		1.08	1.32	0.41	0.72	1.49	1.86	0.61	2.89	5.97	3.88
Source of variation	***df***	***Chl-b***	***Chl a+b***	***Proline***	***MDA***	***RWC***	***TSS***	***GSH***	***AsA***	***RC***	***GY***
Year (Y)	1	0.02^{ns}	0.03^{ns}	0.39**	51.84**	159.16**	0.24^{ns}	0.21**	149.37^{ns}	27.53 ns	362070.72**
Repeat /Y	4	0.05	0.04	0.003	0.46	1.47	0.50	0.02	26.78	51.67 ns	9879.19
Irrigation conditions (IC)	1	0.32**	2.26**	4.78**	622.60**	1908.79**	593.16**	3.96**	18903.74**	1118.30**	12838366.91**
Y × IC	1	0.0003^{ns}	0.002^{ns}	0.001^{ns}	0.28^{ns}	0.47^{ns}	0.03^{ns}	0.003^{ns}	1.06^{ns}	0.23 ns	18790.88ns
Biofertilizer (Biof)	7	0.62**	2.23**	0.58**	63.89**	231.23**	76.32**	0.45**	1011.51**	2987.73 **	610997.21**
Y × Biof	7	0.01^{ns}	0.02^{ns}	0.01^{ns}	1.46^{ns}	5.21^{ns}	0.21^{ns}	0.004^{ns}	18.40^{ns}	1.59 ns	633.13ns
IC × Biof	7	0.02^{ns}	0.03^{ns}	0.03*	5.69**	13.98*	7.80**	0.04**	206.71**	100.83 **	163496.57**
Y × IC × Biof	7	0.0009^{ns}	0.003^{ns}	0.004^{ns}	0.56^{ns}	1.80^{ns}	0.28^{ns}	0.002^{ns}	9.27^{ns}	0.003 ns	132.85ns
Error	60	0.02	0.03	0.01	1.54	4.90	0.48	0.014	38.40	32.52	11473.15
Coefficient of variation (%)		13.25	5.76	3.14	7.85	3.47	4.44	9.77	8.81	16.55	9.99

ADF: Acid detergent fiber, NDF: Neutral detergent fiber, DMD: Dry matter digestibility, TDN: Total digestible nutrient, DMI: Dry matter intake, NEL: Net energy for lactation, RFV: Relative feed value. Chl- a: Chlorophyll-a; Chl-b: Chlorophyll-b; Chl a+b: Total Chlorophyll; MDA: Malondialdehyde; RWC: Relative water content; TSS: Total soluble sugar; GSH: Glutathione; AsA: Ascorbic Acid; RC: Root colonization; GY: Grain yield. *,** and ns, significant at 5% and 1% levels of probability, non-significant, respectively

Rain-fed smooth vetch contained the highest of ADF and NDF in forage (Table 3). Double and triple combination use of biofertilizers (AMF, *Az* and *Th*) reduce the smooth vetch ADF and NDF by 17% and 14% compared with plants inoculated with only AMF or bacteria (Table 3). The average ADF and NDF of smooth vetch plants were 30.13% and 39.01% in 2016 and 32.41% and 41.13% in 2017, respectively (Table 3). The mean comparison revealed that in rain-fed conditions, ADF and NDF significantly increased, but irrigated plants had low ADF and NDF content (Table 3). Insoluble fibers increase in cell walls is one of the physiological responses of plants for avoid moisture loss under water deficit stress (Jahanzad et al. 2013). The application of supplementary irrigation seems to slow this process and prevent a remarkable increase in crude fiber growth rate (Jahansouz et al. 2014).

Table 3- Means (± standard deviation) comparison of the effect of year, irrigation conditions and biofertilizer on some *V. dasycarpa* forage quality traits

Treatments	*ADF (%)*	*NDF (%)*	*DMD (%)*	*TDN (%)*	*DMI (%)*	*RFV (%)*	*NEL (%)*
Year							
2016	30.13±2.37^{b}	39.01±2.69^{b}	65.42±1.85^{a}	62.44±3.06^{a}	3.08±0.21^{a}	156.95±15.02^{a}	1.51±0.05^{a}
2017	32.41±2.31^{a}	41.13±2.72^{a}	63.64±1.80^{b}	59.50±2.98^{b}	2.92±0.19^{b}	144.75±13.50^{b}	1.45±0.06^{b}
Irrigation							
Rain-fed	32.87±1.99^{a}	41.79±2.34^{a}	63.29±1.55^{b}	58.90±2.56^{b}	2.87±0.16^{b}	141.42±11.09^{b}	1.43±0.06^{b}
Supplemental irrigation	29.67±2.11^{b}	38.35±2.33^{b}	65.78±1.64^{a}	63.04±2.72^{a}	3.13±0.19^{a}	160.28±13.39^{a}	1.52±0.06^{a}
Biofertilizer							
C	33.83±2.08^{a}	43.80±2.28^{a}	62.53±1.62^{g}	57.66±2.68^{g}	2.74±0.14^{g}	133.18±10.38^{g}	1.41±0.05^{g}
AMF	31.65±2.11^{c}	39.39±2.42^{d}	64.21±1.65^{e}	60.48±2.73^{e}	3.05±0.18^{d}	152.24±12.89^{d}	1.47±0.06^{e}
Az	32.29±2.38^{b}	41.19±2.35^{c}	63.68±1.58^{f}	59.66±3.07^{f}	2.92±0.17^{e}	144.40±12.74^{e}	1.45±0.05^{f}
Th	32.36±2.03^{b}	42.35±2.11^{b}	63.74±1.85^{f}	59.56±2.62^{f}	2.83±0.14^{f}	140.32±10.44^{f}	1.45±0.05^{f}
AMF + *Az*	28.03±2.27^{g}	37.67±2.40^{g}	67.05±1.77^{a}	65.15±2.94^{a}	3.19±0.21^{a}	166.39±15.01^{a}	1.56±0.06^{a}
AMF + *Th*	29.95±2.24^{f}	38.33±2.08^{f}	65.56±1.74^{b}	62.68±2.89^{b}	3.13±0.19^{b}	159.78±13.72^{b}	1.51±0.06^{b}
Az + *Th*	31.29±2.16^{d}	38.97±2.25^{de}	64.52±1.68^{d}	60.94±2.78^{d}	3.08±0.18^{cd}	154.58±12.67^{c}	1.48±0.06^{d}
AMF + *Az* + *Th*	30.77±1.97^{e}	38.87±2.08^{e}	64.92±1.54^{c}	61.61±2.54^{c}	3.09±0.17^{c}	155.90±12.00^{c}	1.49±0.05^{c}

ADF: Acid detergent fiber; NDF: Neutral detergent fiber; DMD: Dry matter digestibility; TDN: Total digestible nutrient; DMI: Dry matter intake; NEL: Net energy for lactation; RFV: Relative feed value. Means with same letters in each column (for single effect of treatments) are not significantly different based on Duncan's multiple range test p≤0.05. C: Control; AMF: Arbuscular mycorrhizal fungi*; Az: Azotobacter chroococcum*; *Th: Thiobacillus* spp.

Combination use of AMF, with *A. chroococcum* and *Th* exhibited a notable impact on ADF and NDF compared to single use biofertilizers (Table 3). It appears that inoculation with microorganism increase plant cytokinin's, leaf growth and carbon allocation from other parts of the leaf, by delaying leaf senescence (Heydari & Pirzad 2020). Therefore, the mycorrhization plays an important role in maintaining the forage quality by decreasing smooth vetch ADF and NDF content, which improves digestibility. The content of insoluble fibers varies according to ecological conditions. Table 3 shows that 2017 had the highest content of insoluble fibers in neutral and acid detergents. In 2016, high rainfall may be the reason for the low ADF and NDF content in smooth vetch forage compared to 2017 (Table 1).

A means comparison revealed that the highest DMD (65.78%), TDN (63.04%), DMI (3.13%), RFV (160.28%) and NEL (1.52%) were obtained from irrigation (Table 3). When compared with the control treatment, the dual- and triple-use of bio-fertilizers improved forage DMD, TDN, DMI, RFV and NEL content (Table 3). The average amounts of DMD, TDN, DMI, RFV and NEL in the first year of the experiment were 65.42, 62.44, 3.08, 156.95, and 1.51% while in the second year they were 63.64, 59.50, 2.92, 144.75, and 1.45%, respectively (Table 3). Dual-inoculation with "AMF + *A. chroococcum*" increased DMD (Table 3). In contrast to ADF and NDF, forage DMD decreased significantly (Table 3). Due to the negative correlation of DMD with NDF and ADF, the significant decrease of NDF and ADF in inoculated plants, caused to more mineral absorption by the plants and increased DMD (Lithourgidis et al. 2006). It has been noted that the mycorrhizal fungus assists in phytohormone production, which stimulates nutrient absorption and photosynthesis process, which in turn increases DMD content (Saadat et al. 2019). TDN refers to nutrients available for livestock and are related to the ADF content in forages. In parallel to ADF increase, TDN content decreases, which decreases the quality of forage plants (Jahanzad et al. 2013). Water stress during the flowering stage may be due to the reduction in photosynthesis process and dry matter accumulation, which consequently decreases TDN in forage (Balazadeh et al. 2021; Jahansouz et al. 2014). It may be that the implemented biofertilizers, either single or combined, produced a significant increase in total TDN either in leaves or in branches of vetch due to the effect of biofertilizers on increasing nutrient uptake, which improved the growth and development of plants for better

components of TND. Thus, in combined fertilizer treatments and supplementary irrigation, due to reduced NDF content (Table 3), the content of DMI forage and consequently forage production increased.

Among the biofertilizer treatments, the combined treatment of AMF with *A. chroococcum* and/or *Th* had the greatest effect on the increase in RFV (Table 3). High contents of NDF and ADF in control plants led to low RFV forage. Therefore, the improvement of RFV forage is due to the increase in smooth vetch DMI and DMD by biofertilizer application. Because DMI and DMD have a negative correlation with forage NDF and ADF respectively, the RFV index can be used to estimate the intake and energy value of forages using DMD and DMI (Balazadeh et al. 2021; Lithourgidis et al. 2006). When the RFV value is higher than 151, the forage is considered prime (Horrocks & Valentine 1999). In our study, under supplementary irrigation, the RFV values corresponded to prime quality. So, the RFV of irrigated smooth vetch plants saw an increase when compared to rain-fed plants (Table 3).

The NEL is an indicator of the quantity of forage energy obtainable for maintenance and milk production after digestive and metabolic losses and it is shown to be inversely correlated with ADF (Charbonneau et al. 2006). A positive synergistic effect of microorganisms increases the growth, development and transfer of nutrients that results in the highest forage NEL in dual- and triple-inoculated plants and supplementary irrigation. Due to the higher ADF content of smooth vetch plants under rain-fed compared to supplementary irrigation (Table 3), an NEL reduction of forage is predictable. In both rain-fed and supplementary irrigation cultivation, the amount of iron (Fe) and zinc (Zn) in plants combined with biofertilizers increased significantly; however, the amount of Fe and Zn in irrigated plants were higher than in rain-fed plants. The highest amounts of Fe (224.67) and Zn (49.14 mg kg^{-1}) have been found in plants AMF + *A. chroococcum* irrigated plants (Figure 1A, B). Combined use of biofertilzers (AMF, *Az* and *Th*) led to improve the accumulation of Fe and Zn in both rain-fed and irrigated plants (Figure 1A, B). Therefore, given the lower Fe and Zn content of the smooth vetch grains under rain-fed situations, it can be concluded that nutrient uptake potential was low in rain-fed plants due to a rise in water deficit (Figure 1A, B). It has been reported that nutrient solubility reduces as soil moisture decreases (Pirzad & Mohammad Zadeh 2018). The combined use of biofertilizer in our study was effective in improving nutrient uptakes such as Fe and Zn (Figure 1A, B). The researchers found that the application of bio fertilization increases plant growth and development, nutrient absorption, and photosynthetic efficiency significantly due to the increased activity of alkaline phosphatase and acid phosphatase, improving soil EC, synthesizing organic acids, and changing the pH or secretion of enzymes (Heydari & Pirzad 2020).

The results showed that smooth vetch leaf in supplemental irrigation conditions had a higher chlorophyll-a (2.42 mg g^{-1} FW), chlorophyll-b (1.51 mg g^{-1} FW) and total chlorophyll content (3.93 mg g^{-1} FW) than rain-fed plants (Figure 2A-C). In addition, the content of chlorophyll-a, chlorophyll-b and total chlorophyll was higher in AMF, dual, and triple-colonized plants than in the control and other individual biofertilizer applications (Figure 2A-C).

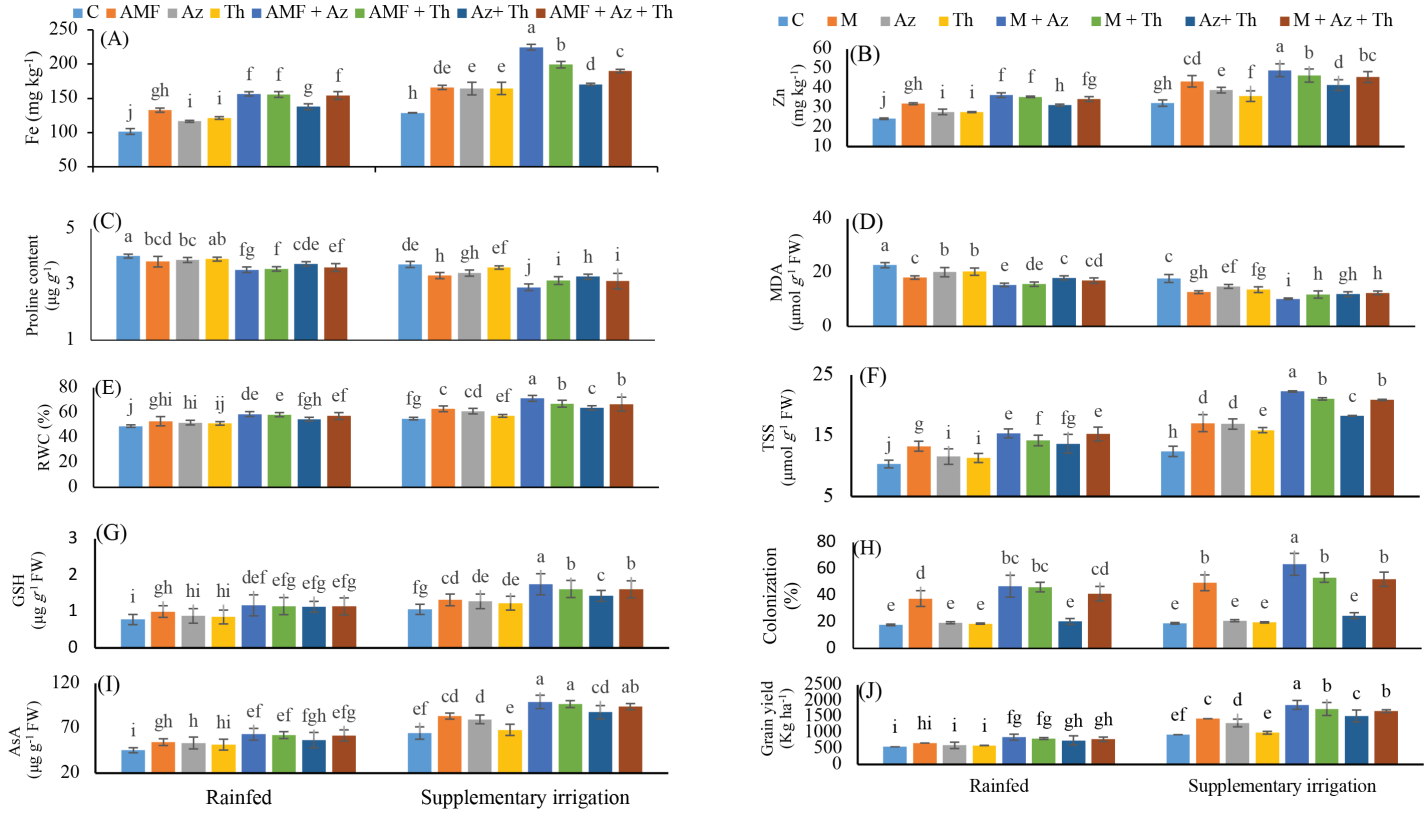

Figure 1- Mean (± standard deviation) comparison of smooth vetch Fe (A), Zn (B), proline (C), MDA (D), RWC (E), TSS (F), GSH (G), root colonization (H), AsA (I) and grain yield (J) as affected by "Irrigation conditions×Biofertilizer". Means with the same letters in each column are not significantly different based on Duncan's multiple range test p≤0.05. C: Control; AMF: Arbuscular mycorrhizal fungi; *Az: Azotobacter chroococcum*; *Th: Thiobacillus* spp

In our experiment, water deficit stress significantly reduced the leaf chlorophyll content (Figure 2 A-C).

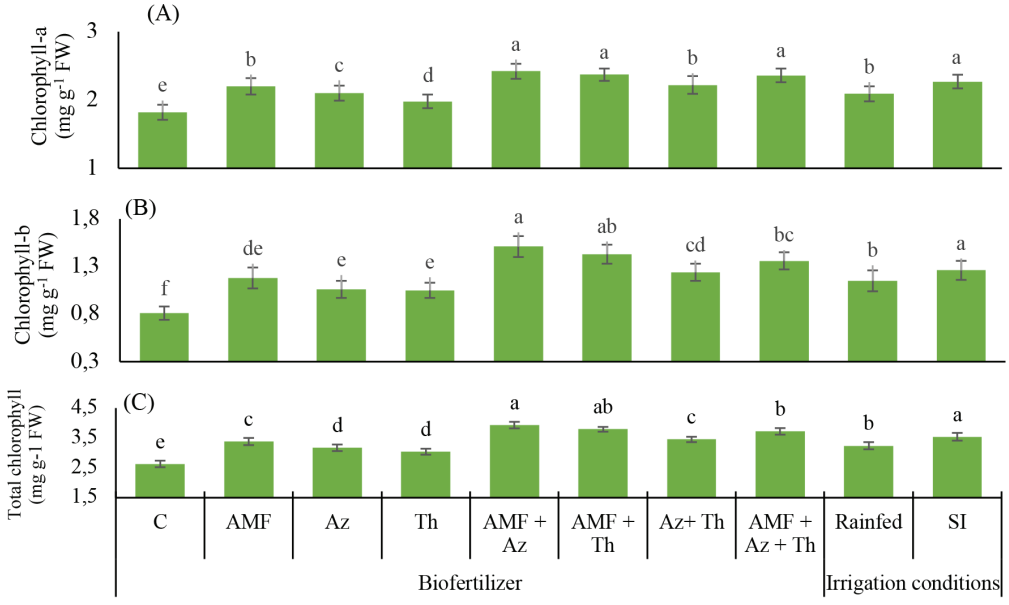

Figure 2- Mean (± standard deviation) comparison of smooth vetch chlorophyll-a (A), chlorophyll-b (B) and total chlorophyll (C) as affected by "Irrigation conditions and Biofertilizer". Means with same the letters in each column are not significantly different based on Duncan's multiple range test p≤0.05. C: Control; AMF: Arbuscular mycorrhizal fungi; *Az: Azotobacter chroococcum*; *Th: Thiobacillus* spp.; SI: Supplementary irrigation

Water is likely to be effective in maintaining active chloroplasts and subsequently performing chlorophyll tasks such as energy absorption and transfer (Saadat et al. 2021). The reduction in chlorophyll content in drought conditions may be due to the impact of water deficiency on the decomposition of chlorophylls and their peroxidation by active oxygen species (Sohrabi et al. 2012b), since the active species of oxygen destroy lipids, proteins and photosynthetic pigments. It has been attributed to a reduction in chlorophyll content in water deficiency conditions to a decreased stability of the chloroplast membrane and its breakdown (Sohrabi et al. 2012a). Under supplementary irrigation conditions, however, the increased chlorophyll content that we observed with the dual- and triple-combination of AMF + *A. chroococcum* and/or *Th* (Figure 2A-C) may have contributed to an increased rate of photosynthesis. Therefore, in explaining the reason for the superiority of the combination of biofertilizers, it can be stated that sufficient nitrogen uptake due to the presence of fungi and nitrogen stabilizing bacteria in biofertilizers caused the plant to have sufficient nitrogen to produce chlorophyll (chlorophyll content has a strong correlation with the amount of nitrogen) (Amirnia et al. 2019).

The concentration of proline and MDA in inoculated plants with combined treatments of AMF plants with *A. chroococcum* and/or *Th* in both rain-fed conditions and supplementary irrigation decreased significantly (Table 4). However, the concentration of proline and MDA was higher in rain-fed plants compared to supplementary irrigation. The lowest amounts of proline (2.9 μg g^{-1} FW) and MDA (10.27±0.28 μmol g^{-1} FW) were obtained in irrigated plants inoculated with "AMF + *A. chroococcum*" (Figure 1C, D). In addition, the concentration of leaf proline and MDA were significantly higher in 2017 than in 2016 (Table 4).

Table 4- Mean (± standard deviation) comparison of the effect of year for some *Vicia dasycarpa* L. traits

Year	*Proline (μg g^{-1} FW)*	*MDA (μmol g^{-1} FW)*	*RWC (%)*	*GSH (μg g^{-1} FW)*	*GY (Kg ha^{-1})*
2016	3.47±0.34[b]	15.10±0.93[b]	60.07±6.77[a]	1.26±0.09[a]	1133.03±88.22[a]
2017	3.60±0.31[a]	16.57±1.02[a]	57.49±6.22[b]	1.17±0.11[b]	1010.20±94.09[b]

MDA: Malondialdehyde, RWC: Relative water content, GSH: Glutathione, GY: Grain yield. Means with the same letters in each column are not significantly different based on Duncan's multiple range test p≤0.05

The plants inoculated with the dual- and triple-combination of "AMF + *A. chroococcum* and/or *Th*", under supplementary irrigation conditions, had the lowest amount of proline prolonged water-deficit stress tolerance (Figure 1C). The accumulation of proline in plants under water shortages has been attributed to various factors, such as the regulatory influence of ABA on light mechanisms (Pirzad & Mohammad Zadeh 2018), and photosynthetic compounds that enhance proline synthesis (Amirnia et al. 2019). It is believed that proline accumulation plays an important function in the stress tolerance of plants as an osmotic adjustment (Mohammadi et al. 2019). The dual- and triple-combined use of AMF, with *Azotobacter* and *Thiobacillus*, exhibited a notable impact on MDA and decrease in both rain-fed and supplemental irrigation conditions (Figure 1D). An increase in MDA, as the main product of lipid peroxidation in the cellular membrane, is associated with a water deficit which leads to excessive oxygen free radicals in the membrane system, so that the membrane lipid is oxidized (Rahimzadeh & Pirzad 2017). The accumulation of MDA may cause damage to the membrane and cells, and MDA level in the plant represents the degree of membrane damage (Mohammadi et al. 2019). However, in all irrigation conditions, the MDA in inoculated plants was lower than in non-inoculated plants, indicating that microorganisms may reduce membrane lipid peroxidation, due to enhanced antioxidant scavenging of ROS (Sohrabi et al. 2012b). AMF + *A. chroococcum* irrigated plants had the highest RWC (71.42%), TSS (22.29 μmol g^{-1} FW), GSH (1.75 μg g^{-1} FW) and AsA (99.34 μg g^{-1} FW) (Figure 1). The combined use of biofertilizers in both irrigated and rain-fed increased RWC, TSS, GSH and AsA than the use of single biofertilizers (Figure 1). In addition, more RWC and GSH were obtained in the first year (Table 4). We found that dual- and triple-inoculation plants of AMF with *A. chroococcum* and/or *Th* had higher RWC, which benefited photosynthetic efficiency (Figure 1E). Reduced growth and root activity, as well as increased evapotranspiration from the plant community, are known to be important factors in RWC reduction (Zhou et al. 2004). It has been stated that the leaf RWC of lentils diminishes as water stress increases (Amirnia et al. 2019). A decrease in the turgor of plant tissues and leaf RWC could be the first effect of water deficit stress, which can have a natural impact on cell growth and size. Biofertilizers enhance water uptake in the host plant by altering root development and spreading the plant's root system (Sohrabi et al. 2012b). Indeed, the use of mycorrhizal fungi and bacteria appears to mitigate the adverse effects of water deficit on plants by increasing leaf water potential, transpiration rate, photosynthetic efficiency, and the rate of CO_2 use in host plants, as well as increasing nutrient absorption, thereby enhancing growth and plant production (Fouad et al. 2014).

When smooth vetch was subjected to rain-fed conditions, the TSS concentration decreased (Figure 1F). It has been reported that limited irrigation causes a reduction in the TSS concentration due to reduced photosynthesis and stomatal closure (Rahimzadeh & Pirzad 2017). Although damage to cell membranes by water deficit stress likely restricts osmotic adjustment, increased leaf water content during water deficit stress may inhibit the formation of osmolytes, such as TSSs (Amirnia et al. 2019).

The higher TSS concentration in biofertilizer-treated plants in both supplementary irrigation and rain-fed conditions (Figure 1F) could be explained by the observed synergic effect of dual- and triple-inoculation on vegetative growth in smooth vetch is likely to be associated with the increased level of photosynthesis induced by these treatments (Fouad et al. 2014). The application of mycorrhizal fungi and bacteria likely improved growth and led to a higher concentration of TSS by supplying water and nutrients.

The decrease in GSH and AsA concentration in rain-fed plants, resulted in enhanced lipid peroxidation (Sohrabi et al. 2012a). To protect the antioxidant system that protects plants from oxidative destruction owing to drought stress, an excessive level of inhibitory AsA is significantly more efficient (Mohammadi et al. 2019). Both AMF and bacteria inoculation significantly increased GSH and AsA concentration compared to the control treatment (Figure 1G, I). It seems that inoculation AMF plants with *A. chroococcum* and/or *Th* have enhanced the accumulation of AsA and GSH as protective compounds to cope with the detrimental effects of water deficiency stress.

Root colonization was the minimum in the rain-fed and irrigated control plants. An increase in root colonization was only observed in the AMF plants. The AMF + *A. chroococcum* irrigated plants had the highest root colonization (63.45%). The combined use of AMF with other biofertilizers improved fungal root colonization in rain-fed plants (Figure 1H). The growth and root colonization decrease under water stress may be caused by changes in the hyphae's morphological features or by reduced spore development and density (Saadat et al. 2021). In addition, water deficit hurts spore germination, hyphal growth, and proliferation in soil, resulting in a reduction in mycorrhizal colonization, suppression of arbuscular formation, and reduced photosynthetic availability, all of which indirectly causes a decrease in colonization under water deficit stress (Heydari & Pirzad 2020). The highest AMF colonization was determined in dual- and triple-inoculation of AMF with *A. chroococcum* and/or *Th* inoculated plants under rain-fed and supplementary irrigation (Figure 1H), The effect of microorganisms on increasing water stress tolerance procedures appears to be connected more to the roots colonized as previously mentioned (Habibzadeh et al. 2015).

The AMF + *A. chroococcum* irrigated plants had the highest grain yield (1873.29 kg ha^{-1}). Grain yield in both rain-fed and irrigated plants demonstrated an effective increase in inoculated plants with dual and triple biofertilizers than in plants only inoculated with AMF or bacteria. The lowest amount of grain yield (555.05 kg ha^{-1}) was obtained under rain-fed conditions and non-inoculated control plants. However, the individual application of biofertilizers had no significant difference when compared to the control in rain-fed conditions (Figure 1J). Furthermore, an increase by 11% in smooth vetch grain yield was observed in the first year compared to the second year (Table 4). The co-inoculation of AMF + *A. chroococcum* enhanced seed yield by 35% and 49 %, respectively, when compared with the control plants (Figure 1J). A reduced grain yield in rain-fed conditions when compared to supplemental irrigation may have arisen from the decrease of pure photosynthesis and nutrients transmitted from leaves to seeds (Pirzad & Mohammad Zadeh 2018). The results show that in combination biofertilizer treatments, due to the mutual interaction of bacteria-fungi, biological nitrogen fixation, increased solubility of non-mobile phosphate, and the production of various plant growth stimulants, stimulate nutrient absorption and, by impacts on photosynthesis processes, improve seed yield components and eventually increase grain yield (Amirnia et al. 2019; Heydari & Pirzad 2021).

4. Conclusions

This study shows that supplemental irrigation improved the forage DMD, TDN, NEL, DMI and RFV content, while higher ADF and NDF, were achieved under rain-fed conditions. Double and triple use of AMF with *A. chroococcum and/or Th* were more efficient for improving DMD, TDN, NEL, DMI and RFV content than the control plants. In both rain-fed and irrigated plants, Fe and Zn accumulated in greater quantities in dual and triple biofertilizers treatments, compared with singly inoculated plants. Dual- and triple-use of AMF with *A. chroococcum* and/or *Th* This resulted in an increase in chlorophyll, RWC, TSS, AsA, and GSH in irrigated plants and reducing proline and MDA. Moreover, in inoculated plants with biofertilizers, the tolerance through boosting non-enzymatic antioxidant synthesis increased, which protects against ROS under rain-fed conditions. Overall, the combined use of AMF + *A. chroococcum* under supplemental irrigation conditions enhanced smooth vetch fodder quality and several physiological characteristics in rain-fed conditions by reducing the adverse effects of ROS production and increasing grain yields.

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References

Amirnia R, Ghiyasi M, Siavash Moghaddam S, Rahimi A, Damalas C A & Heydarzadeh S (2019). Nitrogen-fixing soil bacteria plus mycorrhizal fungi improve seed yield and quality traits of lentil (Lens culinaris Medik). *Journal of Soil Science and Plant Nutrition* 19(3): 592-602. doi.org/10.1007/s42729-019-00058-3

Balazadeh M, Zamanian M, Golzardi F & Torkashvand A M (2021). Effects of limited irrigation on forage yield, nutritive value and water use efficiency of Persian clover (Trifolium resupinatum) compared to berseem clover (Trifolium alexandrinum). *Communications in Soil Science and Plant Analysis 52*(16): 1927-1942. doi.org/10.1080/00103624.2021.1900228

Bates L S, Waldren R P & Teare I D (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil 39*(1): 205-207.

Bremner J M (1996). Nitrogen-total. *Methods of Soil Analysis: Part 3 Chemical Methods* 5: 1085-1121. doi.org/10.1080/00103624.2021.1900228

Carter M R & Gregorich E G (2007). *Soil sampling and methods of analysis* CRC press. doi.org/10.1201/9781420005271

Charbonneau E, Chouinard P Y, Allard G, Lapierre H & Pellerin D (2006). Milk from forage as affected by carbohydrate source and degradability with alfalfa silage-based diets. *Journal of Dairy Science* 89(1): 283-293. doi.org/10.3168/jds.S0022-0302(06)72093-8

Day P R (1965). Hydrometer method of particle size analysis. *Methods of Soil Analysis* 9: 562-566.

Dubois M, Gilles K A, Hamilton J K, Rebers P A & Smith F (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* 28(3): 350-356. doi.org/10.1021/ac60111a017

Fouad M O, Essahibi A, Benhiba L & Qaddoury A (2014). Effectiveness of arbuscular mycorrhizal fungi in the protection of olive plants against oxidative stress induced by drought. *Spanish Journal of Agricultural Research* 12(3): 763-771. doi.org/10.5424/sjar/2014123-4815

Habibzadeh Y, Jalilian J, Zardashti M R, Pirzad A & Eini O (2015). Some morpho-physiological characteristics of mung bean mycorrhizal plants under different irrigation regimes in field condition. *Journal of Plant Nutrition* 38(11): 1754-1767. doi.org/10.1080/01904167.2015.1043374

Haffani S, Mezni M, Nasri M B & Chaibi W (2017). Comparative leaf water relations and anatomical responses of three vetch species (Vicia narbonensis L., V. sativa L. and V. villosa Roth.) to cope with water stress. *Crop and Pasture Science* 68(7): 691-702. doi.org/10.1071/CP17029

Heydari S & Pirzad A (2020). Mycorrhizal fungi and *Thiobacillus* co-inoculation improve the physiological indices of Lallemantia iberica under salinity stress. *Current Microbiology* 77(9): 2523-2534. doi.org/10.1007/s00284-020-02034-y

Heydari S & Pirzad A (2021). Improvement of the yield-related response of mycorrhized *Lallemantia iberica* to salinity through sulfur-oxidizing bacteria. *Journal of the Science of Food and Agriculture* 101(9): 3758-3766. doi.org/10.1002/jsfa.11007

Heydarzadeh S, Jalilian J, Pirzad A, Jamei R & Petrussa E (2022). Fodder value and physiological aspects of rainfed smooth vetch affected by biofertilizers and supplementary irrigation in an agri-silviculture system. *Agroforestry Systems* 96(1): 221-232. doi.org/10.1007/s10457-021-00710-x

Horrocks R D & Valentine J F (1999). Harvested forages. Academic Press.

Houba J, Van der Lee J J, Novozamsky I & Walinga I (1988). Soil and plant analysis, a series of syllabi, Part 5, soil analysis procedures. *Wageningen Agricultural University,* Wageningen.

Jafari A, Connolly V, Frolich A & Walsh E J (2003). A note on estimation of quality parameters in perennial ryegrass by near infrared reflectance spectroscopy. *Irish Journal of Agricultural and Food Research* 42(2): 293-299.

Jahansouz M R, Afshar R K, Heidari H & Hashemi M (2014). Evaluation of yield and quality of sorghum and millet as alternative forage crops to corn under normal and deficit irrigation regimes. *Jordan Journal of Agricultural Sciences* 173(3834): 1-17.

Jahanzad E, Jorat M, Moghadam H, Sadeghpour A, Chaichi M R & Dashtaki M (2013). Response of a new and a commonly grown forage sorghum cultivar to limited irrigation and planting density. *Agricultural Water Management* 117:62-69. doi.org10.1016/j.agwat.2012.11.001

Khan A L, Waqas M, Hussain J, Al-Harrasi A & Lee I J (2014). Fungal endophyte *Penicillium janthinellum* LK5 can reduce cadmium toxicity in *Solanum lycopersicum* (Sitiens and Rhe). *Biology and Fertility of Soils* 50(1): 75-85. doi.org/10.1007/s00374-013-0833-3

Li H, Zhu Y, Hu Y, Han W & Gong H (2015). Beneficial effects of silicon in alleviating salinity stress of tomato seedlings grown under sand culture. *Acta Physiologiae Plantarum* 37(4): 1-9. doi.org/10.1007/s11738-015-1818-7

Lichtenthaler H K & Wellburn A R (1983). *Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents*. *Biochem Soc Trans* 11(5): 591-592. doi.org/10.1042/bst0110591

Lithourgidis A S, Vasilakoglou I B, Dhima K V, Dordas C A & Yiakoulaki M D (2006). Forage yield and quality of common vetch mixtures with oat and triticale in two seeding ratios. *Field Crops Research* 99(2-3): 106-113. doi.org/10.1016/j.fcr.2006.03.008

McGonigle T P, Miller M H, Evans D G, Fairchild G L & Swan J A (1990). A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytologist* 115(3): 495-501. doi.org/10.1111/j.1469-8137.1990.tb00476.x

Mohammadi M, Modarres-Sanavy S A M, Pirdashti H, Zand B & Tahmasebi-Sarvestani Z (2019). Arbuscular mycorrhizae alleviate water deficit stress and improve antioxidant response, more than nitrogen fixing bacteria or chemical fertilizer in the evening primrose. *Rhizosphere* 9: 76-89. doi.org/10.1016/j.rhisph.2018.11.008

Okalebo J R, Gathua K W & Woomer P L (2002). Laboratory methods of soil and plant analysis: a working manual second edition. *Sacred Africa* 21: 25-26.

Olsen S R (1954). Estimation of available phosphorus in soils by extraction with sodium bicarbonate 418 (939). US Department of Agriculture.

Phillips J M & Hayman D S (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* 55(1): 158-161. doi.org/10.1016/s0007-1536(70)80110-3

Pirzad A & Mohammadzadeh S (2018). Water use efficiency of three mycorrhizal Lamiaceae species (Lavandula officinalis, Rosmarinus *officinalis* and Thymus vulgaris). *Agricultural Water Management* 204: 1-10. doi.org/10.1016/j.agwat.2018.03.020

Rahimzadeh S & Pirzad A (2017). Arbuscular mycorrhizal fungi and Pseudomonas in reduce drought stress damage in flax (*Linum usitatissimum* L.): a field study. *Mycorrhiza* 27(6): 537-552. doi.org/10.1007/s00572-017-0775-y

Richards L A (1965). Physical condition of water in soil. Methods of Soil Analysis: Part 1 Physical and Mineralogical Properties. *Including Statistics of Measurement and Sampling* 9(1): 128-152. doi.org/10.2134/agronmonogr9.1

Rowell D L (2014). *Soil science:* Methods & applications. *Routledge*. doi.org/10.4324/9781315844855

Saadat B, Pirzad A & Jalilian J (2019). How do AMF-inoculation and supplemental irrigation affect the productivity of rainfed yellow sweet clover in agrisilviculture systems? *Archives of Agronomy and Soil Science* 65(14): 2043-2058. doi.org/10.1080/03650340.2019.1600674

Saadat B, Pirzad A & Jalilian J (2021). Yield-related biochemical response of understory mycorrhizal yellow sweet clover (Melilotus officinalis L.) to drought in agrisilviculture. *Archives of Agronomy and Soil Science* 67(12): 1603-1620. doi.org/10.1080/03650340.2020.1800645

Sohrabi Y, Heidari G, Weisany W, Ghasemi-Golezani K & Mohammadi K (2012a). Changes of antioxidative enzymes, lipid peroxidation and chlorophyll content in chickpea types colonized by different Glomus species under drought stress. *Symbiosis* 56(1): 5-18. doi.org/10.1007/s13199-012-0152-8

Sohrabi Y, Heidari G, Weisany W, Ghasemi-Golezani K & Mohammadi K (2012b). Some physiological responses of chickpea cultivars to arbuscular mycorrhiza under drought stress. *Russian Journal of Plant Physiology* 59(6): 708-716. doi.org/10.1134/S1021443712060143

Tan M & Yolcu H (2021). Current status of forage crops cultivation and strategies for the future in Turkey: A review. *Journal of Agricultural Sciences* 27(2): 114-121. doi.org/10.15832/ankutbd.903732

Walkley A & Black I A (1934). An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. *Soil Science* 37(1): 29-38.

Wani S P & Gopalakrishnan S (2019). Plant growth-promoting microbes for sustainable agriculture. *In Plant growth promoting rhizobacteria (PGPR): Prospects for sustainable agriculture* pp. 19-45.

Zhou Y H, Yu J Q, Huang L F & Nogués S (2004). The relationship between CO_2 assimilation, photosynthetic electron transport and water-water cycle in chill-exposed cucumber leaves under low light and subsequent recovery. *Plant, Cell & Environment* 27(12): 1503-1514. doi.org/10.1111/j.1365-3040.2004.01255.x

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Investigation of Antibacterial and Antifungal Efficacy of Zinc and Silver Nanoparticles Synthesized from *Nasturtium officinale*

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ABSTRACT

Nanoparticles are nano-sized materials that can be widely used in fields such as medicine, pharmacology, and industry. The use of natural and easily available materials in nanoparticle synthesis is preferable for economic reasons. Plants are extremely suitable for the synthesis of nanoparticles due to their wide availability and the large number of components they contain with various properties. For this purpose, silver nanoparticles and zinc nanoparticles (AgNPs and ZnNPs), two different nanoparticles were synthesized from an edible plant, watercress (*Nasturtium officinale*). Scanning electron microscopy, scanning electron microscopy-energy dispersive X-ray, UV-VIS spectroscopy, X-ray diffraction (XRD), and fourier transform infrared spectrophotometer (FTIR) analyses of these nanoparticles were performed. In addition, the antimicrobial effects of these synthesized nanoparticles were determined using the disk diffusion method. The nanoparticles obtained from *Nasturtium officinale* were effective on Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*), Gram-positive bacteria (*Staphylococcus aureus, Streptococcus pyogenes*), and fungi (*Candida albicans*). In particular, AgNPs with broad-spectrum antimicrobial activity were obtained from the watercress. While ZnNPs showed inhibition effects of 49% on *K. pneumoniae*, 51% on *S. aureus*, and 62% on *C. albicans*, AgNPs showed inhibition effects of 93% on *P. aeruginosa*, 87% on *S. aureus*, 81% on *E. coli*, 80% on *C. albicans*, 72% on *K. pneumoniae*, and 56% on *S. pyogenes*. The results show that *Nasturtium officinale* can be used effectively in the production of new biotechnological products, particularly ones with antimicrobial properties.

Keywords: Antimicrobial activity, Biotechnological products, Green synthesis, Medicinal plants, Metallic nanoparticles

1. Introduction

Plants and their secondary metabolites have important applications in medicine and pharmacology due to their antimicrobial, antioxidant, and anti-inflammatory properties (Punetha et al. 2022; Özkan et al. 2023). *Nasturtium officinale* is a plant belonging to the Brassicaceae family and is a perennial dicotyledonous herb that typically lives in water (Barker 2009). *Nasturtium officinale* is considered to be a traditional medicinal plant due to its many beneficial components such as vitamins, bioactive components, and glucosinolates (Gonçalves et al. 2009; Pourhassan-Moghaddam et al. 2014). In addition, *N. officinale* can be used in the treatment of both rhinitis and urinary tract irritations and is also suitable for use in cosmetic products (Klimek-Szczykutowicz et al. 2018). For these reasons, *N. officinale* is a good option for green nanoparticle synthesis.

Nanoparticles are materials that can find uses from medicine to food. Nanotechnology is an interdisciplinary field of research (Natarajan et al. 2010; Ibrahim 2015). Nanoparticle synthesis is typically carried out by various chemical methods that require the use of expensive or harmful solvents (Saini et al. 2013). For this reason, efforts are being made to find new environmentally friendly methods in the synthesis of metal nanoparticles (Hubenthal 2011; Kouvaris et al. 2012). Silver nanoparticles are relatively more important than other metal nanoparticles due to their chemical stability, conductivity, and antibacterial, antifungal, antiviral, and anti-inflammatory properties (Adebayo-Tayo et al. 2022; Khan et al. 2022) and can be used in the food industry, superconducting materials, and cosmetics (Singhal et al. 2011; Ahmed et al. 2016; Khan et al. 2022). In addition, silver nanoparticles are used in textile and medical devices,

water filtration, and the diagnosis and treatment of some diseases (Geraldes et al. 2015; Khan et al. 2022). ZnO and Zn nanoparticles are added to paints, food supplements, batteries, as well as materials such as plastics, ceramics, cement, and rubber due to their electrical and thermal conductivity, stability, and antimicrobial effects (Moezzi et al. 2012; Sturikova et al. 2018). In particular, environmentally friendly green methods created using plants are preferable due to their low costs. For this purpose, studies have been carried out to synthesize more reliable agents by creating green nanoparticles with antimicrobial properties (Yang et al. 2021). In recent years, particularly, the green synthesis of nanoparticles has attracted widespread attention because the material used is both natural and cheap (Moodley et al. 2020). The chemical properties of the synthesized nanoparticles vary depending on the amount, size, and shape. It is known that many of these properties are controlled by the dispersion of nanoparticles (Heilmann 2003; Slistan-Grijalva et al. 2005; Saini et al. 2013). Due to these different properties, nanoparticles can be used both in the diagnosis of diseases and as an antimicrobial agent (Kouvaris et al. 2012; Parveen et al. 2012) in the medical field, as well as in many fields, such as electronics (Shiju & Guliants 2009; Phillips et al. 2011), the chemical industry (Kim et al. 2010; Kouvaris et al. 2012).

Today, the resistance of bacteria to antibiotics used in the treatment of diseases is an important public health problem (Moodley et al. 2020). The inability to control infections due to antibiotic resistance may cause an increase in patient deaths as well as an increase in healthcare costs (Moodley et al. 2020). The antimicrobial properties of plants and nanoparticles derived from plants have been recently investigated (Ozdek et al. 2020; Subramanyam et al. 2021). For this reason, in this study, AgNPs and ZnNPs green nanoparticles from *Nasturtium officinale* were synthesized and their antimicrobial effects were investigated.

2. Material and Methods

2.1. Materials

Watercress collected from its natural environment in the province of Kayseri was used. The authentication of the plant was performed by Prof. Dr. Hasan AKAN at Harran University and stored Harran University with the herbarium number 6363.

Tested microorganisms; *P. aeruginosa* ATCC 9027, *E. coli* ATCC 11229, *S. aureus* ATCC 25923, *C. albicans* ATCC 10231, *K. pneumoniae* ATCC 13883, strains were purchased from Microbiologics. *S. pyogenes* (ATCC 19615) was obtained from the Ankara Refik Saydam Public Health Center Presidency.

Chemicals; Nutrient agar medium, broth medium (Condalab brand), and erythromycin (15 µg) were purchased from Bioanalyse. $AgNO_3$ and $ZnSO_47H_2O$ were purchased from Sigma-Aldrich (Germany).

2.2. Methods

One hundred g of watercress (*Nasturtium officinale*), which was collected from its natural environment and dried, was taken and mixed with 1000 mL of water in a magnetic stirrer heated at 60 °C for four hours and then passed through filter paper. Prepared 100 mL of 1 mM $AgNO_3$ and 0.1 M 200 mL of $ZnSO_4$ solutions were mixed with 400 mL each of *Nasturtium officinale* extract and incubated for 24 hours. The formation of ZnNPs and AgNPs was confirmed by measuring the absorbance at 400-800 nm (Pugazhendhi et al. 2018). Then, after centrifugation at 5000 rpm for 30 minutes, the solid phase was collected in a tube and dried in an oven at 60 °C. SEM, SEM EDX, XRD, and Fourier transform infrared spectrophotometer (FTIR) analyzes of the synthesized nanoparticles were performed. In addition, solutions of AgNPs and ZnNPs nanoparticles were prepared with distilled water at concentrations of 20 mg/mL. Afterwards, its antimicrobial activity was investigated on Gram-positive bacteria (*S. aureus, S. pyogenes*), Gram-negative bacteria (*E. coli, P. aeruginosa, K. pneumoniae*), and fungi (*C. albicans*).

2.3. UV-VIS spectroscopy, SEM, SEM EDX, XRD, and FTIR analysis

Spectroscopic analyzes were performed using a BIOCHROM S70DUAL UV-VIS spectrophotometer between 300-800 nm. SEM and SEM EDX analyzes were performed using a JEOL JSM 6510 scanning electron microscope (SEM) at the 500V-30kV operating range, x5-x300,000 magnification range. An XRD analysis was performed with a Parallel Beam Scan, and Bragg-Brentano Scan, using a D/teX Ultra detector at 40kV. Descriptive analyzes of the bonds in the molecular structure of nanoparticles were performed using a Perkin Elmer Spectrum 100 FT-IR spectrophotometer.

2.4. Antimicrobial activity

The disk diffusion method was used to determine the antimicrobial activity (NCCLS 1997). Microorganisms from fresh culture were incubated at 37 °C to 0.5 Mcfarland turbidity. The absorbance at 625 nm was adjusted to be 0.08-0.10 in the spectrophotometer. Bacteria and fungi were grown in the prepared broth and then transferred to the solid medium. Then, solutions of AgNPs and ZnNPs at 20 mg/mL concentrations were absorbed into sterile discs. Inhibition zone diameters were measured for *E. coli*, *P. aeruginosa*, *S. aureus*, *S. pyogenes,* and *K. pneumoniae* bacteria after 24 hours of incubation at 37 °C, and after 48 hours of incubation at 30 °C for *C. albicans*. The same procedure was repeated for the positive control erythromycin (15 µg) and the negative control distilled water.

2.5. Statistical analysis

All measurements were made in triplicate and the results are given as mean values ± standard deviations. The antimicrobial activity of AgNPs and ZnNPs nanoparticles was investigated by One-Way ANOVA statistical analysis.. The results showed significant antimicrobial activity. The significance between groups was $p<0.05$.

3. Results and Discussion

3.1. UV-VIS analysis of AgNPs synthesized from Nasturtium officinale

The UV-VIS spectroscopy plot of AgNPs is given in Figure 1.

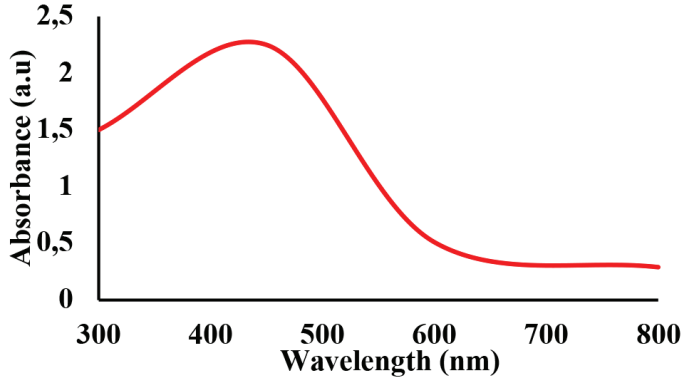


Figure 1- UV-VIS measurement of silver nanoparticle

AgNPs peaked between 400 nm and 800 nm. Both color change and UV-VIS results show that AgNPs were synthesized (Pugazhendhi et al. 2018).

3.2. SEM and SEM EDX analysis of AgNPs synthesized from Nasturtium officinale

The SEM images of AgNPs synthesized from *Nasturtium officinale* are given in Figure 2. The SEM images of AgNPs showed small shapes between 1 µm and 10 µm.

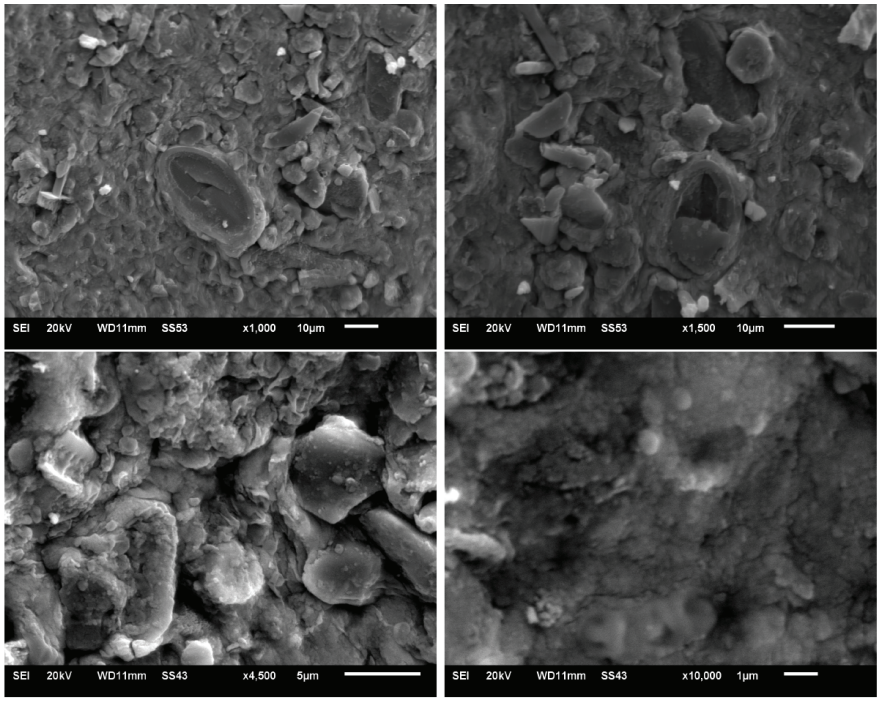
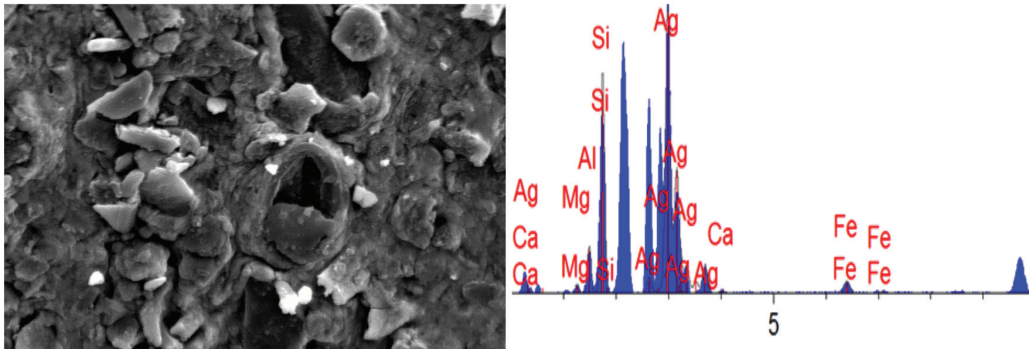


Figure 2- SEM images of AgNPs

The SEM EDX images and a graph of AgNPs are given in Figure 3. The SEM EDX images of AgNPs gave an image similar to small pebbles of different sizes.



Elt.	Line	Intensity (c/s)	Error 2-sig	Conc	Units
Mg	Ka	21.51	2.693	0.767	wt.%
Al	Ka	114.94	3.877	3.388	wt.%
Si	Ka	563.51	6.857	14.667	wt.%
Ca	Ka	84.54	3.866	2.791	wt.%
Fe	Ka	50.13	3.337	2.327	wt.%
Ag	La	901.11	8.940	76.061	wt.%
				100.000	wt.%

Figure 3- SEM EDX image and graph of AgNPs

3.3. XRD analysis of the AgNPs synthesized from Nasturtium officinale

Scanning angle 3-90, scanning speed 10°/min, 2θ (2 theta) versus intensity spectrum graph is given in Figure 4. The 2θ angle was found to be 32.312, 46.30, 67.34, and 76.80. The crystal structure of silver nanoparticles was approved through an XRD analysis as shown in Figure 4. Also, in addition to the Bragg peaks, additional peaks were monitored at 27.83, 54.86, and 57.55. These peaks are the result of organic compounds in charge of the reduction of silver ions (Roopan et al. 2013). The resulting XRD pattern is consistent with previous studies (Kumar et al. 2014; Ibrahim 2015).

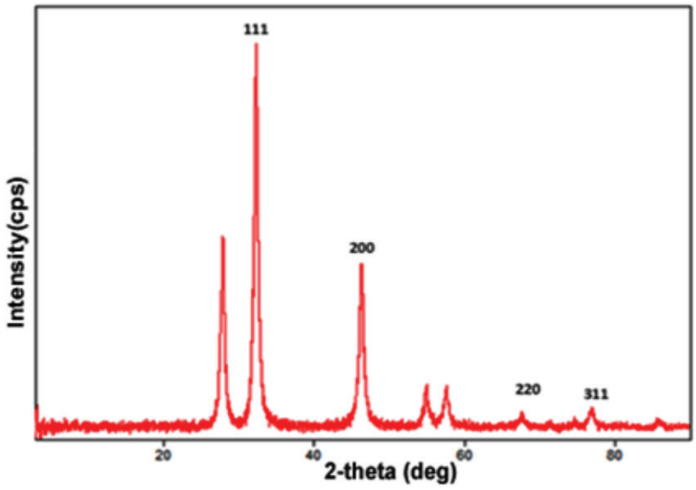


Figure 4- XRD analysis result of AgNPs synthesized from *Nasturtium officinale*
cps: Counts per second, 2-theta: Diffraction angle

3.4. FTIR analysis of the AgNPs synthesized from Nasturtium officinale

The FTIR results of AgNPs nanoparticle gave peaks between 3672-668 (Figure 5). The bands 3672.41, 3187.13, 2926.72, 2323.27, 1995.70, 1761.37, 1633.62, 1473.30, 1398.82, 1130.05, 1020.40, 782.08, 668.78 cm^{-1} vibrated. The vibration of the nanoparticle at 3672 is of the O-H bond, at 3317 of the C-H bond, at 2323.27 the vibration of the C≡C- bond or C≡N bond, the 1995-1633 bands of the C=O bond, at 1130 band of the C-O bond and 782 band of C-H bond (Ciursă et al. 2021; Wang et al. 2022). The FTIR spectra of silver nanoparticles synthesized using *Nasturtium officinale* showed distinct peaks representing functional groups responsible for Ag^{+} reduction. These functional groups can bind with reduced Ag metal to stabilize nanoparticles (Adebayo-Tayo et al. 2022).

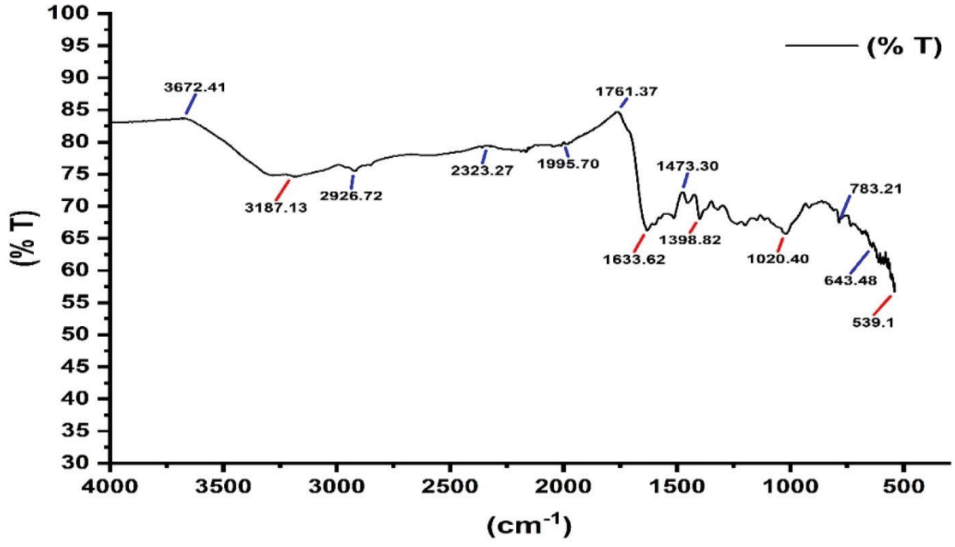


Figure 5- FTIR result of AgNPs
% T: % Transmittance, cm^{-1}: Wavelength

3.5. UV-VIS analysis of ZnNPs synthesized from Nasturtium officinale

The UV-VIS spectroscopy plot of ZnNPs is given in Figure 6.

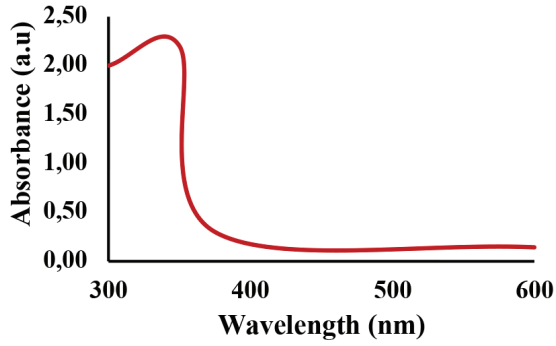


Figure 6- UV-VIS measurement results of zinc nanoparticles

3.6. SEM and SEM EDX analysis of ZnNPs synthesized from Nasturtium officinale

The SEM images of ZnNPs synthesized from *Nasturtium officinale* are given in Figure 7.

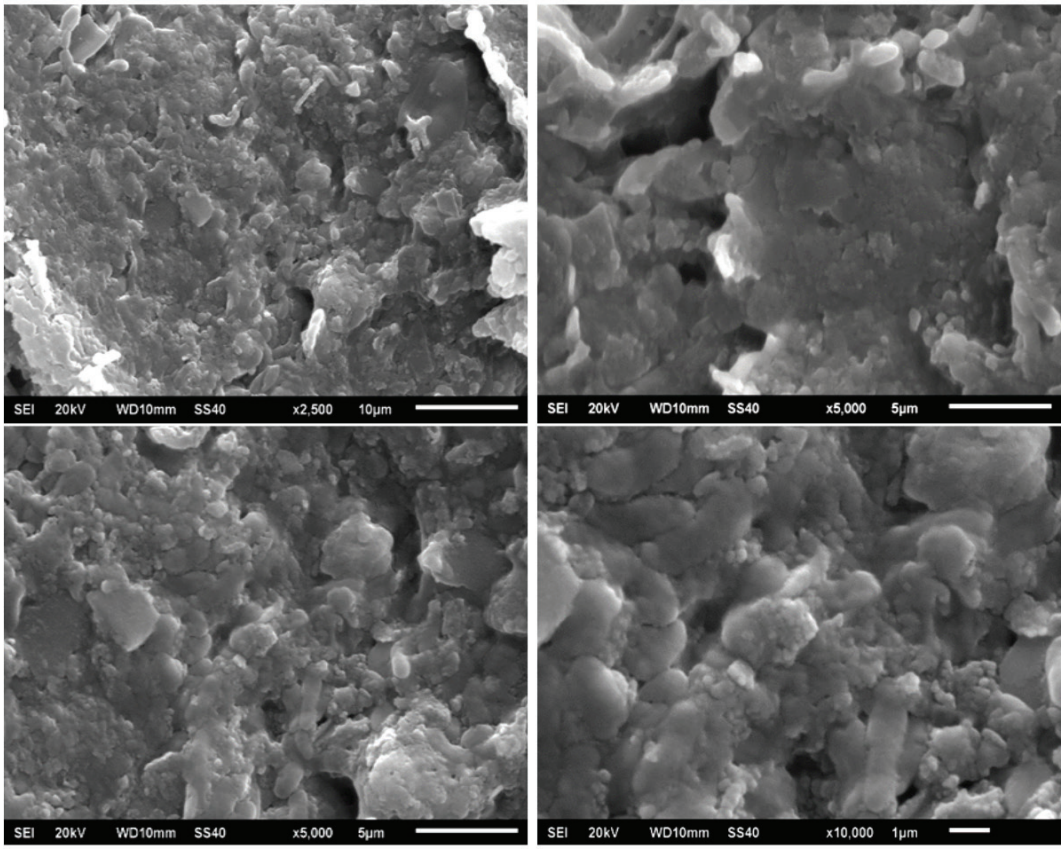
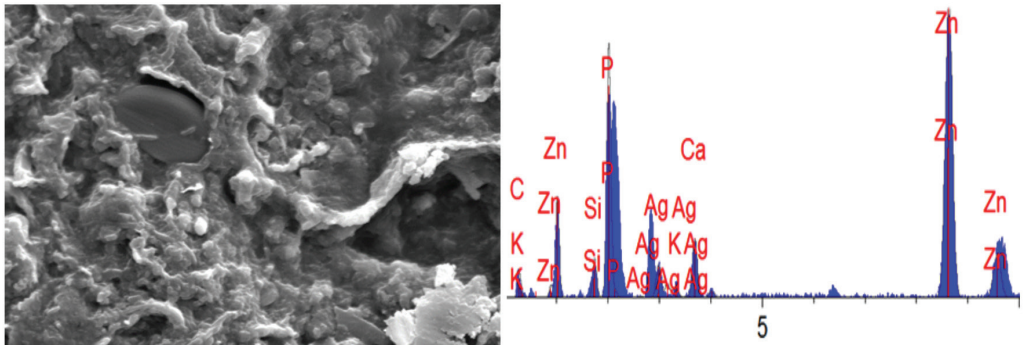


Figure 7- SEM images of ZnNPs

The SEM EDX images and a graph of ZnNPs synthesized from *Nasturtium officinale* are given in Figure 8.



Elt.	Line	Intensity (c/s)	Error 2-sig	Conc	Units
C	Ka	32.87	3.460	10.039	wt.%
Si	Ka	60.38	5.445	1.941	wt.%
P	Ka	408.63	10.059	11.767	wt.%
K	Ka	15.22	4.925	0.352	wt.%
Ca	Ka	114.98	6.361	2.689	wt.%
Zn	Ka	847.70	13.667	68.344	wt.%
Ag	La	61.32	5.770	4.868	wt.%
				100.000	wt.%

Figure 8- SEM EDX image and graph of ZnNPs

When the SEM and SEM EDX images of the synthesized ZnNPs are examined, it is seen that the nanoparticles form bubble-like shapes.

3.7. XRD analysis of the ZnNPs synthesized from Nasturtium officinale

The XRD result of ZnNPs synthesized from *Nasturtium officinale* is given in Figure 9.

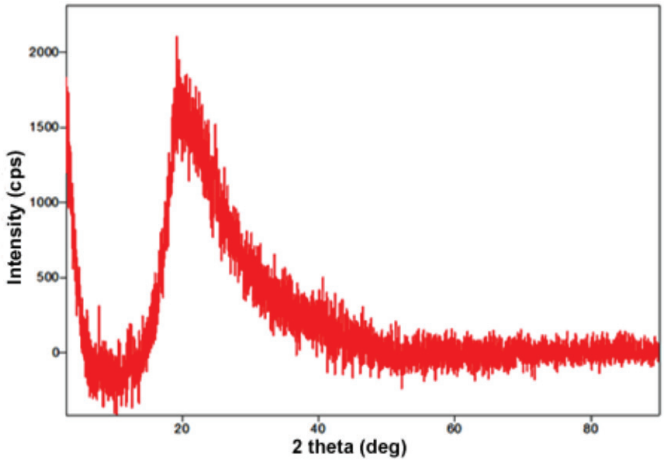


Figure 9- XRD analysis result of synthesized ZnNPs
cps: counts per second, 2-theta: diffraction angle

When the XRD analysis result of synthesized ZnNPS was examined, it was seen that composite material was synthesized in nanostructure (Rangelova et al. 2022).

3.8. FTIR analysis of the ZnNPs synthesized from Nasturtium officinale

When the FTIR result of ZnNPs was examined, it was observed that the nanoparticle gave a peak between 3275-628 (Figure 10). It gave peaks for many identifiable organic compounds. The bands 3275-2924 indicate the presence of a C-H bond, the band 2146 the presence of C≡C bond or C≡N bond, the band 1830 the presence of a C=O bond, the 1622-1579-1480 bands the presence of a C=C bond (double bonds), the 1225 band the presence of C-O bond. In addition, the 1013 band indicates the presence of the C-O bond, and the presence of the 838 band indicates the presence of the C-H bond (Hosseini et al. 2020; Ciursă et al. 2021; Wang et al. 2022).

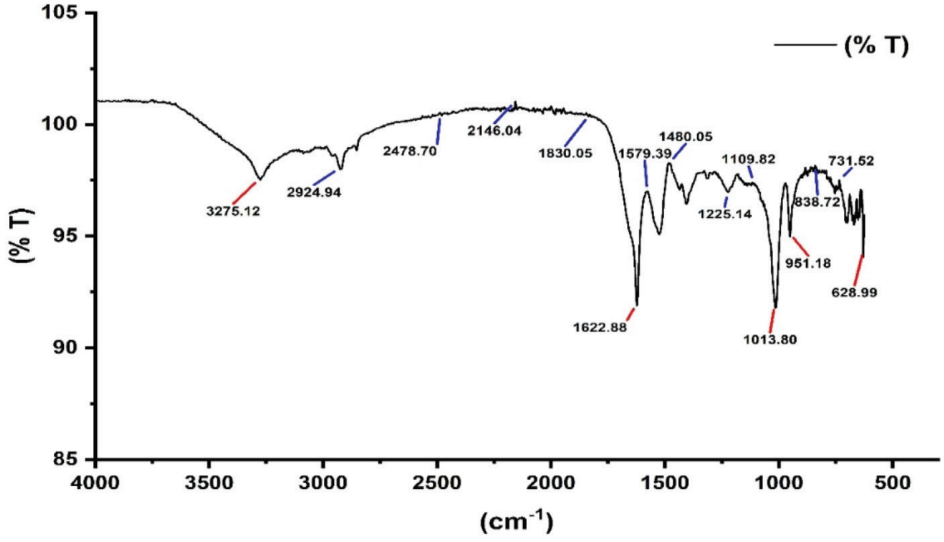


Figure 10- FTIR result of ZnNPs
% T: % Transmittance, cm^{-1}: Wavelength

3.9. Antimicrobial effects of AgNPs and ZnNPs nanoparticles

The antimicrobial analysis results of the prepared nanoparticles and the erythromycin antibiotic are given in Table 1 and Table 2.

Table 1- Antimicrobial activity of AgNPs and ZnNPs nanoparticles

Microorganisms	*Erythromycin (15 µg)*	*AgNPs (20 mg/mL)*	*ZnNPs (20 mg/mL)*
K. pneumoniae	16.43±0.02	11.9±1.3	8.14±1.07
E. coli	14.34±1.4	11.62±0.9	-
S. aureus	18.98±0.5	16.52±2.4	9.82±1.2
P. aeruginosa	12.63±0.02	11.18±0.7	-
S. pyogenes	20.7±0.1	11.64±1.5	-
C. albicans	15.20±1.1	12.18±0.3	9.55±0.42

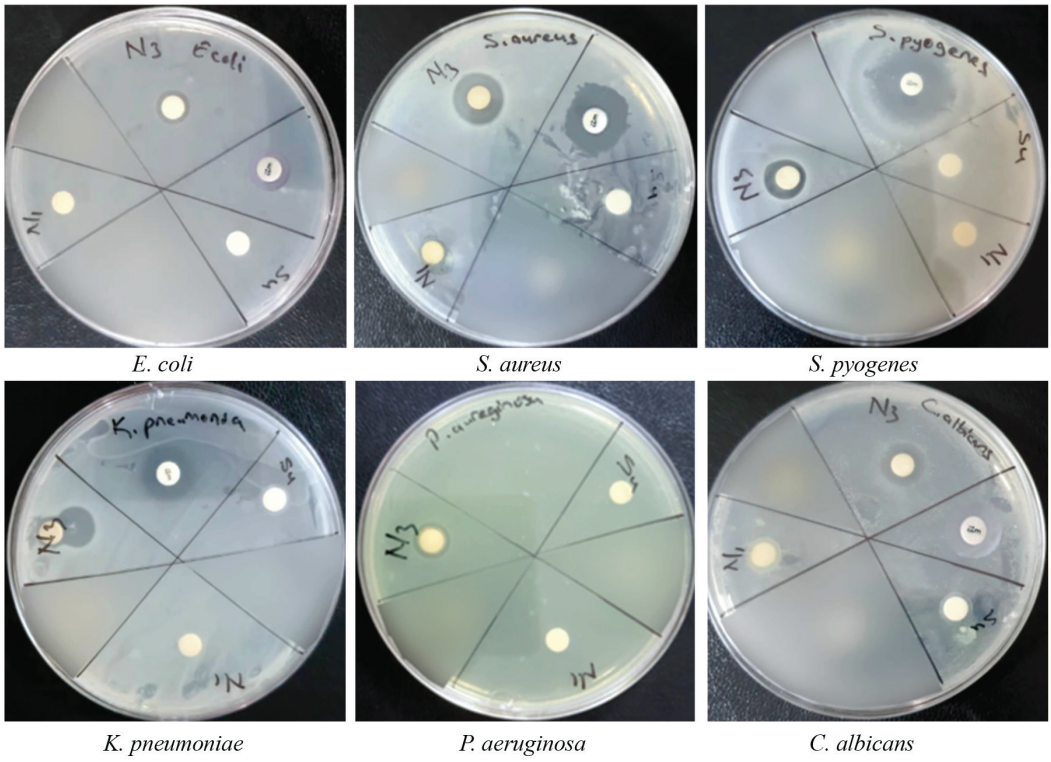
Inhibition zone diameter ± standard deviation (mm)

Table 2- Bacteria and fungus % inhibition values of AgNPs and ZnNPs according to erythromycin antibiotic

Microorganisms	*% inhibition for AgNPs*	*% inhibition for ZnNPs*
K. pneumoniae	72.43	49.54
E. coli	81.03	
S. aureus	87.04	51.74
P. aeruginosa	93.98	
S. pyogenes	56.23	
C. albicans	80.13	62.83

According to the results, watercress nanoparticles synthesized with Zn and Ag have antimicrobial properties.

In experiments investigating the antimicrobial activities of different nanoparticles prepared with watercress, a marsh plant, AgNPs showed the most effective antimicrobial activity (Figure 11). Silver is a non-toxic substance capable of killing many disease-causing microorganisms (Jeong et al. 2005; Ibrahim 2015). Due to the antimicrobial properties they exhibit, it is predicted that silver nanoparticles can be widely used as antimicrobial agents in the future (Rai et al. 2009; Ibrahim 2015). AgNPs synthesized from watercress was highly effective on both gram-negative bacteria (*E. coli, K. pneumoniae, P. aeruginosa*) and Gram-positive bacteria (*S. aureus, S. pyogenes*) as well as fungi (*C. albicans*). The strong antimicrobial effect of synthesized AgNPs showed that *Nasturtium officinale* could be a desirable choice for the synthesis of antimicrobial nanoparticles and biotechnological products. Zn, which is an important mineral in terms of health that participates in the structure of many important enzymes, is one of the prominent minerals in nanoparticle synthesis. The ZnNPs nanoparticle showed an inhibition effect on *K. pneumoniae* and *S. aureus* bacteria and fungi (*C. albicans*). However, it was observed that the antimicrobial effect of ZnNPs synthesized from *Nasturtium officinale* was less than that of AgNPs (Figures 11, 12). The effect of silver and zinc nanoparticles, synthesized with *Nasturtium officinale* on *S. aureus* or *S. aureus* and *E. coli,* respectively, have previously been studied (Sadeghi 2014; Bayrami et al. 2019). However, in this study, the effects on 6 microorganisms were examined and gave very effective results which show that *Nasturtium officinale* is highly effective in the synthesis of antimicrobial products.



E. coli *S. aureus* *S. pyogenes*

K. pneumoniae *P. aeruginosa* *C. albicans*

Figure 11- Antimicrobial effects of AgNPs and ZnNPs nanoparticles (N_1= ZnNPs, N_3= AgNPs, E15: Erythromycin)

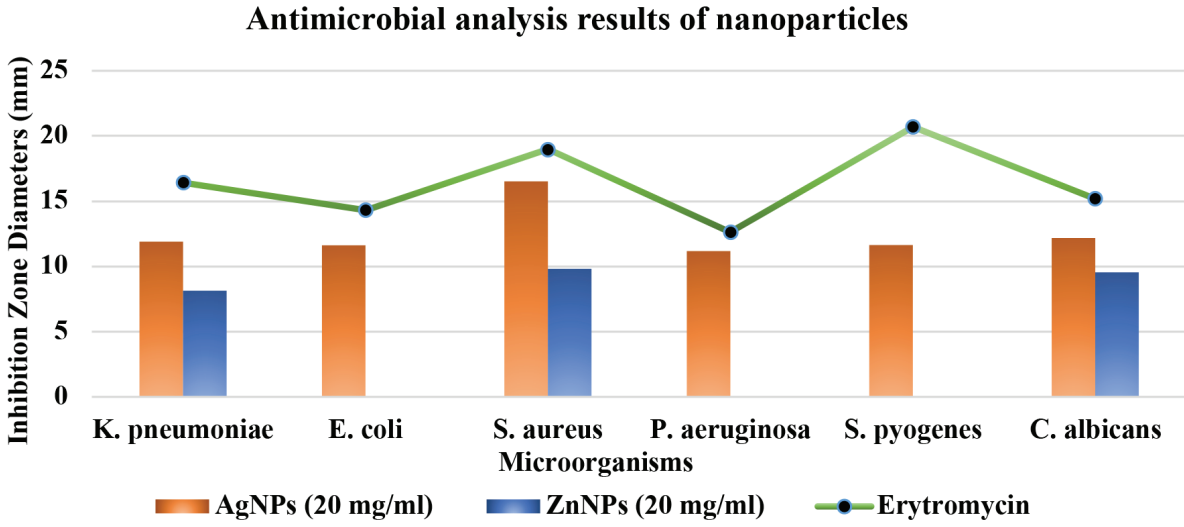


Figure 12- Antimicrobial activity graph of AgNPs, ZnNPs, and erythromycin (mm)
X: Inhibition zone diameters (mm), Y: Bacteria, fungi, and nanoparticles

4. Conclusions

It has been shown that plants that grow spontaneously in nature can be used efficiently in the synthesis of antimicrobial agents. Silver nanoparticles showed a high antimicrobial effect. *Nasturtium officinale* is an extremely suitable plant for the synthesis of nanoparticles with antimicrobial properties. In addition to being natural and inexpensive, antimicrobial agents that are easy to synthesize can have broad-spectrum antimicrobial effects.

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References

Adebayo-Tayo B C, Borode S O & Alao S O (2022). In-Vitro Antibacterial and Antifungal Efficacy of Greenly Fabricated Senna alata Leaf Extract Silver Nanoparticles and Silver Nanoparticle-Cream Blend. *Periodica Polytechnica Chemical Engineering* 66(2): 248-260. doi.org/10.3311/PPCH.18271

Ahmed S, Ahmad M, Swami B L & Ikram S (2016). A review on plants extract mediated synthesis of silver nanoparticles for antimicrobial applications: A green expertise. *Journal of Advanced Research* 7(1): 17-28. doi.org/10.1016/J.JARE.2015.02.007

Barker D J (2009). Pacific Northwest Aquatic Invasive Species Profile: Nasturtium officinale (Watercress). *FSH* 423.

Bayrami A, Ghorbani E, Rahim Pouran S, Habibi-Yangjeh A, Khataee A & Bayrami M (2019). Enriched zinc oxide nanoparticles by Nasturtium officinale leaf extract: Joint ultrasound-microwave-facilitated synthesis, characterization, and implementation for diabetes control and bacterial inhibition. *Ultrasonics Sonochemistry* 58: 104613. doi.org/10.1016/J.ULTSONCH.2019.104613

Ciursă P, Pauliuc D, Dranca F, Ropciuc S & Oroian M (2021). Detection of honey adulterated with agave, corn, inverted sugar, maple and rice syrups using FTIR analysis. *Food Control* 130: 108266. doi.org/10.1016/J.FOODCONT.2021.108266

Geraldes A N, da Silva D F, E Silva L G D A, Spinacé E V, Neto A O & dos Santos M C (2015). Binary and ternary palladium based electrocatalysts for alkaline direct glycerol fuel cell. *Journal of Power Sources* 293: 823-830. doi.org/10.1016/J.JPOWSOUR.2015.06.010

Gonçalves E M, Cruz R M S, Abreu M, Brandão T R S & Silva C L M (2009). Biochemical and colour changes of watercress (Nasturtium officinale R. Br.) during freezing and frozen storage. *Journal of Food Engineering* 93(1): 32-39. doi.org/10.1016/J.JFOODENG.2008.12.027

Heilmann A (2003). Polymer Films with Embedded Metal Nanoparticles. Springer Series in Materials Science 52. doi.org/10.1007/978-3-662-05233-4

Hosseini M A, Malekie S & Ebrahimi N (2020). The analysis of linear dose-responses in gamma-irradiated graphene oxide: Can FTIR analysis be considered a novel approach to examining the linear dose-responses in carbon nanostructures?. Radiation Physics and Chemistry 176: 109067. doi.org/10.1016/J.RADPHYSCHEM.2020.109067

Hubenthal F (2011). Noble Metal Nanoparticles: Synthesis and Optical Properties. *In Comprehensive Nanoscience and Technology* 1: 375-435. doi.org/10.1016/B978-0-12-374396-1.00034-9

Ibrahim H M M (2015). Green synthesis and characterization of silver nanoparticles using banana peel extract and their antimicrobial activity against representative microorganisms. *Journal of Radiation Research and Applied Sciences* 8(3): 265-275. doi.org/10.1016/J.JRRAS.2015.01.007

Jeong S H, Yeo S Y & Yi S C (2005). The effect of filler particle size on the antibacterial properties of compounded polymer/silver fibers. *Journal of Materials Science* 40(20): 5407-5411. doi.org/10.1007/S10853-005-4339-8

Khan S, Almarhoon Z M, Bakht J, Mabkhot Y N, Rauf A, & Shad A A (2022). Single-Step Acer pentapomicum-Mediated Green Synthesis of Silver Nanoparticles and Their Potential Antimicrobial and Antioxidant Activities. Journal of Nanomaterials 2022. https://doi.org/10.1155/2022/3783420

Kim K, Jung B, Kim J & Kim W (2010). Effects of embedding non-absorbing nanoparticles in organic photovoltaics on power conversion efficiency. *Solar Energy Materials and Solar Cells* 94(10): 1835-1839. doi.org/10.1016/J.SOLMAT.2010.05.049

Klimek-Szczykutowicz M, Szopa A & Ekiert H (2018). Chemical composition, traditional and professional use in medicine, application in environmental protection, position in food and cosmetics industries, and biotechnological studies of Nasturtium officinale (watercress) - a review. *Fitoterapia* 129: 283-292. doi.org/10.1016/J.FITOTE.2018.05.031

Kouvaris P, Delimitis A, Zaspalis V, Papadopoulos D, Tsipas S A & Michailidis N (2012). Green synthesis and characterization of silver nanoparticles produced using Arbutus Unedo leaf extract. *Materials Letters* 76: 18-20. doi.org/10.1016/J.MATLET.2012.02.025

Kumar D A, Palanichamy V & Roopan S M (2014). Green synthesis of silver nanoparticles using Alternanthera dentata leaf extract at room temperature and their antimicrobial activity. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 127: 168-171. doi.org/10.1016/J.SAA.2014.02.058

Moezzi A, McDonagh A M & Cortie M B (2012). Zinc oxide particles: Synthesis, properties and applications. *Chemical Engineering Journal* 185-186: 1-22. doi.org/10.1016/J.CEJ.2012.01.076

Moezzi S, Almarhoon Z M, Bakht J, Mabkhot Y N, Rauf A & Shad A A (2022). Single-Step Acer pentapomicum-Mediated Green Synthesis of Silver Nanoparticles and Their Potential Antimicrobial and Antioxidant Activities. *Journal of Nanomaterials* 2022: 10. doi.org/10.1155/2022/3783420

Moodley J S, Krishna S B N, Pillay K & Govender P (2020). Green Synthesis of Metal Nanoparticles for Antimicrobial Activity. *Novel Nanomaterials.* doi.org/10.5772/INTECHOPEN.94348

Natarajan K, Selvaraj S & Ramachandramurty V (2010). Microbial Production of Silver Nanoparticles. *Digest Journal of Nanomaterials and Biostructures* 5: 135-140.

NSSLS (1997). NCCLS Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standard Enclose -A 7 (April 1997 ed.). NCCLS.

Ozdek U, Seçkin H & Çibuk S (2020). Investigation of Antimicrobial Effects of Amygdalus Trichamygdalus (Sweet Almond) and Amygdalus nana L. (Bitter Almond) Plants. *Van Veterinary Journal* 31(1): 22-26. doi.org/10.36483/VANVETJ.651515

Özkan K, Karadağ A & Sağdıç O (2023). Evaluation of the Antioxidant Capacity, Antimicrobial Effect, and In Vitro Digestion Process of Bioactive Compounds of Cherry Laurel Leaves Extracts. *Journal of Agricultural Sciences* 29(1): 352-361. doi.org/10.15832/ANKUTBD.1029306

Parveen S, Misra R & Sahoo S K (2012). Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. *Nanomedicine: Nanotechnology, Biology and Medicine* 8(2): 147-166. doi.org/10.1016/J.NANO.2011.05.016

Phillips J, Bowen W, Cagin E & Wang W (2011). Electronic and Optoelectronic Devices Based on Semiconducting Zinc Oxide. *Comprehensive Semiconductor Science and Technology* 6: 101-127. doi.org/10.1016/B978-0-44-453153-7.00025-0

Pourhassan-Moghaddam M, Zarghami N, Mohsenifar A, Rahmati-Yamchi M, Gholizadeh D, Akbarzadeh A, de La Guardia M & Nejati-Koshki K (2014). Watercress-based gold nanoparticles: biosynthesis, mechanism of formation and study of their biocompatibility in vitro. *Micro & Nano Letters* 9(5): 345-350. doi.org/10.1049/MNL.2014.0063

Pugazhendhi S, Palanisamy P K & Jayavel R (2018). Synthesis of highly stable silver nanoparticles through a novel green method using Mirabillis jalapa for antibacterial, nonlinear optical applications. *Optical Materials* 79: 457-463. doi.org/10.1016/J.OPTMAT.2018.04.017

Punetha A, Kumar D, Suryavanshi P, Chandra Padalıa R & Katanapalya Thimmaiah V (2022). Environmental Abiotic Stress and Secondary Metabolites Production in Medicinal Plants: A. Review. *Journal of Agricultural Sciences* 2022(3): 351-362. doi.org/10.15832/ankutbd.999117

Rai M, Yadav A & Gade A (2009). Silver nanoparticles as a new generation of antimicrobials. *Biotechnology Advances* 27(1): 76-83. doi.org/10.1016/J.BIOTECHADV.2008.09.002

Rangelova N, Aleksandrov L & Yaneva S (2022). Synthesis and structure of amorphous SiO2/ZnO composites with potential application for azo dye degradation. *Materials Today: Proceedings* 61(4): 1272-1279. doi.org/10.1016/J.MATPR.2022.03.064

Roopan S M, Rohit, Madhumitha G, Rahuman A A, Kamaraj C, Bharathi A & Surendra T V (2013). Low-cost and eco-friendly phyto-synthesis of silver nanoparticles using Cocos nucifera coir extract and its larvicidal activity. *Industrial Crops and Products* 43(1): 631-635. doi.org/10.1016/J.INDCROP.2012.08.013

Sadeghi B (2014). Synthesis of silver nanoparticles using leaves aqueous extract of Nasturtium Officinale (NO) and its antibacterial activity. International Journal of Molecular and Clinical Microbiology 4: 428-434.

Saini I, Rozra J, Chandak N, Aggarwal S, Sharma P K & Sharma A (2013). Tailoring of electrical, optical and structural properties of PVA by addition of Ag nanoparticles. *Materials Chemistry and Physics* 139(2-3): 802-810. doi.org/10.1016/J.MATCHEMPHYS.2013.02.035

Shiju N R & Guliants V V (2009). Recent developments in catalysis using nanostructured materials. *Applied Catalysis A: General* 356(1): 1-17. doi.org/10.1016/J.APCATA.2008.11.034

Singhal G, Bhavesh R, Kasariya K, Sharma A R & Singh R P (2011). Biosynthesis of silver nanoparticles using Ocimum sanctum (Tulsi) leaf extract and screening its antimicrobial activity. Journal of Nanoparticle Research 13(7): 2981-2988. doi.org/10.1007/S11051-010-0193-Y/TABLES/1

Slistan-Grijalva A, Herrera-Urbina R, Rivas-Silva J F, Ávalos-Borja M, Castillón-Barraza F F & Posada-Amarillas A (2005). Classical theoretical characterization of the surface plasmon absorption band for silver spherical nanoparticles suspended in water and ethylene glycol. *Physica E: Low-Dimensional Systems and Nanostructures* 27(1-2): 104-112. doi.org/10.1016/j.physe.2004.10.014

Sturikova H, Krystofova O, Huska D & Adam V (2018). Zinc, zinc nanoparticles and plants. *Journal of Hazardous Materials* 349: 101-110. doi.org/10.1016/J.JHAZMAT.2018.01.040

Subramanyam G K, Gaddam S A, Kotakadi V S, Palithya S, Penchalaneni J & Challagundla V N (2021). Argyreia nervosa (Samudra pala) leaf extract mediated silver nanoparticles and evaluation of their antioxidant, antibacterial activity, in vitro anticancer and apoptotic studies in KB oral cancer cell lines. Artificial Cells, Nanomedicine, and Biotechnology 49(1): 635-650. doi.org/10.1080/21691401.2021.1996384

Wang Z, Liu Z, Gao Z, Li X, Eling B, Pöselt E, Schander E & Wang Z (2022). Structure transition of aliphatic m,6-Polyurethane during heating investigated using in-situ WAXS, SAXS, and FTIR. *Polymer* 254: 125072. doi.org/10.1016/J.POLYMER.2022.125072

Yang X, Chung E, Johnston I, Ren G & Cheong Y K (2021). Exploitation of Antimicrobial Nanoparticles and Their Applications in Biomedical Engineering. *Applied Sciences* 11(10): 4520. doi.org/10.3390/APP11104520

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Preparation of Plant-derived Smoke for Stimulating Seed Germination and Quantification of Karrikins Using High Performance Liquid Chromatography

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ABSTRACT

Smoke water (SW) is produced naturally or artificially from burning plant material. It provides the germination of the seeds of many plants and accelerates the growth and development of the plant and is also used in many fields of plant science. SW preparation is a relatively easy and inexpensive method, but a standard method for its preparation has yet to be developed. For this reason, our research aims to develop a low-cost efficient method to produce SW, to standardize it and to measure the amount of the main active biomolecule karrikin (KAR_1) by HPLC. We also aimed to test and compare the best working concentration of SW and commercially available KAR_1 on apricot (*Prunus armeniaca* L.) seeds. The SWs were diluted to 1:100, 1:500, 1:1000, 1:5000 and 1:10000 ratios and KAR_1 to 0.01 µM, 0.1 µM, 1 µM, 5 µM and 10 µM concentrations. In terms of germination, it was determined that the use of 1:1000 (60%) concentration in the SW group and 1 µM (72%) concentration in the KAR_1 group was appropriate. This is the first research in which a standard method was developed for obtaining SW. We believe this study will be a guide to researchers who study with SW, since we obtained the most concentrated KAR_1 according to the literature.

Keywords: Smoke water, KAR_1, Karrikinolide, *Prunus armeniaca*, Plant growth regulator

1. Introduction

Mediterranean-type ecosystems in the world are located on the Pacific coasts of Chile and California, in the western and southern parts of Australia, in the Cape region of South Africa and in the Mediterranean basin (Türkan et al. 1985; Beeby & Brennan 1997). Since fire is a phenomenon that shapes vegetation in Mediterranean-type ecosystems, it has a significant importance in the evolution of plants that spread in these ecosystems, and as a result of natural selection, they have developed some adaptation mechanisms to survive. These adaptation mechanisms are resistant tree bark formation, re-shooting after fire, bradispory that holding the seeds such as inside cones and fruits and releasing the seeds after, fire-induced flowering, easy flammability, early reproductive initiation, and fire-induced germination (Tavşanoğlu & Gürkan 2004). Germination induced by fire occurs in two ways. In species with fire-induced germination, dormancy is provided by the seed coat (testa), which prevents the exchange of water and gases. Temperature shock cracks or melts this hard outer cover (testa), allowing water to pass through and germination is occurred (Christensen 1985; Keeley 1995). The second occurs chemically by the presence of burnt wood in the environment and by means of smoke (Keeley et al. 1985; Keeley & Pizzorno 1986; Keeley & Fotheringham 1997; Keeley & Fotheringham 1998). It has been reported by De Lange & Boucher (1990) that plant-derived smoke promotes seed germination more than temperature. Studies have shown that plant-derived smoke positively affects the seed germination of 1200 plant species from 80 different genera, including Arabidopsis (Chiwocha et al. 2009). It has been found that not only the smoke generated as a result of forest fires promotes seed germination, but also the smoke produced under laboratory conditions promotes seed germination. It has been observed that the smoke obtained by burning the *Themeda triandra* Forssk. plant promotes germination in dormant seeds (Baxter et al. 1994). When dry or wet plant material is burned (active substances

are formed at 160-200 °C), water-soluble volatile compounds that are formed evaporate at high temperatures, and when dissolved in water, they promote the germination of seeds of many species. Besides this germination effect, smoke water (SW) also promotes seedling growth, shoot branching, root formation, flowering, and tolerance in situations of abiotic stress (Brown & Van Staden 1997; De Cuyper et al. 2017). Seventy one compounds have been identified in the active part of plant-derived smoke (Baldwin et al. 1994) from these compounds, butenolides, nitrogen oxides and cyanohydrins were found to have germination- promoting properties (Nelson et al. 2012). These compounds are water-soluble, can maintain their structure for a long time, are heat-resistant and have high activity at low concentrations (Baldwin et al. 1994; Van Staden et al. 2000). Flematti et al. (2004), separated the SW into fractions by liquid chromatography and used each fraction for seed germination test and thus determined the active compound in the SW. This compound is a special type of lactone with the systematic name 3-methyl-2H-furo[2,3-c]pyran-2-one containing only C, H and O, and because of this property, it resembles strigolactones (Flematti et al. 2015). This compound is a substance belonging to the group of karrikins (KAR) in chemical structure and was named karrikinolide. KAR are abbreviated as KAR and are numbered according to their identification in smoke (Figure 1). Six KAR have been discovered so far. These have been named KAR_1, KAR_2, KAR_3, KAR_4, KAR_5 and KAR_6, but KAR_1 is generally the most abundant in smoke and most active in seed germination (Chiwocha et al. 2009; Nelson et al. 2012; Flematti et al. 2015; De Cuyper et al. 2017). There are many studies that SW and KAR germinate seeds, and all studies show that these substances increase the germination rate (Baxter & Van Staden 1994; Tavşanoğlu 2011; Çatav et al. 2012; Chumpookam et al. 2012; Çatav et al. 2014; Kazancı 2014; Kochanek et al. 2016; Tavsanoğlu et al. 2017; Çatav et al. 2018a). KAR stimulate the formation of a new flora in the burned area by promoting the germination of dormant seed by breaking dormancy, and it has also been reported as a result of various studies that it stimulates the germination of parasitic plants such as Striga and Orobanche (De Cuyper et al. 2017).

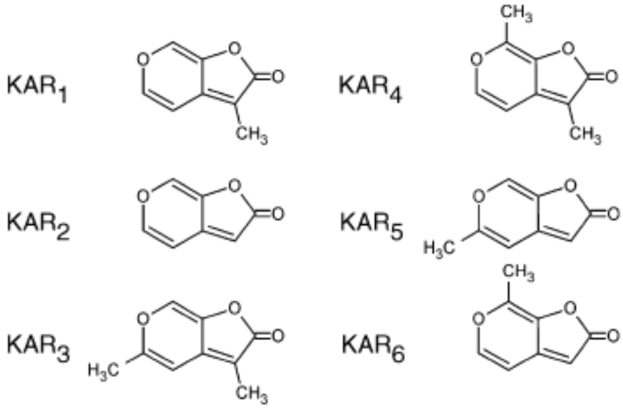

Figure 1- The karrikin family occurring in smoke water (Hrdlička et al. 2019)

Plant-derived smoke compounds cause many changes in seeds, from changes in seed sensitivity to phytohormones and light requirements, testa morphology and permeability properties (Chiwocha et al. 2009). Since KAR_1 stimulates the germination of many species and acts at very low concentrations (<1 ppb or 1 nM), it is hypothesized that it may act by affecting the production or metabolism of other phytohormones. The phytohormones gibberellic acid (GA) and abscisic acid (ABA) are widely accepted as essential endogenous regulators, playing mostly antagonistic roles in plant growth processes and environmental responses. Auxin, one of the phytohormones, is effective in elongation, cell, and tissue differentiation in plants. Of the natural auxins, indole-3-acetic acid (IAA) is the richest auxin in plants and the only endogenous molecule that directly activates auxin signals (Xu et al. 2021). The signaling pathway of protein degradation from phytohormones (GA, IAA and ABA) mainly includes the phytohormone receptor (GID1 for GA, TIR1/AFB for IAA and PYR/PYL/PCAR for ABA), F-box protein (SLY/GID2 for GA, TIR1 for IAA and PP2Cs for ABA), transcription repressor protein (DELLA for GA, AUX/IAA for IAA and SnRK2s for ABA), and transcription factor (GAMYB for GA, ARF for IAA and TFs for ABA). Transcription repressor proteins can interact with various transcription factors and change their activity. When the receptor detects and binds to phytohormones (GA, IAA, and ABA), its structure changes. The N-terminal of the receptor wraps the phytohormone and interacts with the transcription repressor protein. Then, the phytohormone-receptor-transcription repressor protein complex binds to the F-box protein and is subsequently degraded by ubiquitination of the transcription repressor protein (leading to disinhibition of transcription repressor protein and activating phytohormones response genes). The released transcriptional factors then mediate the expression of genes (*EXP2* for GA, *IAA1* for IAA and *ABI3* for ABA) that cause the physiological and morphological responses of seeds or plants to KARs (Figure 2) (Sirko et al. 2021, Xu et al. 2021).

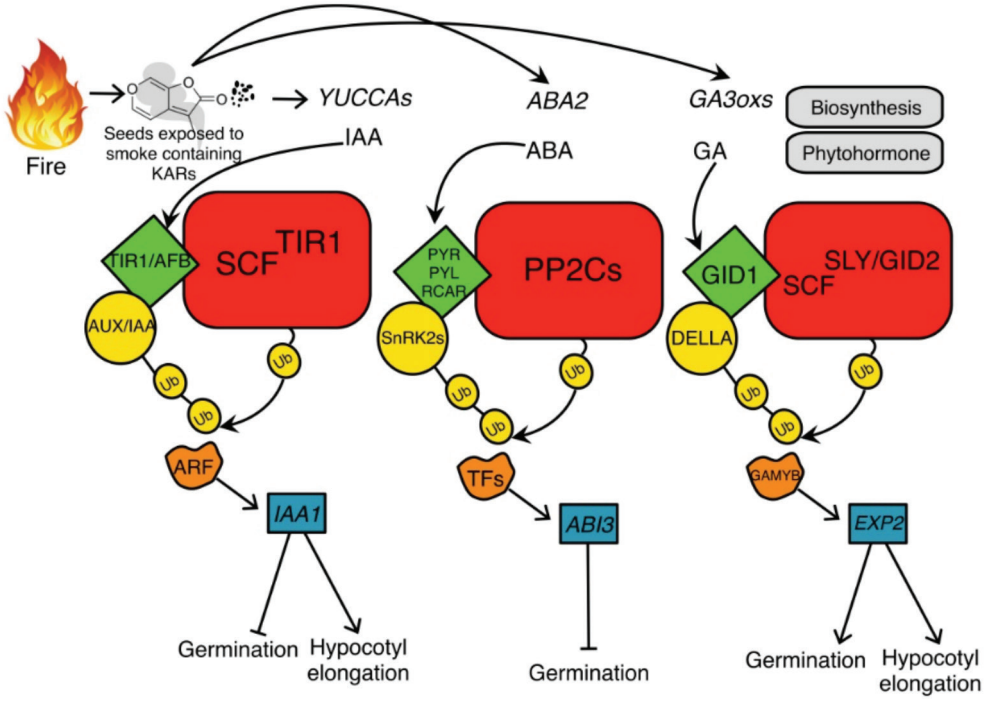

Figure 2- Karrikins regulate seed germination and hypocotyl elongation by affecting phytohormones
IAA inhibits germination and promotes hypocotyl elongation. ABA inhibits seed germination. GA promotes both seed germination and hypocotyl elongation

There are many studies that SW and KAR_1 affect GA, ABA and IAA metabolism (Grossmann 1990; Van Staden et al. 1995; Bewley 1997; Kucera et al. 2005; Merritt et al. 2006; Daws et al. 2007; Stevens et al. 2007; Commander et al. 2009; Nelson et al. 2009). However, this mechanism is still not fully known. KARs are water-soluble substances. KARs have seed germination promoting activity at very low concentrations, usually below 10^{-9} mol L^{-1} (Light et al. 2009; Nelson et al. 2012). However, it is known that SW tends to have a "dual regulatory" effect on germination, as higher concentrations of SW inhibit germination, while lower concentrations have a germination promoting effect (Light et al. 2002). 3,4,5-Trimethylfuran-2(5H)-one (2,3,4-trimethylbut-2-enolide), a compound isolated from plant-derived smoke, was found to be responsible for its germination inhibiting activity (Light et al. 2010). Therefore, in order to maximize its stimulant biological activity, the SW must be diluted with water before use, usually at ratios of 1:250, 1:500, 1:1000, 1:1500 and 1:2000 (v/v), depending on the plant species (Van Staden et al. 2004).

SW is a material that is cheap, economical and easy to use, used in very low concentrations and stored for many years. Different researchers have conducted various studies to obtain SW. Many researchers have tried to prepare SW by using different plant materials, burning plant materials at different temperatures and times, and attempted to establish the active application range. Knowing the KAR concentration in the SW is crucial for biological studies. There remains no standard method for obtaining SW and using concentration. Although this importance is known, an optimum, fast and cheap method has yet to be developed. Our research aims to develop a standard method for the preparation of SW (optimal burning time and temperature) and to find the most active range for germination to compare the SW obtained for the germination test with the commercially available KAR_1 substance statistically. In addition, the research aims to determine the KAR_1 concentration in the obtained SW with HPLC device and discuss it in relation to other studies in the literature. The most important point that distinguishes this study from other studies is that it is the first study in terms of developing a standard method for obtaining SW. In addition, since the amount of KAR_1 in the SW obtained by this method is higher than other studies in the literature, we believe that it will be a source literature for future studies.

2. Material and Methods

2.1. Material

In the research, the seeds of the Şalak apricot variety of *Prunus armeniaca* L., belonging to the Rosaceae family, were used as material (Figure 3). The seeds were obtained from Iğdır University Agricultural Application and Research Center (TUAM). After the fleshy parts of the apricot was separated and washed, it was dried in a cool and shaded place.

Figure 3- Tree, fruit and seed form of the material used, respectively

2.2. Methods

2.2.1. Smoke water preparation

SW was obtained by burning 1 kg of *Medicago sativa* L. straw in 1 L sterile distilled water in a Carbolite brand ELF 11/6B model laboratory oven, allowing the smoke to dissolve in the water in the erlen (Figure 4). The *Medicago sativa* L. straw was burned at 275 ºC for 60 minutes until it turned to ash. In order for the smoke to dissolve more in water, an ice pack was placed under the filtering flask. The obtained SW was stored at +4 ºC until used.

Figure 4- A) The process of burning the *Medicago sativa* L. straw in the laboratory oven, B) The smoke water obtained

2.2.2. Measurement of karrikin content in smoke water by HPLC

A HPLC analysis was carried out at Iğdır University Research Laboratory Practice and Research Center. The HPLC was performed with an Agilent 1260 Infinity Series device containing a Diode Array detector. A Zorbax C18 (4.6×250 mm) reverse phase column with a diameter of 5 micrometers and an injection volume of 20 micrometers was used as the column. The column was eluted with 50% acetonitrile at 1 mL/d 30 ºC for 10 minutes and then with 50% H_2O for 10 minutes. UV absorbance was measured at 325/4 nm wavelength. KAR_1 (Toronto Research Chemicals Canada) was then added to the HPLC device library. KAR_1 was introduced to the device at a concentration of 5.08076 ng μL^{-1}. Then, in order to measure the amount of KAR in the SW, the SW was introduced to the device; the retention time and the amount of KAR in SW given by the device were evaluated.

2.2.3. Sterilization of seed and other materials to be used in the study

The sterilization of seeds was carried out according to Kemeç Hürkan and Akı (2022). Before sterilization, 2 mg of KAR_1 was dissolved in 2 mL of chloroform solvent, and then diluted (0.01 µM, 0.1 µM, 1 µM, 5 µM, 10 µM) from the stock solution (1000 ppm) was used. SW was used by dilution from the stock solution (1:100, 1:500, 1:1000, 1:5000, 1:10000) obtained after burning the plant material. SW was passed through filter paper before being used in the study. SW and KAR_1 were sterilized by being passed through a membrane filter (0.22 µm) before use. Glass materials (petri dishes, magentas, measuring tape, flask, beaker, bottles, etc.), filter papers, forceps, scalpels and distilled water to be used in the study were sterilized in an autoclave at 121 °C under 1.2 atmospheres pressure for 15 minutes.

2.2.4. Germination of seeds

Seeds were sown under aseptic conditions, and a sterile cabinet with laminar flow and HEPA filter was used for this. After the testa part of the sterilized seeds was peeled, they were transferred to petri dishes with filter paper inside. Each group consisted of 50 seeds and seeds were sown in 10 replications, with 5 seeds per petri dish.

Then, according to the experimental groups, each of them was wetted with the previously prepared solutions (Figure 5).

The control group was only wetted with sterile distilled water. Petri dishes were wrapped with cling film to prevent the moist filter papers from drying out. The seeds were stored under dark conditions at 4 ºC±1 (wet stratification in cold) until germination. Seeds germinated after 1 week and germinated seeds were recorded for statistical data.

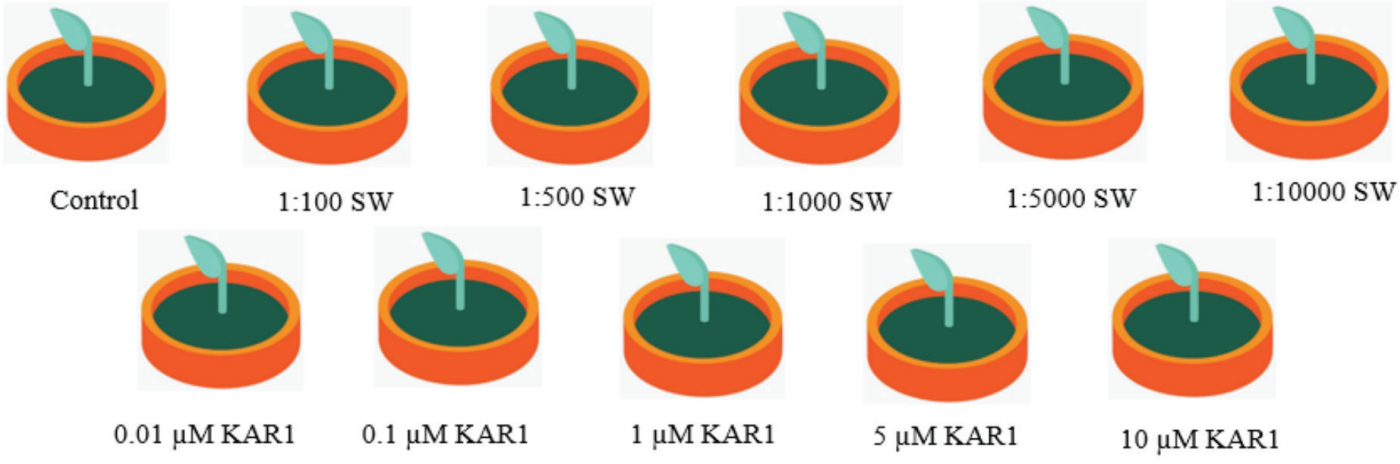

Figure 5- Experimental groups prepared for germination study
SW: Smoke water, KAR_1: $Karrikin_1$

2.2.5. Statistical analysis

All of the data obtained from this study were evaluated by making ANOVA in the XLSTAT 2021 statistical package program according to the randomized plots trial design. After the statistically significant transactions were determined, the differences between the averages were determined using the Duncan test at the p=0.05 level. The obtained data are given in tables as mean ± standard deviation.

3. Results and Discussion

3.1. Results

3.1.1. HPLC measurements

The KAR_1 substance added to the device library to measure the amount of KAR in the SW by HPLC gave a clear peak in 4.287 minutes (Figure 6A). Then, SW was introduced to the device to measure the amount of KAR_1 in the SW and it was determined that the KAR_1 concentration was calculated as 8.70398 ng/µl in 4.328 minutes (Figure 6B). Since SW consists of 71 compounds, unlike KAR_1, the HPLC peak was flactual.

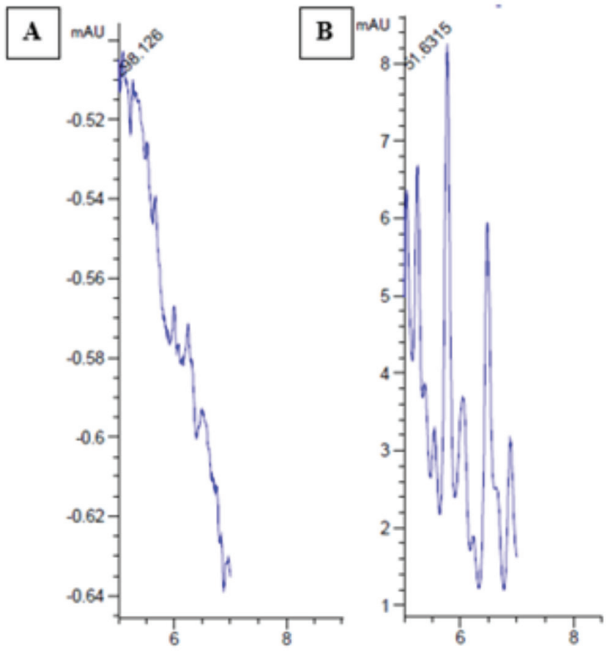

Figure 6- A) Retention time of KAR_1 substance, B) Retention time of smoke water

3.1.2. Seed germination percentage test

The seeds in the experimental groups started to germinate after 3 days. After one week, the germination rates were determined.

As a result of the statistical analysis, the difference between the groups in terms of germination was found to be significant ($p<0.05$). The highest rate of germination was 1:1000 SW (60%) and 1 µM KAR_1 (72%) (Table 1). It was observed that the germination increased as the concentration decreased in the SW substance at the concentrations tried throughout the study, and the germination increased as the concentration increased in the KAR_1 substance. For this purpose, for concentration optimization in germination, experimental groups were formed at 1:5000 and 1:1000 concentrations in the SW group and at 5 µM and 10 µM concentrations in the KAR_1 group, and germination rates were determined. According to the study, it was observed that germination decreased at the concentrations tested. In terms of germination, it was determined that the concentration of 1:1000 in the SW group and 1 µM in the KAR_1 group was appropriate.

Table 1- Effects of experimental groups on germination

Experimental groups	*Germinated seed (%)*
control	34.00±0.229^{ef}
1:100 SW	40.00±0.387^{cdef}
1:500 SW	50.00±0.403^{bcd}
1:1000 SW	60.00±0.387^{ab}
1:5000 SW	28.00±0.245^{fg}
1:10000 SW	30.00±0.512^{fg}
0,01 µM KAR_1	46.00±0.229^{bcde}
0,1 µM KAR_1	54.00±0.391^{bc}
1 µM KAR_1	72.00±0.332^{a}
5 µM KAR_1	38.00±0.350^{def}
10 µM KAR_1	18.00±0.350^{g}
p<0.002	

Values with different superscript letters in the same column are significantly different from each other (p<0.05; Duncan's test). SW: Smoke Water, KAR_1: $Karrikin_1$

4. Discussion

We think that the amount of KAR in the SW content may vary depending on the burned material, the burning temperature, the burning time and the amount of the burned material. In the majority of studies, forest floor vegetation such as *T. triandra* Forssk, *Heteropogon contortus* Beauv. ex Roemer & J. A. Schultes, *Tristachya leucothrix* Trin. ex Nees, *Hyparrhenia hirta* (L.) Staph, *Aristida junciformis* Trin. & Rupr, *Cymbopogon validus* Stapf ex Burtt Davy, *Cynodon dactylon* (L.) Pers., leaves and wood parts of plants in the form of shrubs and trees such as *Eucalyptus lanceolatus* Labill., *Eucalyptus camaldulensis* Dehnh., *Saraca asoca* (Roxb.) Willd., *Morus alba* L., *Ficus religiosa*. (L.) Forssk, *Passerina vulgaris* Meisn., straw, urban waste plant materials, paper, sugar cane, cellulose, glucose, xylose and glycine were used as the burning material (Flematti et al. 2009; Kochanek et al. 2016; Gupta et al. 2019; Hrdlička et al. 2019; Shabir et al. 2021). In our research, *Medicago sativa* L. straw was used as the burning material. Straw contains 30-40% cellulose and 20-30% xylose (Artık et al. 1993; Yüksel 2017). According to Flematti et al. (2004), synthetic cellulose, glucose, xylose and glycine were burned and the amount of KAR in their content was determined. They found KAR in xylose + glycine > xylose > cellulose > glucose, respectively (Flematti et al. 2011). According to the literature, it is thought that if the plant material is burned at 180-200 ºC for 30 minutes, it will be sufficient to release the substances that will promote germination (Flematti et al. 2015). The burning temperature and time typically used are between 180-200 ºC and 10-30 minutes (Brown & Van Staden 1997; Van Staden et al. 2004; Downes et al. 2013; Çatav et al. 2018a; Çatav et al. 2018b; Shabir et al. 2021), and in another study 450-730 ºC for 2-40 minutes (Kochanek et al. 2016). In most studies, the burning temperature and time were not specified, and the plant material is burned until ashes. In our research, the plant material was burned at 275 ºC for 60 minutes. We prepared a comprehensive table includes all the parameters and results which have been used to obtain SW in the literature (Table 2). According to comparison, we obtained the highest KAR_1 concentration in SW and believe that this may be due to the material we burned (*Medicago sativa* L. straw), the burning temperature, the burning time and the amount of the burned material. In addition, by placing an ice pack under the filtering flask where the smoke is dissolved, the faster and more effective dissolution of the gases in the water showed that more KAR_1 substance is held in the SW. In the literature, it is seen that the amount of KAR_1 obtained in the study (Gupta et al. 2019) in which plant material was burned at a rate of 1/1, like our study, is the closest result to our study. In other studies, even if proportionally more plant material was burned, the amount of KAR_1 obtained was found to be very low. This may be due to the difference in burning time and temperature. According to the study conducted by Kochanek et al. (2016), it is thought that the plant material should burn slowly, under low temperature and with large raw material quantities in order for the KAR_1 substance to be more concentrated in the SW. On the contrary, it is thought that if the plant material is burned quickly, under higher temperature or with a small amount of raw material, the KAR_1 substance is consumed more quickly and deteriorates, and it is not formed effectively. It is therefore estimated to be present only in low concentrations in SW mixtures produced under these conditions.

Table 2- Comparison of the literature and the data obtained in this study

		The amount of KAR_1 in the smoke water	*[1]Present study: Literature ratio*	*Temperature (°C)*	*Duration (min.)*	*Burned material*	*Amount of material burned*
The data of this research		8.70398 ng μL^{-1}		275	60	Medicago sativa	1 kg/1 L
References	Gupta et al. (2019)	1.71148±2300 ng μL^{-1} (2018 data)	5:1	The plant material was burn up until ashes Fynbos leaves *Passerina vulgaris*, *Themeda triandra*		*Themeda triandra*, Heteropogon *contortus*, *Tristachya leucothrix*, *Hyparrhenia hirta*, *Aristida junciformis*, *Cymbopogon validus*	26 kg/26 L
		0.00123±3.2 ng μL^{-1} (1993 data)	7076:1				5 kg/500 mL
		0.00488±1.4 ng μL^{-1} (1998 data)	1784:1				
	Hrdlička et al. (2019)	0.00176±4.3 ng μL^{-1}	4945:1	-	45	Fynbos leaves	
		0.00488±1.4 ng μL^{-1}	1784:1			*Passerina vulgaris*, *Themeda triandra*	5 kg/500 mL
		0.00949±1.5 ng μL^{-1}	917:1			*Themeda triandra*	
		0.00141±0.4 ng μL^{-1}	6173:1			*Themeda triandra*	10 kg/500 mL
		0.01117±3.7 ng μL^{-1}	779:1			Fynbos leaves	5 kg/500 mL
		0.01525±7.9 ng μL^{-1}	571:1	The plant material was burn up until ashes		Commercial smoke water (brand and model not specified)	
	Kochanek et al. (2016)	0.069±8.8 ng μL^{-1}	126:1	590	28-29	Pyrolytic liquid (wood vinegar)	250 kg/20 L

[1]KAR_1 ratios obtained in this study according to the literature

At high concentrations (1:100 or less dilution) SW inhibits germination. However, lower concentrations (1:1000 dilution) significantly increase germination compared to control (Light et al. 2002). Consistent with the literature data, in our study, it was observed that for apricot seeds, germination increased as SW concentration decreased, and germination increased as KAR_1 concentration increased. The best germination optimization for SW was obtained at a concentration of 1:1000, and for KAR_1 at a concentration of 1mM. A decrease in the germination percentage was observed at 1:5000 and 1:10000 SW concentrations. We think that this is because as the dilution rate increases, the density of the KAR_1 substance decreases as well as the 3,4,5-Trimethylfuran-2(5H)-one substance present in its content, and it slows down the germination rate. A decrease in germination percentage was also observed at 5 mM and 10 nM KAR_1 concentrations. We think that the reason for this is that the increased concentration creates a toxic effect for the seed and thus slows the germination rate.

5. Conclusions

With the SW preparation method designed in this study, the results show that low cost, simple and very high concentrations of SW can be obtained. Unlike other systems, adjusting the burning temperature and time allows for more control and a more effective performance. In this way, much more plant growth regulators will be produced in quantity than other plant growth regulators that can be stored and used for many years. Commercially available plant growth regulators (GA, auxin, cytokinin, ABA, strigolactone, etc.) are both very expensive, not stored for long periods, and are also sensitive to heat. SW has the potential to be used in many fields of plant sciences such as agriculture, horticulture and laboratories. SW is an economic substance that supports seed germination, shoot, root and plant growth even at very low concentrations. We think that this study will help other researchers working in this field in terms of SW preparation method and optimization. SW can potentially be used in plant tissue culture, molecular biology, plant physiology, agriculture, and plant protection. In addition, farmers may see the benefits of it in agriculture if the SW generating apparatus is made for large-scale commercial use.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: Y.K.H., C.A., Design: Y.K.H., C.A., Data Collection or Processing: Y.K.H., Analysis or Interpretation: Y.K.H., C.A., Literature Search: Y.K.H., Writing: Y.K.H.

Conflict of Interest: No conflict of interest was declared by the authors.

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References

Artık N, Velioğlu S & Kavalcı B (1993). Şeker alkollerden ksilitol; özellikleri üretimi ve gıdalarda kullanımı. Gıda 18(2): 101-109.

Baldwin I T, Staszak K L & Davidson R (1994). Up in smoke: I. Smoke-derived germination cues for postfire annual, *Nicotiana attenuata* torr. Ex. Watson. Journal of Chemical Ecology 20(9): 2345-2371. doi.org/10.1007/BF02033207

Baxter B J M & Van Staden J (1994). Plant-derived smoke: an effective seed pre-treatment. *Plant Growth Regulation* 14(3): 279-282. doi.org/10.1007/BF00024804

Baxter B J M, Van Staden J, Granger J E & Brown N A C (1994). Plant-derived smoke and smoke extracts stimulate seed germination of the fire-climax grass *Themeda triandra*. *Environmental and Experimental Botany* 34(2): 217-223. doi.org/10.1016/0098-8472(94)90042-6

Beeby A & Brennan A M (1997). First Ecology. Chapman & Hall.

Bewley J (1997). Seed germination and dormancy. *Plant Cell* 9(7): 1055-1066 doi.org/10.1105/tpc.9.7.1055

Brown N A C & Van Staden J (1997). Smoke as a germination cue: A review. Plant Growth Regulation 22(2): 115-124. doi.org/10.1023/A:1005852018644

Çatav Ş S, Bekar I, Ateş B S, Ergan G, Oymak F, Ülker E D & Tavşanoğlu Ç (2012). Germination response of five eastern Mediterranean woody species to smoke solutions derived from various plants. *Turkish Journal of Botany* 36(5): 480-487. doi.org/10.3906/bot-1111-12

Çatav Ş S, Elgin E S, Dağ Ç, Stark J L & Küçükakyüz K (2018a). NMR-based metabolomics reveals that plant-derived smoke stimulates root growth via affecting carbohydrate and energy metabolism in maize. *Metabolomics* 14(11): 1-11. doi.org/10.1007/s11306-018-1440-y

Çatav Ş S, Küçükakyüz K, Akbaş K & Tavşanoğlu Ç (2014). Smoke-enhanced seed germination in Mediterranean Lamiaceae. *Seed Science Research* 24(3): 257-264. doi.org/10.1017/S0960258514000142

Çatav Ş S, Küçükakyüz K, Tavşanoğlu Ç & Pausas J G (2018b). Effect of fire-derived chemicals on germination and seedling growth in Mediterranean plant species. *Basic and Applied Ecology* 30: 65-75. doi.org/10.1016/j.baae.2018.05.005

Chiwocha S D S, Dixon K W, Flematti G R, Ghisalberti E L, Merritt D J, Nelson D C, Stevens J C (2009). Karrikins: A new family of plant growth regulators in smoke. *Plant Science* 177(4): 252-256. doi.org/10.1016/j.plantsci.2009.06.007

Christensen N L (1985). Shrubland fire regimes and their evolutionary consequences. *The Ecology of Natural Disturbance and Patch Dynamics* 85-100.

Chumpookam J, Lin H L & Shiesh C C (2012). Effect of smoke-water on seed germination and seedling growth of papaya (Carica papaya cv.Tainung No.2). *HortScience* 47(6): 741-744. doi.org/10.37855/jah.2012.v14i02.23

Commander L, Merritt D, Rokich D & Dixon K (2009). Seed biology of Australian arid zone species: germination of 18 species used for rehabilitation. *Journal of Arid Environments* 73(6): 617-625. doi.org/10.1016/j.jaridenv.2009.01.007

Daws M, Davies J, Pritchard H, NAC B & Van Staden J (2007). Butenolide from plant-derived smoke enhances germination and seedling growth of arable weeds species. *Plant Growth Regul* 51(1): 73-82. doi.org/10.1007/s10725-006-9149-8

De Cuyper C, Struk S, Braem L, Gevaert K, De Jaeger G & Goormachtig S (2017). Strigolactones, karrikins and beyond. *Plant Cell and Environment* 40(9): 1691-1703. doi.org/10.1111/pce.12996

De Lange J H & Boucher C (1990). Autecological studies on Audouinia capitata (Bruniaceae). I. Plant-derived smoke as a seed germination cue. *South African Journal of Botany* 56(6): 700-703. doi.org/10.1016/s0254-6299(16)31009-2

Downes K S, Light M E, Pošta M, Kohout L & Van Staden J (2013). Comparison of germination responses of Anigozanthos flavidus (Haemodoraceae), Gyrostemon racemiger and Gyrostemon ramulosus (Gyrostemonaceae) to smoke-water and the smoke-derived compounds karrikinolide (KAR_1) and glyceronitrile. *Annals of Botany* 111(3): 489-497. doi.org/10.1093/aob/mcs300

Flematti G R, Dixon K W & Smith S M (2015). What are karrikins and how were they "discovered" by plants?. *BMC Biology* 13(1): 1-7. doi.org/10.1186/s12915-015-0219-0

Flematti G R, Ghisalberti E L, Dixon K W & Trengove R D (2004). A compound from smoke that promotes seed germination. *Science* 305(5686): 977. doi.org/10.1126/science.1099944

Flematti G R, Ghisalberti E L, Dixon K W & Trengove R D (2009). Identification of alkyl substituted 2H-furo[2,3-c]pyran-2-ones as germination stimulants present in smoke. *Journal of Agricultural and Food Chemistry* 57(20): 9475-9480. doi.org/10.1021/jf9028128

Flematti G R, Scaffidi A, Dixon K W, Smith S M & Ghisalberti E L (2011). Production of the seed germination stimulant karrikinolide from combustion of simple carbohydrates. *Journal of Agricultural and Food Chemistry* 59(4): 1195-1198. doi.org/10.1021/jf1041728

Grossmann K (1990). Plant growth retardants as tools in physiological research. *Physiologia Plantarum* 78(4): 640-648. doi.org/10.1111/j.1399-3054.1990.tb05254.x

Gupta S, Hrdlicka J, Ngoroyemoto N, Nemahunguni N K, Gucky T, Novak O, Kulkarni M G, Doležal K &Van Staden J (2019). Preparation and standardisation of smoke-water for seed germination and plant growth stimulation. *Journal of Plant Growth Regulation* 39(1) 338-345.

Hrdlička J, Gucký T, Novák O, Kulkarni M, Gupta S, Van Staden J & Doležal K (2019). Quantification of karrikins in smoke water using ultra-high performance liquid chromatography-tandem mass spectrometry. *Plant Methods* 15(1): 1-12. doi.org/10.1186/s13007-019-0467-z

Kazancı D D (2014). Determination of post-fire germination properties of Mediterranean plants. M.Sc. Thesis, Hacettepe University.

Keeley J E (1995). Seed-Germination patterns in fire-prone mediterranean-climate regions. *Ecology and Biogeography of Mediterranean Ecosystems in Chile California and Australia* 239-273.

Keeley J E & Fotheringham C J (1997). Trace gas emissions and smoke-induced seed germination. *Science* 276(5316): 1248-1250.

Keeley J E, Morton B A, Pedrosa A & Trotter P (1985). Role of allelopathy, heat and charred wood in the germination of chaparral herbs and suffrutescents. *Journal of Ecology* 73(2): 445-458.

Keeley Jon E & Fotheringham C J (1998). Smoke-induced seed germination in California chaparral. *Ecology* 79(7): 2320-2336. doi.org/10.1890/0012-9658(1998)079[2320:SISGIC]2.0.CO;2

Keeley S C & Pizzorno M (1986). Charred Wood stimulated germination of two fire-following herbs of the california chaparral and the role of hemicellulose. *American Journal of Botany* 73(9): 1289-1297. doi.org/10.1002/j.1537-2197.1986.tb10870.x

Kemeç Hürkan Y & Akı C (2022). Surface sterilization optimization in seeds of şalak apricot variety (Prunus armeniaca L. cv. Şalak). *Journal of the Institute of Science and Technology* 12(3): 1358-1363. doi.org/ 0.21597/jist.1132037

Kochanek J, Long R L, Lisle A T & Flematti G R (2016). Karrikins identified in biochars indicate post-fire chemical cues can influence community diversity and plant development. *PLoS ONE* 11(8): 1-19. doi.org/10.1371/journal.pone.0161234

Kucera B, Cohn M & Leubner Metzger G (2005). Plant hormone interactions during seed dormancy release and germination. *Seed Science Research* 15(4): 281-307.

Light M E, Daws M I & Van Staden J (2009). Smoke-derived butenolide: Towards understanding its biological effects. *South African Journal of Botany* 75(1): 1-7. doi.org/10.1016/j.sajb.2008.10.004

Light M E, Gardner M J, Jäger A K & Van Staden J (2002). Dual regulation of seed germination by smoke solutions. *Plant Growth Regulation* 37(2): 135-141. doi.org/10.1023/A:1020536711989

Light Marnie E, Burger B V, Staerk D, Kohout L & Van Staden J (2010). Butenolides from plant-derived smoke: natural plant-growth regulators with antagonistic actions on seed germination. *Journal of Natural Products* 73(2): 267-269. doi.org/10.1021/np900630w

Merritt D, Kristiansen M, Flematti G R, Turner S & EL G (2006). Effects of a butenolide present in smoke on light-mediated germination of Australian Asteraceae. *Seed Science Research* 16(1): 29-35. doi.org/10.1079/SSR2005232

Nelson D C, Flematti G R, Ghisalberti E L, Dixon K W & Smith S M (2012). Regulation of seed germination and seedling growth by chemical signals from burning vegetation. *Annual Review of Plant Biology* 63: 107-130. doi.org/10.1146/annurev-arplant-042811-105545

Nelson D C, Riseborough J A, Flematti G R, Stevens J, Ghisalberti E L, Dixon K W & Smith S M (2009). Karrikins discovered in smoke trigger arabidopsis seed germination by a mechanism requiring gibberellic acid synthesis and light. *Plant Physiology* 149(2): 863-873. doi.org/10.1104/pp.108.131516

Shabir S, Ilyas N, Asif S, Iqbal M, Kanwal S & Ali Z (2021). Deciphering the role of plant-derived smoke solution in ameliorating saline stress and improving physiological, biochemical, and growth responses of wheat. *Journal of Plant Growth Regulation* 41(7): 2769-2786. doi.org/10.1007/s00344-021-10473-5

Sirko A, Wawrzyńska A, Brzywczy J & Sieńko M (2021). Control of aba signaling and crosstalk with other hormones by the selective degradation of pathway components. *International Journal of Molecular Sciences* 22(9): 4638. doi.org/10.3390/ijms22094638

Stevens J, Merritt D, Flematti G, Ghisalberti E & Dixon K (2007). Seed germination of agricultural weeds is promoted by the butenolide 3-methyl-2H-furo[2,3-c]pyran-2-one under laboratory and field conditions. *Plant Soil* 298(1): 113-124

Tavşanoğlu Ç (2011). Fire-related cues (heat shock and smoke) and seed germination in a *Cistus creticus* population in Southwestern Turkey. *Ekoloji* 20(79): 99-104. doi.org/10.5053/ekoloji.2011.7913

Tavsanoğlu C, Ergan G, Çatav S S, Zare G, Küçükakyüz K & Özüdoğru B (2017). Multiple fire-related cues stimulate germination in Chaenorhinum rubrifolium (Plantaginaceae), a rare annual in the Mediterranean Basin. *Seed Science Research* 27(1): 26-38. doi.org/10.1017/S0960258516000283

Tavşanoğlu Ç, Gürkan B (2004). Akdeniz havzasında bitkilerin kuraklık ve yangına uyumları. *OT Sistematik Tanik* Dergisi 11(1): 119-132.

Türkan İ, Tokur S & Öztürk M (1985). Akdeniz ekosistemleri. Akdeniz Ekosistemleri, Doğa Bilim Dergisi 9: 3.

Van Staden J, Brown N A C, Jäger A K & Johnson T A (2000). Smoke as a germination cue. *Plant Species Biology* 15(2): 167-178. doi.org/10.1046/j.1442-1984.2000.00037.x

Van Staden J, Jager A & Strydom A (1995). Interaction between a plant-derived smoke extract, light and phytohormones on the germination of light-sensitive lettuce seeds. *Plant Growth Regul* 17: 213-218. doi.org/10.1007/BF00024728

Van Staden J, Jäger A K, Light M E & Burger B V (2004). Isolation of the major germination cue from plant-derived smoke. *South African Journal of Botany* 70(4): 654-659. doi.org/10.1016/S0254-6299(15)30206-4

Xu K, Wu N, Yao W, Li X, Zhou Y & Li H (2021). The biological function and roles in phytohormone signaling of the f-box protein in plants. *Agronomy* 11(11): 1-17. doi.org/10.3390/agronomy11112360

Yüksel F (2017). Buğday Samanının Fungal İnokulasyon Yoluyla Lignoselülozik İçeriğinin Parçalanması Ve In-Situ Naylon Torba Tekniği İle Yem Değerinin Belirlenmesi: I. Kuru Madde Parçalanabilirliği. *Yemler ve Hayvan Besleme Bilim Dalı* 32(1): 27-33.

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The Effect of the Addition of Fermented Natural Lactic Acid Bacterial Liquid and Some Lactic Acid Bacterial Inoculants on Alfalfa Silage Quality, *In Vitro* Digestibility and Gas Production

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ABSTRACT

This study was conducted to evaluate the effect of adding fermented natural lactic acid bacteria (LAB) liquid, also known as pre-fermented juice (PFJ) obtained from different sources and some LAB inoculants to alfalfa (*Medicago sativa* L.) silage on fermentation, *in vitro* organic matter digestibility (IVOMD) and *in vitro* gas production. The silages were prepared in laboratory conditions using 1.5 L glass jars. In the study, the treatments were: the ensiled pure alfalfa plant as a control group; alfalfa plant with the addition of 2% molasses; alfalfa plant +2% molasses, with the addition of PFJ prepared from alfalfa; alfalfa plant +2% molasses, with the PFJ prepared from meadow grass; alfalfa plant +2% molasses, with the PFJ prepared from maize; alfalfa plant +2% molasses with addition of homofermentative LAB inoculant; alfalfa plant +2% molasses with addition of heterofermentative LAB inoculant. Homofermentative and heterofermentative LAB inoculants were added to the alfalfa plant at the level of 10^8 cfu/kg. When the dry matter, ash, acid detergent fiber, neutral detergent fiber, IVOMD, metabolizable energy, and methane values of the prepared silages were examined, the differences between the groups were found to be statistically significant ($p<0.05$). When the fermentation characteristics (pH, NH_3-N, CO_2, LA, AA, BA, mold) of the silages were prepared by adding PFJ and some LAB inoculants to the alfalfa plant, the differences between groups were found to be statistically significant ($p<0.05$). When examined in terms of all parameters, it was determined that the addition of PFJ (3% molasses) prepared from meadow grass +2% molasses to alfalfa plant had positive effects on silage quality, fermentation characteristics and IVOMD.

Keywords: Epiphytic lactic acid bacteria, Fermentation, Legume silage

1. Introduction

Silage is a preferable roughage source in terms of nutrients obtained by fermenting green forage plants and some aqueous industrial residues under anaerobic conditions. Due to the low dry matter and water-soluble carbohydrate (WSC) content in the structure of legume roughages, they are very difficult to ensilage due to their high buffering capacity (McDonald et al. 1991). Plants with high buffering capacity need more WSC and longer fermentation time to ferment. During ensiling, more acid formation is required to lower the pH of the environment. Since the pH values of silages obtained from plants with high buffering capacity are also high, the loss of nutrients is higher in silages made from such plants. During silage fermentation, the use of lactic acid bacteria (LAB) inoculants and easily soluble sources has become widespread in silage material in order to increase the lactic acid content of the silage, decrease the silage pH value, improve aerobic stability and reduce the fermentation losses of the silage (Arriola et al. 2011). The primary purposes of the use of silage additives are to reduce the loss of nutrients, to improve the feed value and quality of the obtained silage, to regulate the fermentation flow, to increase the evaluation levels of the silages by the animals by showing a probiotic effect, and to provide that the silage quality is preserved for a long time after the silage is opened (Gül & Coşkuntuna 2016). The role of LAB in the ensiling process is not only to reduce the pH level by breaking down the WSC but also to prevent the proliferation and activities of microorganisms that compete with the nutrients in the plant but are not desired to be present in the silo environment by forming antifungal and antimicrobial products

such as bacteriocin, hydrogen peroxide, lactate peroxidase or 1.2-propanediol (Davies et al. 1996). As an alternative to commercial LAB inoculants, a new silage additive has recently been developed called fermented LAB liquid. Fermented LAB liquid has important advantages such as being of biological origin as a silage additive, being very easy and economical to prepare and use, being safe, not having any toxic effects, not causing corrosion in the machines used in silage making, not causing environmental pollution and being a natural product (Jin-ling et al. 2013).

This study was conducted to evaluate the effect of adding fermented natural LAB liquid from different sources and some LAB inoculants to alfalfa silage on fermentation, *in vitro* organic matter digestibility (IVOMD), and gas production.

2. Material and Methods

2.1. Study design and silage preparation

In this study, fermented natural LAB liquid formed from alfalfa, meadow grass, and maize plants by adding molasses was prepared using the method reported by Masuko et al. (2002). For this purpose, 2000 mL of distilled water was added to 1000 g of fresh alfalfa, meadow grass, and maize plants and shredded for 2 minutes using a blender. The obtained plant liquid mixtures were filtered using two layers of cheesecloth. The bottles were closed by adding 3% molasses and were incubated at 30 °C for 72 hours. In ensiling, 1 mL of fermented natural LAB liquid was added to 1 kg of alfalfa plants. The alfalfa plant was used as silage raw material in the study. To ensure homogenization in all silage groups prepared in the study, 50 mL/kg of distilled water was added. The total LAB count in fresh silage material was determined by the method reported by Güney and Ertürk (2020) as 3 repetitions for each group according to the tempo automatic bacteria counter-test method. The buffering capacity of the fresh alfalfa used in the study was determined according to the method reported by Playne and McDonald (1966). In the study, the treatments were: the ensiled pure alfalfa plant as a control group; alfalfa plant with the addition of 2% molasses; alfalfa plant +2% molasses, with the addition of pre-fermented juice (PFJ) prepared from alfalfa; alfalfa plant +2% molasses, with the PFJ prepared from meadow grass; alfalfa plant +2% molasses, with the PFJ prepared from maize; alfalfa plant +2% molasses with addition of homofermentative LAB (HoLAB) inoculant; alfalfa plant +2% molasses with addition of heterofermentative LAB (HetLAB) inoculant. Homofermentative and heterofermentative LAB inoculants were added to the alfalfa plant at the level of 10^8 cfu/kg. HoLAB used in the study included the strains such as (*Lactobacillus plantarum* DSM 18112, *Lactobacillus plantarum* DSM 18113, *Lactobacillus plantarum* DSM 18114, *Lactobacillus plantarum* ATCC 55943, *Enterococcus faecium* ATCC 55593, *Enterococcus faecium* ATCC 53519) whereas HetLAB included the strains such as (*Lactobacillus buncheri* ATCC PTA-2494).

Each trial group of silages was compressed into 1.5 liter glass jars with 4 repetitions and ensiled up in an airtight manner. Silages were stored at room temperature for 60 days in a dark environment.

2.2. Fermentation profile analysis

The silages were opened at the end of the 60 day fermentation period. The 3-5 cm part at the top of the jars was discarded, 100 mL of distilled water was added to the homogeneously taken 25 g silage sample and shredded for 2 minutes using a blender, and the pH value of the shredded silage liquid was recorded by measuring rapidly with pH meter measuring device (Polan et al. 1998). The liquid in the blender was filtered and taken into 10 mL tubes; 0.1 mL of 1M HCl was added to the samples to be analyzed for ammonia nitrogen and 25% 0.25 mL of metaphosphoric acid was added to the samples to be analyzed for lactic acid and volatile fatty acid and stored in a deep freezer until analysis. Ammonia nitrogen analyzes of the silage samples were performed according to the method reported by Broderick and Kang (1980), lactic acid and volatile fatty acids (butyric, acetic, and propionic acid) concentrations were determined using a high pressure liquid chromatography device (HPLC) according to the method reported by Suzuki and Lund (1980). For this purpose, it was utilized HPLC device [Shimadzu LC-20 AD HPLC pump, Shimadzu SIL-20 ADHT Autosampler, Shimadzu SPD M20A Detector (DAD), Shimadzu CTO-20ac Colum oven, Icsep Coregel (87H3 colon)]. The silages obtained in the study were subjected to an aerobic stability test (determination of CO_2 production values) for 5 days (Ashbell et al. 1991).

The nutrient contents such as the dry matter, ash, and crude protein analyzes of the silages obtained from alfalfa used as silage material in the study were performed according to AOAC (2005), while ADF and NDF analyzes were performed according to Van Soest et al. (1991). The nutrient analyzes were carried out after the ensiled materials and the obtained silages were dried at room temperature and then ground in a laboratory mill to pass through a 1 mm sieve. The gas production values of the silages and alfalfa herbage were determined through the method described by Menke and Steingass (1988) using four glass syringes as replicate. The rumen fluid used in the analysis was taken with the help of a rumen pump from 2 rams who were given training food (60% forage, 40% concentrate) for

2 weeks. The IVOMD (g/kg OM) and metabolizable energy (ME) (MJ/kg DM) of silages were calculated using equations reported by Menke et al. (1979) as:

$ME(MJ/kgDM)=2.20+0.136\times Gp+0.057\times CP+0.0029\times CP^2$,

$IVOMD(\%)=14.88+0.889\times Gp+0.45\times CP+0.0651\times XA$,

Where; CP is CP in g/100 g DM, crude ash in g/100 g DM and gas production is the net gas production (mL) from 200 mg DM after 24 h of incubation. After recording 24 h gas production values, gas inside the syringe was taken by three-way syringe system and total gas was injected into computer-assisted infrared methane gas meter (Sensor Europe GmbH, Erkrath, Germany) and then methane content was determined as a percentage of 24 h the total amount of gas formed (Goel et al. 2008).The amount of yeast and mold contained in the silages was determined according to the method reported by Filya et al. (2000).

2.3. Statistical analysis

In the study, one way analysis of variance (one-way ANOVA) was used to determine whether the data obtained from the groups were widely different. Duncan's multiple comparison tests were used to control the significance of the difference between the groups, and for this purpose, the SPSS (1991) package program was used.

3. Results and Discussion

The amount of LAB, lactic acid, acetic acid, LA/AA ratio, pH, yeast, and mold values of fermented natural LAB liquid obtained from different sources are given in Table 1. It was determined that the differences were found to be statistically significant between the groups ($p<0.05$). When the LAB value was examined, it was determined that the lowest LAB value was obtained from PFJ prepared from maize, while the highest value was obtained from PFJ prepared from alfalfa. In addition to mineral needs, LAB also need a source of carbon and nitrogen. The higher number of LABs in alfalfa can be explained by the fact that the nitrogen in the structure of alfalfa is higher than that of maize and meadow grass. Total LAB values in fermented natural LAB liquids were higher than the values obtained from the studies of Koç et al. (2017), Aydin and Denek (2019) (5.39 cfu/g), and was found to be similar to the values obtained from the study conducted by Aydın (2019) (10.48 - 11.62 cfu/g).

Table 1- Determined values of naturally fermented LAB liquid prepared from different sources with adding 3% molasses

Plants	*LAB*	*LA*	*AA*	*LA/AA*	*pH*	*Yeast (cfu/g)*	*Mold (cfu/g)*
Alfalfa	10.08^{a}	146.61^{a}	39.22^{a}	3.73^{b}	3.74^{a}	5.58^{c}	3.79^{a}
Meadow grass	9.44^{b}	54.01^{c}	11.16^{c}	4.65^{a}	3.68^{b}	6.96^{a}	0.02^{b}
Maize	8.72^{c}	69.13^{b}	20.61^{b}	3.35^{c}	3.59^{c}	6.72^{b}	0.02^{b}
SEM	0.197	14.340	4.121	0.193	0.022	0.213	0.628
p value	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

$^{a-c}$Values within a same column with different superscripts differ significantly at $p<0.05$; LAB: Lactic acid bacteria, LA: Lactic acid g/kg DM, AA: Acetic acid g/kg DM, LA/AA: Lactic acid/acetic acid ratio

When the LA and AA values were examined, it was seen that the highest was obtained from PFJ prepared from alfalfa, and the lowest from PFJ prepared from meadow grass. The lactic acid production level of LAB differed between species (Evren et al. 2011). The highest LA and AA values in PFJ prepared from alfalfa were due to the high LAB number of PFJs prepared from alfalfa, and it was concluded that microbial species in PFJ prepared from alfalfa better-evaluated molasses. It was determined that although the LAB number of PFJ prepared from maize was lower than the LAB number of PFJ prepared from meadow grass. The fact that the lactic acid and acetic acid values for PFJ prepared from maize were higher than that from meadow grass may be because the LAB species in the maize plant produce higher lactic acid. The LA (154.2 g/kg DM) and AA (36 g/kg DM) values found in the PFJ prepared by Bureenok et al. (2005) by adding 5% molasses were compatible with the results in this study.

When the LA/AA ratios were examined, it was seen that the L/A ratios were between (3.35 and 4.5). The lowest LA/AA ratio (3.35) was obtained from PFJ obtained from maize, and the highest was obtained from meadow grass PFJ (4.45). It was reported that homolactic fermentation occurred when the LA/AA ratio was greater than 3.0, while the heterolactic fermentation occurred when the LA/AA ratio was less than 3.0 (Zhang et al. 2015). In this study, the fact that all LA/AA ratios were greater than 3 revealed that homolactic activity was more intense in fermentation. When the pH values of PFJs were examined, it was determined that the lowest value was obtained from PFJs obtained from maize plant and the highest value was obtained from alfalfa PFJ. The higher the buffering capacity of a plant against acidification, the slower the pH decreases in silage. During the ensiling of the alfalfa plant, which has a high buffering capacity, more acid formation is required to decrease the pH of the environment (Atalay 2015). In addition, differences in pH values in PFJs may depend on the type and amount of LAB used, plant species, easily soluble carbohydrate source and amount, and fermented incubation time. It was reported by Can (2010) and Denek et al. (2011) that they found the pH values in PFJ, prepared by adding molasses to barley, wheat and meadow grass were in the range of 3.75-3.84, similar to this study. Tao et al. (2017) and Wang et al. (2009) found pH values were in the range of 3.78 and 3.75 in the PFJs prepared by adding glucose to the alfalfa plant. These pH values were compatible with the present study results.

When the yeast value was examined, it was seen that the highest value was obtained from PFJ from meadow grass and the lowest from PFJ from alfalfa. When the mold values were examined, the highest value was determined for PFJ prepared from alfalfa, and the lowest for PFJ prepared from meadow grass and maize. When the total yeast and mold values were examined, the lowest value was obtained for PFJ prepared from maize and meadow grass, and the highest was obtained for PFJ prepared from alfalfa. The amount of acetic acid produced by heterolactic LAB fermentation has an inhibitory effect on the reproduction and activity of yeasts. The report of Ali et al. (2020) supports the report that high acetic acid and the lowest total yeast mold values were observed in PFJs obtained from alfalfa. The results of the study were found to be similar to the yeast and mold values (5.34, 4.18 cfu/g) in the PFJ prepared from the alfalfa plant containing 2% glucose, by Tao et al. (2017).

LAB value ($1.2*10^5$ cfu/mL), buffering capacity value (680 meq kg/DM), yeast value ($4.9x10^5$ cfu/mL), mold value ($8x10^3$ cfu/mL) of fresh alfalfa plant used as silage raw material were determined in the study. The reports on LAB numbers ($1x10^4$, $3x10^5$, and $4.32x10^4$ cfu/mL) in alfalfa plant by Bureenok et al. (2005), Ohshima et al. (1997) and Wang et al. (2009) were found to be compatible with the present study. In the earlier studies, it was stated that the number of LAB infecting the plant before harvesting could vary from $1x10^1$ cfu/mL to $1.0x10^7$ cfu/mL, and there were differences in the number and types of LAB infecting the plants to be ensiled. Among the reasons for these differences, there were reports that many factors such as ultraviolet rays, environment temperature, environmental humidity, and many reasons related to the plant itself were effective and that the decomposition of silage plants increased the number of bacteria on the plant (Jones & Gogerddan 1994; Jones 1995). The buffering capacity of the alfalfa plant in our study with the value of 680 meq/kg DM was lower than the values of the buffering capacities of the alfalfa (720, 728 meq/kg DM) reported by Turan and Önenç (2018) and Çotuk (2016) in their studies and higher than the buffering capacity of alfalfa plant (583, 425), reported by Sun et al. (2021), in their study. Besides, Ohshima et al. (1997) found that the buffering capacity of the alfalfa plant was 683 meq/kg DM and it was found similar to the result of our study.

In the study, alfalfa silage nutrient contents and IVOMD, ME, and *in vitro* CH_4 values of treatments with added fermented natural LAB liquids from different sources, or HoLAB, or HetLAB, and 2% molasses are given in Table 2.

Table 2- Nutrient contents and IVOMD, ME, *in vitro* CH_4 values of silage groups

Groups	*DM*	*CA*	*CP*	*ADF*	*NDF*	*IVOMD*	*ME*	*CH_4*
Control	26.40^{e}	10.97^{bc}	18.04^{c}	32.54^{a}	46.20^{a}	55.90^{b}	8.35^{bc}	14.34^{a}
2% molasses added to group	30.41^{bc}	10.88^{c}	18.91^{bc}	32.05^{ab}	45.86^{a}	56.75^{b}	8.35^{bc}	13.96^{a}
Alfalfa PFJ +2% molasses	31.14^{ab}	10.96^{bc}	19.60^{ab}	30.13^{c}	44.82^{a}	55.68^{b}	8.21^{c}	12.81^{b}
Meadow grass PFJ +2% molasses	31.94^{a}	10.88^{c}	19.55^{ab}	30.37^{bc}	44.45^{a}	60.80^{a}	9.00^{a}	14.07^{a}
Maize PFJ +2% molasses	30.44^{bc}	10.89^{c}	19.20^{ab}	30.21^{c}	45.44^{a}	60.43^{a}	8.94^{a}	13.91^{a}
HoLAB +2% molasses	30.17^{cd}	11.31^{ab}	19.45^{ab}	30.60^{bc}	43.19^{b}	59.92^{a}	8.86^{ab}	13.75^{a}
HetLAB +2% molasses	29.35^{d}	11.44^{a}	20.15^{a}	31.25^{abc}	40.59^{b}	57.87^{ab}	8.55^{abc}	14.15^{a}
SEM	0.331	0.058	0.168	0.463	0.468^{a}	0.526	0.079	0.132
p value	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

$^{a-e}$Values with different letters in the same column were found to be different (p<0.05); DM: Dry matter; CA: Crude ash DM%; CP: Crude protein, DM%; ADF: Acid detergent insoluble fiber, DM %; NDF: Neutral detergent insoluble fiber, %DM; IVOMD: *In vitro* organic matter digestibility g/kg OM, ME: Metabolizable energy MJ/kg DM, CH_4: *In vitro* methane (%)

The fermentation characteristics of the silages obtained with using the fermented natural LAB liquids from different sources, or some LAB inoculants are given in Table 3 and the correlations of the values are given in Table 4.

Table 3- Fermentation characteristics of the experimental silages

Groups	*pH*	*NH_3N*	*CO_2*	*LA*	*AA*	*BA*	*Mold*	*Yeast*
Control	5.58^{a}	15.26^{a}	6.77^{b}	9.56d	13.70^{ab}	3.44^{a}	1.70^{a}	<10
2% molasses added to group	4.53^{bcd}	8.44^{cd}	4.52^{c}	26.82^{bc}	16.67^{a}	0.00^{b}	1.00^{d}	<10
Alfalfa PFJ +2% molasses	4.71^{b}	8.84^{cd}	1.66^{d}	11.40^{d}	6.23^{c}	0.00^{b}	1.01^{d}	<10
Meadow grass PFJ +2% molasses	4.35^{d}	7.31^{d}	4.49^{c}	38.91^{a}	15.65^{a}	0.00^{b}	1.60^{b}	<10
Maize PFJ +2% molasses	4.45^{cd}	9.88^{bc}	1.74^{d}	31.29^{ab}	16.58^{a}	0.00^{b}	1.30^{c}	<10
HoLAB +2% molasses	4.50^{bcd}	9.01^{bcd}	9.31^{a}	15.90^{cd}	7.85^{bc}	0.00^{b}	1.30^{c}	<10
HetLAB +2% molasses	4.67^{bc}	11.10^{b}	3.54^{c}	7.82d	19.88^{a}	0.00^{b}	0.00^{e}	<10
SEM	0.078	0.519	0.520	2.490	1.182	0.242	0.100	<10
p value	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<10

$^{a-e}$Values with different letters in the same column were found to be different (p<0.05); NH_3-N/TN: Ammonia nitrogen; CO_2: Carbon dioxide g/kg KM; LA: Lactic acid g/kg DM; AA: Acetic acid g/kg DM; BA: Butyric acid g/kg DM

Table 4- Correlation of the analysis in the silages

Fermentation parameters		*pH*	*NH_3N*	*LA*	*AA*	*BA*	*CO_2*	*Mold*	*IVOMD*	*ME*	*Methane*
pH	**PK**	1	0.854**	-0.527**	-0.091	0.936**	0.249	0.211	-0.496**	-0.458*	-0.003
	P		0.000	0.004	0.644	0.000	0.201	0.282	0.007	0.014	0.987
NH_3N	**PK**		1	-0.374*	0.240	0.796**	0.235	0.065	-0.341	-0.295	0.129
	P			0.050	0.219	0.000	0.228	0.742	0.076	0.127	0.513
LA	**PK**			1	0.443*	-0.333	-0.107	0.384*	0.530**	0.523**	0.114
	P				0.018	0.084	0.589	0.043	0.004	0.004	0.564
AA	**PK**				1	-0.034	-0.189	-0.271	0.265	0.278	0.501**
	P					0.863	0.336	0.163	0.173	0.152	0.007
BA	**PK**					1	0.333	0.426*	-0.332	-0.295	0.145
	P						0.083	0.024	0.085	0.128	0.463
CO_2	**PK**						1	0.311	0.064	0.079	0.195
	P							0.107	0.746	0.690	0.321
MOLD	**PK**							1	0.143	0.187	0.060
	P								0.467	0.340	0.760
IVOMD	**PK**								1	0.987**	0.243
	P									0.000	0.213
ME	**PK**									1	0.321
	P										0.095

PK: Pearson correlation, *Correlation is significant at 0.05 level, **Correlation is significant at 0.01 level, P: Significance degree

The differences between the groups were found to be statistically significant ($p<0.05$) when the DM, CA, ADF, NDF, IVOMD, ME, and CH_4 values of the silages were examined. When the DM values were examined, increases were observed in all trial groups compared to the control group. The highest DM value (31.93) was obtained from the group with PFJ +2% molasses added to the meadow grass. Henderson et al. (1982) determined the reason for the increase in silage DM level due to the inhibition of butyric acid bacteria and many types of microorganisms according to the decrease in pH level in the silo. Also, the increase in DM value in the group with the addition of HoLAB supports the report that DM loss decreases due to the increase in the amount of lactic acid in the environment and conversion of sugars such as glucose and fructose into lactic acid by homofermentative LAB in the silo (Kung 2018). When the CA values of the silages were examined, increases were observed in the HetLAB +2% molasses added group. When the CP values were examined, increases were observed in all trial groups compared to the control group. The reason is that readily fermentable soluble carbohydrate sources have a positive effect on silage fermentation and reduce proteolysis (Okuyucu 2018; Bingöl et al. 2009; Görü & Seydoşoğlu 2021). When the ADF values of the silages were examined, a decrease was observed in all trial groups compared to the control group, and a decrease was observed in the NDF value in the HetLAB +2% molasses added group ($p<0.05$). In the studies carried out by Nsereko et al. (2008) and Ding et al. (2019), it was reported that as it was known that LAB cannot degrade the polysaccharides that form the cell wall, in recent researches it was found that some special HetLAB strains such as *L. buchneri*, *L. reuteri*, *L. crispatus,* and *L. brevis* produced ferulate esterase, which could reduce the cell wall coverage, and this result was compatible with the result of our study that the lowest NDF content was in the HetLAB +2% molasses added group. Drouin et al. (2019), in their study of alfalfa plant with *Pediococcus pentosaceus* and *Lactobacillus plantarum* inoculant, reported that they increased the breakdown of hemicellulose polysaccharides, one of the plant cell wall elements and this result supported the current study.

In the study, when the IVOMD and ME values of the silage groups were examined, increases were observed compared to the control group, but the highest was obtained in the group with meadow grass PFJ +2% molasses ($p<0.05$). The high *in vitro* digestibility of organic matter of supplemented silages can be explained by the lowest content of NDF and ADF relativeto control group. In addition, this increase supports the report of Okuyucu (2018) that the main fermentation product in silages is LA and that LA is fermented in the rumen and evaluated by ruminants, and accordingly, it increases the IVOMD and ME values. A positive correlation was observed between LA and IVOMD (R=0.530), and between LA and ME (R=0.523). When the CH_4% value of the silages was examined, a decrease was observed in the PFJ +2% molasses added group compared to the other groups ($p<0.05$). The reason for this may be the decrease in methane production parallel with the decrease in *in vitro* organic matter digestibility.

In the study, when the fermentation characteristics (pH, NH_3-N, CO_2, LA, AA, BA, mold) of alfalfa silage groups were examined, the differences between the groups were found to be statistically significant ($p<0.05$). When yeast values were examined in all silages, no yeast was detected in all trial groups. The pH values of the silage groups were determined in the range of (4.35-5.58). While a decrease was observed in all trial groups compared to the control group, the lowest was determined in the group with meadow grass PFJ +2% molasses. It was concluded that this decrease in the additive groups was due to higher lactic acid content in these groups than in the control group. The pH values in the whole additive group were found to be by the statement of Kung and Shaver (2001) that the pH value for quality legume silages should be in the range of 4.3-4.7. The pH values of silages were affected by many factors such as the type of LAB used as the inoculant source, the buffering capacity of the plant, the content of WSC, the structure of the microbial flora present in the plant, and the process applied in the preparation of the silage. When Table 4 was examined, a negative correlation (R=-0.527) was observed between pH and LA. Luo et al. (2021) reported that pH values were in the range (of 4.48-4.84) in their study by adding 1-3 percent molasses to the alfalfa plant, and this report was similar to the current study.

In the study, when the silage NH_3-N values were compared with the control group, a decrease was observed in all trial groups ($p<0.05$). In the study, the lowest ammonia nitrogen value was determined in the group with PFJ +2% molasses. This is because the protease enzymes in the plant break down the proteins in the plant into peptides, amides, and ammonia during the proteolysis event. Due to the decrease in the efficiency of proteolytic enzymes as a result of the lactic acid production rate and rapid pH decrease in the environment, proteolysis decreases, so the degradation of proteins also decreases (Reich & Kung Jr 2010). Carpintero et al. (1979) reported that the proportion of NH_3-N should be lower than 11% of silage total N to be evaluated in the good-quality silage class. The statement reported by Bureenok et al. (2011) that the addition of molasses to alfalfa silage reduced the ammonia nitrogen value was compatible with the result of this study.

The LA value of the silages increased in all trial groups compared to the control group ($p<0.05$). When the LA value of the silages (Table 3) was examined, the highest lactic acid content and the lowest pH value were observed in the meadow grass PFJ +2% molasses added silage group. There was determined, a negative correlation (R=-0.527) between pH and LA, and a positive correlation (R=0.443) between LA and AA. In quality silage, the lactic acid level should be 65-70% of the total silage acids (Kung & Shaver 2001). In this study, the amount of lactic acid remained below the specified ratio in the control group, while the highest ratio was determined in the silage group with meadow grass PFJ -2% molasses. Gao et al. (2021) reported that LA values increased due to the addition of molasses to alfalfa silage and this statement was compatible with the present study.

When the AA values of the silages were examined, the highest acetic acid value (19.88) was determined in the heterofermentative inoculant +2% molasses added group. When BA values were examined, BA was detected in the control group, but not in the additive groups. In this study, it is thought that the high CP ratio and low DM and WSC content of the alfalfa plant used as silage material cause insufficiency in the production of lactic acid, which is necessary to inhibit the growth of clostridial bacteria (Weinberg et al. 1988). As a result of this state, saccharolytic clostridia transform the WSC and organic acids in the plant into butyric acid (Ohshima 1997). The low LA and high pH explains the presence of butyric acid in the control group.

When the CO_2 and mold values of the silages were examined, a decrease was observed in all experimental groups compared to the control group ($p<0.05$). In the study, the highest CO_2 value of the silages was determined in the homofermentative inoculant +2% molasses added to the group, while the lowest value was found in the alfalfa PFJ +2% molasses added group. When compared with all groups, there were obtained the highest acetic acid, the lowest yeast, and mold content and the lowest CO_2 ratio in the heterofermentative inoculant +2% molasses added to the group. These were supported by the results reported by Ali et al. (2020) that the amount of acetic acid produced by heterolactic LAB fermentation in silages has an inhibitory effect against microorganisms that cause silage deterioration, prevents the reproduction and activity of yeasts, reduces CO_2 production, in other words, and improves aerobic stability values.

4. Conclusions

This study was conducted to evaluate the effect of adding fermented natural LAB liquid from different sources and some LAB inoculants to alfalfa silage on fermentation, IVOMD, and gas production. It was seen that the pH, CO_2, yeast, and mold values of the silages decreased in all trial groups compared to the control group. In terms of all parameters, it was concluded that adding meadow grass PFJ +2% molasses had positive effects on silage quality, fermentation characteristics and *in vitro* organic matter digestion, and this fermented natural LAB liquid can be efficiently used as silage additive.

Ethical Statement: In this study, it was reported by the Harran University Animal Experiments Local Ethics Committee that there is no need for an ethics committee document. (decision no: 2022/006/08, date: 14.09.2022).

Data availability: Data are available on request due to privacy or other restrictions.

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References

Ali N, Wang S, Zhao J, Dong Z, Li J, Nazar M & Shao T (2020). Microbial diversity and fermentation profile of red clover silage inoculated with reconstituted indigenous and exogenous epiphytic microbiota. *Bioresource Technology* 314: 123606. doi.org/10.1016/j.biortech.2020.123606

AOAC (2005). Official methods of the association of official analytical chemists. Association of Official Analytical Chemists.

Arriola K G, Kim S C & Adesogan A T (2011). Effect of applying inoculants with heterolactic or homolactic and heterolactic bacteria on the fermentation and quality of corn silage. *Journal of Dairy Science* 94(3): 1511-1516. doi.org/10.3168/jds.2010-3807

Ashbell G, Weinberg Z G, Azrieli A, Hen Y & Horev B (1991). A simple system to study the aerobic determination of silages. *Canadian Agricultural Engineering* 34: 171-175.

Atalay A İ (2015). The use of locust bean fracture as a silage additive for grass. Kahramanmaraş Sütçü İmam University, Graduate School of Natural and Applied Sciences. Doctoral thesis.

Aydın S S (2019). The effect of lyophilized and frozen natural lactic acid bacteria juice on the count of lactic acid bacteria and quality of alfalfa silage. Ph.D. Thesis, Harran University, Graduate School of Health Sciences, Şanlıurfa, Türkiye. (in Turkish)

Aydin S S & Denek N (2019). The effect of fermented lactic acid juice prepared with different levels of sucrose and incubation times on the alfalfa silage quality. *Harran Üniversitesi Veteriner Fakültesi Dergisi* 8(1): 44-51.

Bingöl N T, Bolat D, Karslı M A & Akça İ (2009). Effects of molasses addition into barley-sainfoin mixture at varying levels on silage quality and digestibility. *Atatürk Üniversitesi Veteriner Bilimleri Dergisi* 4(1): 23-30.

Broderick G A & Kang J H (1980). Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. *Journal of Dairy Science* 63(1): 64-75. doi.org/10.3168/jds.S0022-0302(80)82888-8

Bureenok S, Namihira T, Tamaki M, Mizumachi S, Kawamoto Y & Nakada T (2005). Fermentative quality of guinea grass silage by using fermented juice of the epiphytic lactic acid bacteria (FJLB) as a silage additive. *Asian-Australasian Journal of Animal Sciences* 18(6): 807-811.

Bureenok S, Suksombat W & Kawamoto Y (2011). Effects of the fermented juice of epiphytic lactic acid bacteria (FJLB) and molasses on digestibility and rumen fermentation characteristics of ruzigrass (Brachiaria ruziziensis) silages. *Livestock Science* 138(1-3): 266-271. doi.org/10.1016/j.livsci.2011.01.003

Can L (2010). The effects of inoculants and/or enzymes on the fermentation, aerobic stability and in vitro organic matter digestibility characteristics of triticale: Hungarian vetch silages. MSc Thesis. Namık Kemal University, Graduate School of Natural and Applied Sciences, Tekirdağ, Türkiye. (in Turkish)

Carpintero C M, Henderson A R & McDonald P (1979). The effect of some pre-treatments on proteolysis during the ensiling of herbage. *Grass and Forage Science* 34(4): 311-315. doi.org/10.1111/j.1365-2494.1979.tb01483.x

Çotuk G (2016). Effect of wheat bran and pudding added to alfalfa silage on fermentation, aerobic stability and in vitro digestibility. MSc Thesis. Namık Kemal University. *Graduate School of Natural and Applied Sciences*.

Davies D R, Merry R J & Bakewell E L (1996). The effect of timing of slurry application on the microflora of grass, and changes occurring during silage fermentation. *Grass and Forage Science* 51(1): 42-51. doi.org/10.1111/j.1365-2494.1996.tb02036.x

Denek N, Can A, Avci M, Aksu T & Durmaz H (2011). The effect of molasses-based pre-fermented juice on the fermentation quality of first-cut lucerne silage. *Grass and Forage Science* 66(2): 243-250. doi.org/10.1111/j.1365-2494.2011.00783.x

Ding Z T, Xu D M, Bai J, Li F H, Adesogan A T, Zhang P, & Guo X S (2019). Characterization and identification of ferulic acid esterase-producing Lactobacillus species isolated from Elymus nutans silage and their application in ensiled alfalfa. *Journal of Applied Microbiology* 127(4): 985-995. doi.org/10.1111/jam.14374

Drouin P, Mari L J & Schmidt R J (2019). Lactic acid bacteria as microbial silage additives: current status and future outlook. In New Advances on Fermentation Processes. *IntechOpen* 266.

Evren M, Apan M, Tutkun E & Evren S (2011). Geleneksel fermente gıdalarda bulunan laktik asit bakterileri. *Elektronik Mikrobiyoloji Dergisi* 9(1): 11-17.

Filya I, Ashbell G, Hen Y & Weinberg Z G (2000). The effect of bacterial inoculants on the fermentation and aerobic stability of whole crop wheat silage. *Animal Feed Science and Technology* 88(1-2): 39-46. doi.org/10.1016/S0377-8401(00)00214-5

Gao R, Wang B, Jia T, Luo Y & Yu Z (2021). Effects of different carbohydrate sources on alfalfa silage quality at different ensiling days. *Agriculture* 11(1): 58. doi.org/10.3390/agriculture11010058

Goel G, Makkar H P, & Becker K (2008). Effects of Sesbania sesban and Carduus pycnocephalus leaves and Fenugreek (Trigonella foenum-graecum L.) seeds and their extracts on partitioning of nutrients from roughage-and concentrate-based feeds to methane. *Animal Feed Science and Technology* 147(1-3): 72-89. doi.org/10.1016/j.anifeedsci.2007.09.010

Görü N & Seydoşoğlu S (2021). Determination of silage quality of some winter cereals (oat, barley, rye and triticale) mixed with common vetch (Vicia sativa L.). *SDU Journal of the Faculty of Agriculture* 16(1): 26-33.

Gül S & Coşkuntuna L (2016). Çayır Otu Silajlarına Farklı Katkı Maddesi İlavesinin Silaj Fermantasyonu ve Aerobik Stabilite Üzerine Etkileri.

Güney F & Ertürk Ö (2020). Determination of the effects of propolis ethanolic extract on some properties of fruit yogurt during storage. *Mustafa Kemal University Journal of Agricultural Sciences* 25(2): 145-152. doi.org/10.37908/mkutbd.694712

Henderson A R, McDonald P & Anderson D H (1982). The effect of silage additives containing formaldehyde on the fermentation of ryegrass ensiled at different dry matter levels and on the nutritive value of direct-cut silage. *Animal Feed Science and Technology* 7(3): 303-314. doi.org/10.1016/0377-8401(82)90023-2

Jin-ling H, Li-ke W & Si-fa D (2013). Effects of previously fermented juice on nutritive value and fermentative quality of rice straw silage. *Journal of Northeast Agricultural University* (*English Edition*) 20(2): 48-52. doi.org/10.1016/S1006-8104(17)30019-3

Jones R (1995). Role of biological additives in crop conservation. Biotechnology in the Feed Industry, *Proc. of the 11th Annual Symposium Nottingham Univ* 627.

Jones R & Gogerddan P (1994). The importance of quality fermentation in silage making and future trends in forage production, Alltech. *8th Annual Europan Lecture Tour* 9(33): 5.

Koç F, Aksoy S O, Okur A A, Çelikyurt G, Korucu D & Özduven M L (2017). Effect of pre-fermented juice, Lactobacillus plantarum and Lactobacillus buchneri on the fermentation characteristics and aerobic stability of high dry matter alfalfa bale silage. *Journal of Animal and Plant Sciences* 27(5): 1426-1431.

Kung L (2018). Silage fermentation and additives. *Archivos Latinoamericanos de Producción Animal* 26(3-4): 61-66.

Kung L & Shaver R (2001). Interpretation and use of silage fermentation analysis reports. *Focus on Forage* 3(13): 1-5.

Luo R, Zhang Y, Wang F, Liu K, Huang G, Zheng N & Wang J (2021). Effects of sugar cane molasses addition on the fermentation quality, microbial community, and tastes of alfalfa silage. *Animals* 11(2): 355. doi.org/10.3390/ani11020355

Masuko T, Hariyama Y, Takahashi Y, Cao LM, Goto M & Ohshima M (2002). Effect of addition of fermented juice of epiphytic lactic acid bacteria prepared from timothy (Phleum pratense) and orchardgrass (Dactylis glomerata) on fermentation quality of silages. *Journal of Japanese Society of Grassland Science* 48(2): 120-125.

McDonald P, Henderson A R & Heron S J E (1991). The biochemistry of silage (2nd ed.) additives. *Chalcombe Publ* 340.

Menke K H, Raab L, Salewski A, Steingass H, Fritz D, & Schneider W (1979). The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. *The Journal of Agricultural Science* 93(1): 217-222. doi.org/10.1017/S0021859600086305

Menke K H & Steingass H (1988). Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Animal Research and Development* 28: 7-55. doi.org/10.2508/chikusan.79.483

Nsereko V L, Smiley B K, Rutherford W M, Spielbauer A, Forrester K J, Hettinger G H & Harman B R (2008). Influence of inoculating forage with lactic acid bacterial strains that produce ferulate esterase on ensilage and ruminal degradation of fiber. *Animal Feed Science and Technology* 145(1-4): 122-135. doi.org/10.1016/j.anifeedsci.2007.06.039

Ohshima M, Kimura E & Yokota H (1997). A method of making good quality silage from direct-cut alfalfa by spraying previously fermented juice. *Animal Feed Science and Technology* 66: 129-137. doi.org/10.1016/S0377-8401(96)01111-X

Okuyucu B (2018). The effects of lactic acid bacteria and enzyme mixture inoculants on the fermentation, aerobic stability, and feed value of alfalfa silage. MSc Thesis. Namık Kemal University, Graduate School of Natural and Applied Sciences, Tekirdağ, Türkiye. (in Turkish)

Playne M J & McDonald P (1966). The buffering constituents of herbage and silage. Journal of the *Science of Food and Agriculture* 17(6): 264-268. doi.org/10.1002/jsfa.2740170609

Polan C E, Stieve D E & Garrett J L (1998). Protein preservation and ruminal degradation of ensiled forage treated with heat, formic acid, ammonia, or microbial inoculant. *Journal of Dairy Science* 81(3): 765-776. doi.org/10.3168/jds.S0022-0302(98)75633-4

Reich L J & Kung Jr L (2010). Effects of combining Lactobacillus buchneri 40788 with various lactic acid bacteria on the fermentation and aerobic stability of corn silage. *Animal Feed Science and Technology* 159(3-4): 105-109. doi.org/10.1016/j.anifeedsci.2010.06.002

SPSS (1991). Inc. Statistical package for the social sciences (SPSS/PC+) *IL*.

Sun L, Jiang Y, Ling Q, Na N, Xu H, Vyas D, Adesogan A T & Xue Y (2021). Effects of adding pre-fermented fluid prepared from red clover or lucerne on fermentation quality and in vitro digestibility of red clover and lucerne silages. *Agriculture* 11(5): 454. doi.org/10.3390/agriculture11050454

Suzuki M & Lund C W (1980). Improved gas-liquid chromatography for simultaneous determination of volatile fatty acids and lactic acid in silage. *Journal of Agricultural and Food Chemistry* 28(5): 1040-1041.

Tao L, Zhou H, Zhang N, Si B, Tu Y, Ma T & Diao Q (2017). Effects of different source additives and wilt conditions on the pH value, aerobic stability, and carbohydrate and protein fractions of alfalfa silage. *Animal Science Journal* 88(1): 99-106. doi.org/10.1111/asj.12599

Turan A & Önenç S S (2018). Effect of cumin essential oil usage on fermentation quality, aerobic stability and in vitro digestibility of alfalfa silage. *Asian-Australasian Journal of Animal Sciences* 31(8): 1252. doi.org/10.5713%2Fajas.17.0834

Van Soest P V, Robertson J B & Lewis B A (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* 74(10): 3583-3597. doi.org/10.3168/jds.S0022-0302(91)78551-2

Wang J, Wang J Q, Zhou H & Feng T (2009). Effects of addition of previously fermented juice prepared from alfalfa on fermentation quality and protein degradation of alfalfa silage. *Animal Feed Science and Technology* 151(3-4): 280-290. doi.org/10.1016/j.anifeedsci.2009.03.001

Weinberg Z G, Ashbell G & Azrieli A (1988). The effect of applying lactic bacteria at ensilage on the chemical and microbiological composition of vetch, wheat and alfalfa silages. *Journal of Applied Microbiology* 64(1): 1-7. doi.org/10.1111/j.1365-2672.1988.tb02423.x

Zhang Q, Yu Z & Wang X (2015). Isolating and evaluating lactic acid bacteria strains with or without sucrose for effectiveness of silage fermentation. *Grassland Science* 61(3): 167-176. doi.org/10.1111/grs.12097

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The Effects of Hazelnut Husk Supplementation on Silage Quality, Deterioration, and *In Vitro* Digestion Parameters in Second Crop Maize

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ABSTRACT

This research investigates the effects of hazelnut husk on the low dry matter (DM) maize silage quality, microbial profile, deterioration, fiber components, and digestion parameters. Second crop maize was harvested at the milk stage of maturity (26.61% DM) and ensiled in laboratory silos with or without ground hazelnut husk. A total of 18 jars of silage were made utilizing two treatments (control silage and 15% hazelnut-contained silage), three different opening dates, and three replicates. All silage analyses were completed in all of the opening periods. Hazelnut husk increased the ($p<0.05$) silage DM, pH, ash, protein, and cellulose fractions content while only decreasing ($p<0.05$) hemicellulose. The lactic acid, propionic acid, acetic acid, and butyric acid in silages were reduced ($p<0.05$) by the hazelnut husk. The addition of hazelnut husk to the silages increased ($p<0.05$) the population of lactobacilli but had no influence ($p>0.05$) on the yeast-mold population. Hazelnut husk increased ($p<0.05$) aerobic stability in maize silages. Hazelnut husks reduced *in vitro* gas production value, digestible organic matter, metabolic energy, and net energy lactation values, but increased protozoa in the rumen ($p<0.05$). The hazelnut husk demonstrated a potential hygroscopic property in low DM maize silage by increasing silage DM and improving fermentation efficiency, as well as air stability during feedout.

Keywords: Hazelnut husk, Maize silage, Low dry matter, Fermentation, Air stability, Nutritive value

1. Introduction

The hazelnut tree (Corylus avellana, Betulaceae) is a valuable tree in the Mediterranean basin, particularly Turkey, which produces 60% of the world's hazelnuts. Turkey is the leading producer, producing approximately 776,000 t hazelnuts in shell in 2019, followed by Italy, Azerbaijan, and the United States, which produced approximately 98,530; 53,739; and 39,920 t, respectively. France and Spain, for example, have much smaller production capacities of 11,660 t and 12,370 t. The European Union's total hazelnut production capacity is 160,683 t. Germany, Italy, and France are major European importers. Total hazelnut production surpassed 1 million tons, demonstrating the global popularity of hazelnuts (FAOSTAT 2020). The husk, or green, leafy covering of hazelnuts, covers up to one-third of the hazelnut's exterior surface (Dok 2014). After harvest, the green leafy coverings of hazelnuts are separated from the shell in the first step of processing (Solmaz 2017) and are typically discarded (Piccinelli et al. 2016). The majority of this waste is ligno-cellulose shells, which are obtained after cracking the kernel and are used as a heat source, mulch, and furfural production in the industrial production of pigment. Commercially viable compounds found in hazelnut side products could be used to create valuable coproducts. Hazelnut shells contain phenolic compounds that have natural antioxidant properties (Stévigny et al. 2007). Candellone et al. (2019) suggested using hazelnut skin for its natural antioxidant properties observed that including hazelnut skin in the diet of rabbits improved their oxidative status and immune function. Cetinkaya and Kuleyin (2016) investigated the digestibility of different hazelnut internal skins in order to recommend hazelnut fruit skin as a roughage replacement in ruminant nutrition. Cetinkaya and Kuleyin (2016) proposed that the internal fruit skin of various hazelnut species could be used as a substitute roughage for ruminant nutrition due to

its crude and digestible nutritive qualities. Caccamo et al. (2019) investigated the impact of hazelnut skin in dairy ewes' diets on the biological and sensory qualities of ovine cheeses, finding that the by-product had a significant impact on the cheeses' lipid content, fatty acids (FAs), and gustatory profiles. Another study found that substituting hazelnut skin for dried beet pulp had no effect on feed intake or milk output in lactating ewes, but did improve the atherogenic index and health-promoting unsaturated FAs in ewe milk while lowering the milk protein percentage and viable cells (Campione et al. 2020). Furthermore, Renna et al. (2020) found that hazelnut skin contains a high concentration of antioxidants and that feeding this byproduct to dairy cows can significantly increase the vitamin E content of the milk. According to Renna et al. (2020), despite being a minor percentage of dry matter (DM) intake, using hazelnut skin in the cow's diet improves the sustainability of the acquired milk in terms of the food-feed challenge and lowers the daily cost of the diet. Maize (*Zea mays*) silage is a common feed source in many animal agricultural practices. It meets a significant portion of the energy and fiber daily requirements of dairy cows when ensiled at optimal DM (28-35%) concentrations (Bell et al. 2007). However, in some situations, such as during the prolonged rainy season (Haigh 1997; Bell et al. 2007) or when maize is sown as a second crop in midsummer, achieving an optimal DM content before harvest can be difficult (Khorvash et al. 2006; Ranjbari et al. 2007). In all these situations, effluent generation has been considered a nutrient loss, so finding alternative solutions for raw sewage reduction or elimination should be a top priority. Since the maize plant is not optimal for wilting, one technique is to combine additives with ensiled low-DM maize (Muck et al. 2018). Various cereal straws, wheat bran, soy bran flakes, dried meadow grass, dried alfalfa grass, legume stalks, soybean husks, cereals, dried sugar beet pulp, bentonite, zeolite, whey powder, dried molasses, newspapers or waste papers are the additives for this purpose (McDonald et al. 1991; Jones & Jones 1996; Khorvash et al. 2006; Barmaki et al. 2018).

So far, most of the trials (Cetinkaya & Kuleyin 2016; Caccamo et al. 2019; Campione et al. 2020; Renna et al. 2020) have investigated the nutritional value of hazelnut inner skin as a diet ingredient. To our knowledge, there has yet to be a study on the nutritional benefits of the hazelnut's outer skin when utilized as a silage additive. For this reason, this study investigates how incorporating hazelnut husk into second crop maize forage affected silage fermentation, microbiological structure, aerobic stability, cell wall components, and *in vitro* digestion parameters.

2. Material and Methods

In the study, the hazelnut husk was obtained from Düzce Province's Gölyaka District, Kuyudüzü District. The husks were crushed and passed through a 2 mm sieve before being used in the experiment. A maize variety called DKC-7211 (Monsanto Gıda ve Tarım Tic. Ltd. Sti.) with a growth period of 120 days was used as silage material in 2018. A maize plant was cultivated in the area between latitudes 40°13' 35.1" north and longitudes 28°51' 48.8" east. In 2018, the total precipitation was 65.5 mm, the average temperature was 24 °C, and the relative humidity was 64%. According to the soil analysis results, the soil was heavy and medium textured, had a pH of 7.9, and was not saline. The soil had a low organic matter content but was free of lime, was rich in available phosphorus and potassium, and had a sufficient quantity of nitrogen. To determine plant height, ten plants from a specific row were cut. Five out of ten sampled plants were separated into stem, leaf, and ear fractions to determine their percentages in a whole-plant weight. Forage samples were collected from a 5.7 m^2 center area of each plot to assess forage yields. 500 g of fresh plant material was collected and dried for 48 hours at 75 °C to calculate hay yields. As a result of these measurements, the plant height was 2.78 m, the forage yield was 5990 kg/da, the DM yield was 1660 kg/da, and the ear/stalk ratio was 29.2%.

Maize was harvested when it reached the milk stage of maturity and chopped through a 2 cm screen with a standard forage harvester. The freshly chopped raw maize plant was then immediately delivered to the laboratory. It is homogeneously combined with ground hazelnut husk at a rate of 15% and ensiled into 1.5-liter special laboratory type jars (Weck, Wehr, Oflingen, Germany). For each application, two treatments (control and 15% husk supplemented), three opening dates (8, 21, and 60 days), and a total of 18 jars of silage were prepared as three parallels. For the control and hazelnut husk supplemented groups, each jar was filled with approximately 1.3 and 1.1 kg (fresh weight) of chopped forage, respectively. The packing densities were 230 and 248 kg/DM/m^3, respectively. On the eighth, twenty-first, and sixty-first days after ensiling, three jars from each treatment (control silages and silages with 15% husk supplemented) were opened, and pH, DM, and microbiological tests were performed on all silages on the same day. The 40 g wet silage sample was mixed with 360 mL of distilled water, shaken for 3 minutes in a stomacker, then filtered using Whatman paper. It was then centrifuged at 12000 rpm for 15 minutes before being transferred to sterile eppendorf tubes. The samples were kept at -20 °C in a deep freezer. A pH meter (Sartorius PB-20, Goettingen, Germany) was used to measure the silage pH directly from the silage juice. Each replicate's subsamples were composited, and the DM was measured by oven-drying at 65 °C for 48 hours. The dried samples were then milled to pass through a 1-mm screen in a laboratory mill (Elmeksan, E.M.S. 101-TIP, Turkey). Analytical DM was prepared for chemical analysis by oven-drying previously dried silage samples at 100 °C for 4 hours. All samples were analyzed for ash

(method 942.05, AOAC 2002), and ether extract (method 942.05, AOAC 2002). The sodium sulfite addition method without amylase was used to analyze neutral detergent fiber (NDF) and acid detergent fiber (ADF), and the results were given with the remaining ash (Van Soest et al. 1991). The difference between NDF and ADF was used to calculate hemicellulose (HCEL). The DM disappearance (DMD) in silages in the form of gases and effluents was quantified by weight difference, with respect to Cai and Ohmomo (1995). Gas chromatography was used to assess the organic acid and ethanol content of the fresh forage and silages (Agilent Technologies 6890N Network GC System, 7683 B Series Injector). In the analysis, a capillary column (Stabilwax®-DA; Crossbond "Carbowax"-PEG for acidic chemicals, 30 m, 0.25 mm ID, 0.25 m df, maximum program temperature of 260 °C) suited for the specified gas chromatography was utilized. The lactic acid content of the fresh forage and silages were determined using the spectrophotometric method described earlier by Barker and Summerson (1941). Fresh forage and silage microbiological analyses were performed in quadruplicate (per treatment for each replicate) and the results were presented on a fresh and wet silage basis. Lactobacilli colonies were cultivated on Rogosa agar (Oxoid CM627, Oxoid, Basingstoke, U.K.), and yeast and mold were cultivated on malt extract agar (Difco, Detroit, MI) that had been acidified with lactic acid to pH 4.0. Plates were incubated at 30 °C for 3 days. The $\log_{10}$ transformation was applied to all microbiological data. An aerobic deterioration test (5 days of air exposure) was performed at the end of the ensiling period, according to Ashbell et al. (1991). As previously described, silage samples were examined for pH, yeast, and mold.

An *in vitro* ruminal fermentation study was conducted to evaluate the rumen fermentation properties of hazelnut husk as a maize silage supplement. Rumen fluid was taken from ruminally cannulated 600 kg Holstein non-lactating dairy cows (n=2) before morning feeding. The animals were cared for and handled in accordance with the Turkish Directorate of Provincial Agriculture and Forestry's welfare and ethics standards for experimental animals. The donor cows were fed a 50/50 combination of maize silage and concentrate to ensure that the rumen fluid had a balanced cellulolytic and amylolytic activity. Rumen fluid was taken before morning feeding and immediately transported to the laboratory after being placed in a warm Thermos flask (39 °C). Rumen fluid was squeezed through cheesecloth and placed in a 39 °C Erlenmeyer flask. For *in vitro* rumen fermentation, the Menke and Steingass (1988) method was used to create a buffer combination (comprising micro-and macro-elements, a reducing agent, and a resazurin reduction indicator). In a warmed bottle (39 °C), particle-free rumen fluid and buffer were combined and continuously gassed with CO_2. As incubation vessels, glass syringes (Fortuna®, Häberle Labortechnik, Germany) were used. Each syringe contained 30 mL of rumen fluid-buffer medium. Each syringe had about 200 mg of dry feed sample. The samples were incubated in triplicate. As blanks, triplicates of bottles with no substrate were employed. The total volume of gas produced was measured at 3, 6, 12, 24, 48, and 96 hours. The quantities of generated gas were computed in the Neway computer program using the model $y= a+b (1-e^{-ct})$ provided by Orskov and McDonald (1979).

In the model;

a = gas amount of easily soluble fractions, mL

b = gas production amount of insoluble fractions, mL

c = gas production rate of insoluble fractions (b) ($hour^{-1}$)

a+b = potential gas production, mL

t = incubation time, hours (h)

y = amount of gas produced during t

The organic matter digestibility and metabolic energy (ME) contents of silages were calculated according to the formulas reported by Menke & Steingass (1988) for forages.

OMD (%)= 15.38+0.8453xGP+0.0595xHP+0.0675xCA (1.1)

ME (MJ/kg DM)= $2.20+0.1357xGP+0.0057xCP+0.0002859xEE^2$ (1.2)

GP: net gas production (mL) after 24 hour incubation period

CP: Crude protein value (%) in DM

EE: Ether extract value (%) in DM

CA: Crude ash value (%) in DM

The following formula was used to calculate the net energy lactation (NE_L) content of silages (Menke et al. 1979).

NE_L (MJ/kg DM)= 0,101xGP+0,051xCP+0,112xEE (1.3)

GP: net gas production (mL) after 24 hour incubation period

CP: Crude protein value (%) in DM

EE: Ether extract value (%) in DM

The protozoa count was performed after 96 hours of incubation. To count protozoa, one milliliter of rumen fluid was combined with 49 milliliters of rumen protozoa counting solution (2.02% formalin and 15.15% glycerol). Using the Boyne et al. (1957) approach, diluted ruminal fluid samples were counted using a Fuchs Rosenthal counting chamber.

The data from silage quality were analyzed using a completely randomized design with three replications and the SAS GLM procedure for analysis of variance (Statistical Analysis System, version 6.0). Tukey's test was used to test for differences between means, when $p<0.05$, differences between treatments were regarded as significant, and when $0.05<p<0.10$, considered to be significant.

3. Results

The chemical composition of fresh maize and hazelnut husk are shown in Table 1. Fresh maize chemical structure is distinguished by a low DM (26.6), crude protein (6.7%), fat (2.0%) and low fibrous fractions (43.7, 26.7 and 2.7% for NDF, ADF, and ADL, respectively). Hazelnut husk's chemical structure is distinguished by a low protein content (6.3%) and crude fat (0.81 percent), as well as a high pH (7.9), DM (77%) and fibrous fractions (67.6, 53.4, and 26.7% for NDF, ADF, and ADL, respectively).

Table 1- The chemical composition of fresh maize and hazelnut husk

Item	*Maize*	*Hazelnut husk*
pH	5.95	7.90
DM,%	26.61	76.98
OM, % DM	94.59	93.67
CP, % DM	6.74	6.27
EE, % DM	2.01	0.81
CCEL, % DM	21.80	31.48
CA, % DM	5.41	5.81
NDF, % DM	43.76	67.55
ADF, % DM	26.68	53.36
ADL, % DM	2.71	26.65
HCEL, % DM	17.08	14.19

DM: Dry matter, OM: Organic matter, CP: Crude protein, EE: Ether extract, CCEL: Crude cellulose, CA: Crude ash, NDF: Neutral detergent fiber, ADF: Acid Detergent Fiber, ADL: Acid Detergent lignin, HCEL: Hemicellulose

The results obtained from the chemical composition of silages are given in Table 2. The DM content of silages varied between 25.35% and 33.05% in control and hazelnut husk treated silages, respectively. Hazelnut husk had a high absorbency potency, which increased ($p<0.05$) the DM content of low DM maize silage (25.5%) by adding 15% hazelnut husk (32.5%). While opening days had no effect on the DM content of the control silages ($p>0.05$), there was an increasing trend in the DM content of the hazelnut husk-treated silages ($p=0.07$). The highest OM content was found in control silages ($p<0.05$). The crude ash content of hazelnut-treated silages was found to be higher than that of control silages ($p<0.05$). The highest ($p<0.05$) crude protein content was found in hazelnut husk treated silages on the 21st day of ensiling. On day 60, the crude protein content of all silages was comparable ($p>0.05$). The ether extract content of the silages did not differ between treatments ($p>0.05$). Regardless of treatment, the NDF content of maize silages decreased as the ensiling dates progressed ($p<0.05$). The control silages had the lowest ($p<0.05$) levels of CCEL, NDF, ADF, and ADL. The HCEL content of the hazelnut husk-treated silages was lower ($p<0.05$) than that of the control silages.

Table 2- The effect of the hazelnut husk additive on the chemical composition (% DM) of maize silages

Parameters	*Treatment*	*Ensiling days*			*SEM*	*P value*		
		8	21	60		Day	Trt	Day*Trt
DM, %	Control	25.77c	25.80c	25.35c	0.328	0.07	*	*
	Hazelnut husk	31.12b	32.50ab	33.05a				
DMD	Control	2.67c	2.43d	3.47a	0.046	*	*	*
	Hazelnut husk	2.82b	1.86e	2.43d				
OM	Control	94.41a	94.46a	94.48a	0.093	NS	*	NS
	Hazelnut husk	93.93b	94.12ab	93.94b				
CA	Control	5.59b	5.54b	5.52b	0.093	NS	*	NS
	Hazelnut husk	6.05a	5.87ab	6.07a				
CP	Control	6.28c	6.99b	7.19b	0.112	*	*	*
	Hazelnut husk	6.86b	7.62a	7.11b				
EE	Control	3.56	3.90	4.10	0.344	NS	NS	NS
	Hazelnut husk	2.98	3.92	3.76				
CCEL	Control	24.95d	24.00e	22.29f	0.189	*	*	*
	Hazelnut husk	31.51a	30.51b	28.55c				
NDF	Control	47.01b	45.53b	39.77c	0.794	*	*	*
	Hazelnut husk	53.93a	49.12b	48.17b				
ADF	Control	28.24c	28.25c	26.28c	0.469	*	*	*
	Hazelnut husk	40.70a	36.52b	36.12b				
ADL	Control	2.79e	3.09d	2.02f	0.04	*	*	*
	Hazelnut husk	10.25a	9.25b	8.15c				
HCEL	Control	18.77a	17.28a	13.49b	0.697	*	*	*
	Hazelnut husk	13.23b	12.60b	12.05b				

SE: Standard error, DM: Dry matter, DMD: Dry matter disappearance, OM: Organic matter, CA: Crude ash, CP: Crude protein, EE: Ether extract, CCEL: Crude cellulose, NDF: Neutral detergent fiber; ADF: Acid detergent fiber, ADL: Acid detergent lignin, HCEL: Hemicellulose, NS: Not significant, *p<0.05. The differences between the averages indicated with different letters on the same row are significant (p<0.05)

The results obtained from the fermentative properties and microbial structure of silages are given in Table 3. Hazelnut husk-treated silages had the highest (p<0.05) pH and the lowest (p<0.05) levels of lactic acid, acetic acid, propionic acid, and butyric acid. On the 60th day of ensiling, the control silages had the lowest (p<0.05) silage pH. The final pH of the silages ranged from 3.64 to 3.78, indicating that they had all been well preserved. There was no difference in the ethanol content between treatments (p>0.05). After 60 days of ensiling, hazelnut husk application increased LAB counts (p<0.05), but had no effect on mold counts (p>0.05). Although no yeast growth was detected in any of the silages, the mold counts increased in control silages as the ensiling period progressed (p<0.05).

Table 3- The effect of the hazelnut husk additive on the pH, organic acids (g/kg DM), ethanol (g/kg DM) concentrations and microbial structure (cfu/g DM) of maize silages

Parameters	*Treatment*	*Ensiling days*			*SE*	*P value*		
		8	21	60		Day	Trt	Day*Trt
pH	Control	3.84a	3.79b	3.64c	0.020	*	*	*
	Hazelnut husk	4.00a	3.85b	3.78b				
Ethanol	Control	0.09	0.10	0.12	0.002	NS	NS	NS
	Hazelnut husk	0.08	0.10	0.10				
LA	Control	43.21d	50.85bc	56.33a	0.563	*	*	*
	Hazelnut husk	40.33e	48.26c	52.72b				
AA	Control	26.39a	17.73c	13.44de	0.382	*	*	*
	Hazelnut husk	23.08b	14.92d	11.63e				
PA	Control	2.04a	1.03d	1.23e	0.029	*	*	*
	Hazelnut husk	1.82b	0.94d	0.96d				
BA	Control	0.24ab	0.25ab	0.26a	0.006	*	*	*
	Hazelnut husk	0.18c	0.18c	0.22b				
LAB	Control	9.14a	8.34b	7.00c	0.104	*	*	*
	Hazelnut husk	9.07a	8.99a	7.42c				
Yeast	Control	0	0	0	0	NS	NS	NS
	Hazelnut husk	0	0	0				
Mold	Control	3.37	4.77	4.97	0.355	NS	NS	NS
	Hazelnut husk	4.51	4.25	4.15				

DM: Dry matter, LA: Lactic acid, AA: Acetic acid, PA: Propionic acid, BA: Butyric acid, SE: Standard Error, NS: Not significant, *p<0.05. cfu: colony-forming unit, LAB: Lactic acid bacteria. The differences between the averages indicated with different letters on the same row are significant (p<0.05)

Changes in pH, CO_2 production, yeast and mold number, and visual assessment of mold were evaluated as maize silage deterioration markers (Table 4). When the deterioration characteristics of maize silages were compared, hazelnut husk treated silages had the lowest ($p<0.05$) CO_2 production and visible molding, while there was no treatment effect ($p>0.05$) on mold and yeast count.

Table 4- Effect of hazelnut husk additive on the deterioration of maize silages

Parameters	*Control*	*Hazelnut Husk*	*SE*	*P value*
pH	3.64b	3.79a	0.022	*
CO_2 (g/kg DM)	3.42a	1.79b	0.651	*
LAB (cfu/g DM)	7.26b	8.51a	0.225	*
Mold+Yeast (cfu/g DM)	7.18	7.59	0.115	0.07
Visible molding**	3	2		

SE: Standard error, CO_2: Carbon dioxide, DM: Dry matter, LAB: Lactic acid bacteria, cfu: Colony-forming unit, *p<0.05.
**It is a visual assessment of the molding stage of silages with numbers ranging from 1 to 5. 1: no mold, 2: very little mold in spots, 3: speckled mold on the surface, 4: locally moldy areas, the surface partly covered with mold, and 5: a silage whose surface is totally covered with mold, has a strong odor, and the particles are stuck together. These assessments were completed by two people and then averaged. The differences between the averages indicated with different letters on the same row are significant (p<0.05)

Table 5 shows the ruminal fermentation pattern markers as pH value and *in vitro* gas production at various hours, as well as OMD, ME, and NEL values and protozoa counts in rumen fluid at 96 hours. Total gas production gradually increased in all treatment groups from 3 to 96 hours ($p<0.05$). Starting at 3 hours and continuing until 96 hours, the addition of hazelnut husk decreased ($p<0.05$) total gas production. Adding hazelnut husks reduced the amount of easily fermentable DM ("a" value) slightly ($p>0.05$, Table 5). The "b" value, which was 45.23 mL in the control silage, was reduced to 31.89 mL with the addition of husk, while the a+b value was reduced from 50.28 mL to 35.92 mL with the addition of hazelnut husk ($p<0.05$, Table 5). The addition of hazelnut husk to maize silage had no effect ($p>0.05$, Table 5) on the gas production rate constant ("c"). When compared to control silages, the addition of hazelnut husk to maize silage decreased the *in vitro* OMD, ME, and NEL while increasing the protozoa count ($p<0.05$).

Table 5- Effect of hazelnut husk additive on the *in vitro* gas production (mL) and parameters, rumen pH, organic matter digestion (OMD, %), metabolic energy (ME, MJ/kg DM) and net energy lactation levels (NEL, MJ/kg DM), protozoa populations (cfu/g) of maize silages

Parameters	*Control*	*Hazelnut husk*	*SE*	*P value*
Incubation time (hour)				
3	10.67a	7.64b	0.399	*
6	16.67a	11.17b	0.660	*
12	26.17a	15.95b	0.623	*
24	35.83a	24.29b	0.712	*
48	45.17a	31.23b	1.136	*
72	48.83a	33.79b	1.552	*
96	50.42a	35.54b	1.934	*
Gas production parameters				
a	5.04	4.03	0.631	NS
b	45.23a	31.89b	1.489	*
a+b	50.28a	35.92b	0.099	*
c	0.18	0.05	1.972	NS
pH	6.61	6.64	0.044	NS
OMD (%)	53.67a	44.24b	0.706	*
ME (MJ/kg DM)	7,95a	6.31b	0.111	*
NE_L (MJ/kg DM)	4.45a	3.24b	0,080	*
Protozoa (cfu/g)	5,42b	5.59a	0.030	*

SE: Standard error, cfu: Colony-forming unit, NS: Not significant, *$p<0.05$. a: the volume of gas formed at the beginning (mL), b: the volume of gas formed over time (mL), a+b, total gas output (mL), c: Constant gas output rate (mL/hour), The differences between the averages indicated with different letters on the same row are significant ($p<0.05$)

4. Discussion

Hazelnut husk is rich in complex carbohydrates, has a high DM content (76.98%, Table 1), and is largely composed of cellulose (Jones & Jones 1996). As a result, it may be an effective supplement to improve the DM content of the silage material. This research examined how hazelnut husk affected silage fermentation, microbial structure, aerobic stability, cell wall components, and the *in vitro* digestibility of second crop maize forage harvested at low DM (26.61%).

The chemical content of maize silage studied in this investigation was comparable to that reported by others working with low DM maize silage (Özdüven et al. 2009; Barmaki et al. 2018, Altınçekiç & Filya 2018). According to Xu et al. (2012), total carbohydrate was the most abundant component in all hazelnut shells, accounting for 93.4% to 96.7%, with crude fiber accounting for more than 85%. Protein was the second most abundant component in the shells (2.1-4.0%), followed by ash (0.8-2.0%) and oil (0.3-0.7%). Renna et al. (2020) investigated the use of hazelnut skin as a dietary source for dairy cows. They observed that hazelnut skin has high ether extract (224 g/kg DM), NDF (530 g/kg DM), and ADF (492 g/kg DM) concentrations, but low CP (62 g/kg DM) and lignin concentrations (24 g/kg DM). According to Ozcan & Kılıc (2018), hazelnut husk pellets contained 9.32% DM crude protein and 0.84% DM ether extract. In one experiment, various hazelnut hull varieties were evaluated as a source of ruminant feed (Cetinkaya &

Kuleyin 2016). In that same study, the chemical composition did not differ between varieties with the exception of crude fat, which ranged from 17% to 21%, and DM, CP, NDF, ADF, and ADL, which were 91.1, 5.9, 30.3, 48.6, and 25.4%, respectively. In our study, we discovered that the crude fat content of hazelnut outer skin is lower (0.81% of DM, Table 1) than the findings of Renna et al. (2020) and Cetinkaya & Kuleyin (2016), but similar to the findings of Xu et al. (2012) and Ozcan & Kılıc (2018). Crude protein, on the other hand, was comparable to Renna et al. (2020), but lower than Ozcan and Kılıc's (2018) findings and higher than Xu et al. (2012) and Cetinkaya & Kuleyin's (2016) findings.

The addition of dry ingredients to moist silage resulted in an increase in DM content due to the dilution effect (Khorvash et al. 2006). Higher DM content in silages may be important not only for silage quality, but also for maintaining high feed consumption in dairy cows, as moist silage has been shown to reduce feed intake (Lahr et al. 1983). Silage fermentation tends to generate volatiles, which causes the apparent DM of the silage to decline, particularly in comparison to fresh material (Porter & Murray 2001). Higher DM preservation as a result of hazelnut husk application could be attributed to the additive's increased fermentation efficiency (Woolford 1983). This also supports the finding that hazelnut husk-treated silages had the lowest ($p<0.05$) DMD, particularly on the 21st day of ensiling (Table 2). All silages had a higher CP content (7.1%) than fresh forage (6.74%), indicating that the ensiling process protects forage by allowing for controlled fermentation with less proteolysis in silages (McDonald et al. 1991). Cell wall decomposition in silages is thought to be caused by the acidic content in the silage, and hydrolysis of hemicellulose (Kung et al. 2003) can increase the availability of energy for fermentation in the silo (McDonald et al. 2001).

In the current study, using small-scale experimental silos in a laboratory setting, low pH values indicated good fermentation for maize silages with DM content as low as 26% DM, even without additive. Lactic acid bacteria use water-soluble carbohydrates (WSC) to yield LA during the ensiling process. The acidity of the silage finally rises, and the pH falls as a result (McDonald et al. 1991). Silages harvested at an early stage of maturity, on the other hand, can be difficult to achieve low pH and good fermentation on a commercial scale without the use of an additive due to their low WSC and DM content (McDonald et al. 1991, Khorvash et al. 2006). Paydaş et al. (2019) obtained similar results by adding 15% pistachio outer shell to maize silage, which reduced the ammonia nitrogen, butyric acid, propionic acid, and lactic acid concentrations.

Control silages were found to be less stable after air exposure when compared to hazelnut husk treated silages (Table 4), most likely due to the high lactic acid content and mold count (Table 3), as well as the high moisture content of control silages (Table 2). When the forage is ensiled below 30% DM, an additive may be required to prevent the growth of clostridia. If the DM content is low, effluent will be produced. The effluent from silage production can become highly harmful as it is a perfect medium for microorganisms, but it also represents a loss of energy and nutrients (Woolford 1983). When the silages were exposed to air, the number of molds increased in comparison to the number of mold + yeast in the silages that were not exposed to air (Table 3). Sugar and lactic acid consumption by molds raise the pH and accelerate the process of aerobic instability, resulting in high DM losses during the feed out phase (McDonald et al. 1991).

Since we found no previous research on the use of hazelnut husks as a silage additive and how it affects the nutritive value of silage, we used straws, hay, and the hulls of some plants with similar nutritive values to hazelnut husk to compare the study findings. Notable change in gas volume were observed between treatments, as seen in Table 5. The reduction in gas production observed in hazelnut husk treated silages in the current study was similar to that observed in Babaeinasab et al. (2015), who used a potato and wheat straw mixture in maize silage, and Denek et al. (2017), who used pistachio byproduct in maize silage and revealed that additives that increased silage DM content decreased *in vitro* gas production value. Niderkorn et al. (2020) found that including hazelnut pericarps (Corylus avellana L.), which are high in procyanidin type condensed tannins, in a basal diet reduced total gas production. Although the tannin content of hazelnut husk and its effect on rumen microbiota were not examined in this study, Xu et al. (2012) investigated the tannin content of hazelnut shell from different cultivars and harvest years and discovered that condensed tannins ranged from 3.7 to 8.3 (% leucocyanidin equivalent), and total tannin content ranged from 3.6 to 7.9 (mg TAE g^{-1}). Because condensed tannin has been suggested to have biological functions on rumen fermentation, a decrease in gas production from diets supplemented with condensed tannin extract was predicted due to its impact on rumen microbes (Bueno et al. 2020). The current study found that adding hazelnut husks reduced the "a" value slightly ($p>0.05$, Table 5) which was expected due to high fibrous fractions of hazelnut husk (Table 1). The "b" value, which indicates the gas volume produced by the silage's slow fermenting fraction, and the "a+b" values, which represent total gas production and describe the gas volume that varies with time, were both dramatically reduced in hazelnut husk-supplemented silages ($p<0.05$, Table 5). By adding bay leaf and molasses, Atalay (2009) obtained comparable results with alfalfa silage. The tannin in the bay leaf, according to the author, has a negative effect on rumen microbes and causes the production of indigestible compounds with tannin

during the ensiling and fermentation processes. The "c" constant (0.049 mL/hour, Table 5) values of the silages obtained in this investigation were also determined by Babaeinasab et al. (2015), Sariçiçek and Kiliç (2009), and Salem et al. (2013). However, the results of Babaeinasab et al. (2015) potato pulp and straw added to maize silage and Sariçiçek and Kiliç (2009) straw added to maize silage were found to be higher than the results of this study. In the current study, the addition of hazelnut husk reduced the organic matter digestion of silages by 17.6% when compared to the control silage ($p<0.05$). This is due to higher NDF, ADF, and ADL contents in hazelnut husk supplemented silages ($p<0.05$, Table 2) than in control silages, while crude protein content was lower ($p<0.05$, Table 2), resulting in a decrease in *in vitro* gas production value and organic matter digestion. According to Rezaei et al. (2014), while supplying more crude protein to rumen bacteria enhances gas production, the provision of extra cell wall components reduces gas production. Furthermore, gas production is directly related to the amount of carbohydrates available to rumen bacteria (Blummel & Orskov 1993). Similar to our results, chestnut tannin reduced the organic matter digestibility of alfalfa silage by 5.1% (Tabacco et al. 2006). The current study found that the reduction in ME and OMD content in hazelnut husk treated silages was similar to the Paydaş et al. (2019) study, which revealed that the *in vitro* OMD and ME of maize silage prepared with 15% pistachio outer shell (52.63% and 7.96 MJ/kg DM, respectively) were lower than the control silages (54.07% and 8.17 MJ/kg DM, respectively). In another study, adding lucerna hay to low DM maize silage reduced apparent nutrient digestibilities (Barmaki et al. 2018). Similar results were also obtained in other studies by Atalay (2009), Babaeinasab et al. (2015), and Denek et al. (2017). While some absorbent additives reduce silage effluent production, they may also reduce the nutritious content of the silage (Khorvash et al. 2006). The addition of different absorbent additions, such as milled barley (5-15%) and powdered whey (5-15%), to low DM maize silage, however, increased its nutritional value (Khorvash et al. 2006). In the current experiment, the presence of rumen protozoa in control silages was determined to be 5.42 cfu/g (Table 5). This value was discovered to be compatible with Benchaar et al. (2007) findings. However, it was found to be greater than the reports of Chahaardoli et al. (2018), but Zhang et al. (2016) were found to be lower than the report. Protozoa account for more than half of the rumen microbes in terms of biomass. They have a numerical value of 10^5 to 10^7 per milliliter of rumen fluid (Williams & Coleman 1997). Protozoa consume starch in the rumen and inhibit rumen bacteria from using it excessively, i.e., converting it to volatile FAs. Furthermore, certain protozoa consume cellulolytic bacteria (Galindo et al. 2016). Protozoa are known to coexist with 9-25% of ruminal methanogens (McAllister & Newbold 2008). Higher protozoa levels in hazelnut supplemented silages were associated with lower silage ME, NEL, and OMD levels ($p<0.05$, Table 5). These findings could support the theory that an increase in the number of protozoa in the rumen can reduce cellulolytic bacteria, affect rumen pH stabilization, increase free ammonia levels and methanogenesis, and reduce the digestive efficiency of various diets, particularly those high in fiber content (Makkar 2005). However, definite conclusions are difficult to draw because various factors influence silage digestibility, energy content, and the number of microbial community. The state of various microorganisms in the rumen, as well as how ammonia and volatile FAs are regulated in the rumen, should be evaluated.

5. Conclusions

According to the results of the current study, when hazelnut husk was added at a rate of 15% to the second-crop maize plant, which was harvested at 26.61% DM, the DM was increased to 33%, which is the ideal level for silage fermentation. Hazelnut husk demonstrated a potential hygroscopic property for maize silage that increased silage DM. Simultaneously, the addition of hazelnut husk reduced silage DM losses, propionic and butyric acid, and mold-yeast activity, while also improving aerobic stability. Hazelnut husk enhanced the cell wall components of maize silage due to its fibrous structure. The hazelnut husk addition increased silage fermentation characteristics and provided the desired attributes in quality silage. As a result of its chemical features, hazelnut husk may represent a new silage addition capable of raising the low DM of maize used as a second crop to the optimal DM ratio. However, the addition of hazelnut husk reduced the ME, NEL, and OMD levels of maize silage, which are indicators of silage nutritional value. Future research in large-scale silos under field conditions will be beneficial in determining how hazelnut husk affects silage quality. Furthermore, animal feeding studies would be desirable in demonstrating the benefits of the hazelnut husk additive on silage quality.

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References

Altınçekiç E & Filya İ (2018). Effect of using bacterial inoculant and organic acid on the aerobic stability and feed value of small bale maize silages containing low dry matter. *Turkish Journal of Agriculture - Food Science and Technology* 6(7): 887-892. doi.org/10.24925/turjaf.v6i7.887-892.1873

AOAC (2002). Official Methods of Analysis. *Association of Official Analytical Chemists International*.

Ashbell G, Weinberg Z G, Azrieli A, Hen Y & Horev B (1991). A simple system to study te aerobic determination of silages. *Canadian Agricultural Engineering* 33: 391-399.

Atalay AI (2009). Investigation on the use of molasses and laurus nobilis mixture in alfalfa silage and the effect on the silage quality. Master's thesis. *Kahramanmaras Sutcu Imam University Institute of Natural and Applied Sciences Department of Animal Science*.

Babaeinasab Y, Rouzbehan Y, Fazaeli H & Rezaei J (2015). Chemical composition, silage fermentation characteristics, and *in vitro* ruminal fermentation parameters of potato-wheat straw silage treated with molasses and lactic acid bacteria and corn silage. *Journal of Animal Science* 93(9): 4377-4386. doi.org/10.2527/jas.2015-9082

Barker SB & Summerson WH (1941). The colorimetric determination of lactic acid in biological material. *Journal of Biological Chemistry* 138: 535-554. doi.org/10.1016/S0021-9258(18)51379-X

Barmaki S, Alamouti A A, Khadem A A & Afzalzadeh A (2018). Effectiveness of chopped lucerne hay as a moisture absorbent for low dry-matter maize silage: Effluent reduction, fermentation quality and intake by sheep. *Grass and Forage Science* 73: 406-412. doi.org/10.1111/gfs.12343

Bell JF, Offer NW & Roberts DJ (2007). The effect on dairy cow performance of adding molassed sugar beet feed to immature forage maize at ensiling or prior to feeding. *Animal Feed Science and Technology* 137(1-2): 84-92. doi.org/10.1016/j.anifeedsci.2006.11.002

Benchaar C, Petit HV, Berthiaume R, Ouellet DR, Chiquette J & Chouinard PY (2007). Effects of essential oils on digestion, ruminal fermentation, rumen microbial populations, milk production, and milk composition in dairy cows fed alfalfa silage or corn silage. *Journal of dairy science*, 90(2), 886–897. https://doi.org/10.3168/jds.S0022-0302(07)71572-2

Blummel M & Orskov E R (1993). Comparison of *in vitro* gas production and nylon bag degradabilities of roughages in predicting food intake of cattle. *Animal Feed Science and Technology* 40(2-3): 109-119. doi.org/10.1016/0377-8401(93)90150-I

Boyne A, Eadie JM & Raitt K (1957). The development and testing of a method of counting rumen ciliate protozoa. *The Journal of General Microbiology* 17(2): 414-423. doi.org/10.1099/00221287-17-2-414

Bueno ICS, Brandi RA, Fagundes GM, Benetel G & Muir JP (2020). The role of condensed tannins in the *in vitro* rumen fermentation kinetics in ruminant species: feeding type involved?. *Animals* 10(4): 635. doi.org/10.3390/ani10040635

Caccamo M, Valenti B, Luciano G, Priolo A, Rapisarda T, Belvedere G, Marino VM, Esposto S, Taticchi A, Servili M & Pauselli M (2019). Hazelnut as ingredient in dairy sheep diet: Effect on sensory and volatile profile of cheese. *Frontiers in Nutrition* 6 (125): 1-10. doi.org/10.3389/fnut.2019.00125

Cai Y & Ohmomo S (1995). Effect of lactic acid bacteria and cellulose on the changes in microflora during fermentation, gas production and dry matter loss in silage. *Animal Science and Technology* 66: 941-948.

Campione A, Natalello A, Valenti B,Luciano G, Rufino-Moya PJ, Avondo M, Morbidini L, Pomente C, Krol B, Wilk M, Migdal P & Pauselli M (2020). Effect of feeding hazelnut skin on animal performance, milk quality, and rumen fatty acids in lactating ewes. *Animals* 10(4): 588. doi.org/10.3390/ani10040588

Candellone A, Peiretti PG, Bergagna S & Meineri G (2019). Effect of linseed diet and hazelnut skin supplementation on oxidative status and blood serum metabolites in rabbits. *Progress in Nutrition* 21(3): 631-640. doi.org/10.23751/pn.v21i3.7725

Cetinkaya N & Kuleyin YS (2016). Evaluation of hazelnut Hulls as an alternative forage resource for ruminant animals. *International Journal of Agricultural and Biological Engineering* 10(5): 319-322. doi.org/10.5281/zenodo.1125401

Chahaardoli A, Soroor M N & Foroughi A (2018). The effects of Anise (*Pimpinella anisum*) essential oil and extract on *in vitro* rumen fermentation parameters and protozoa population of sheep. *International Journal of Veterinary Science and Medicine* 7(1): 21-27.

Denek N, Aydin SS & Can A (2017). The effects of dried pistachio (Pistachio vera L.) by-product addition on corn silage fermentation and *in vitro* methane production. *Journal of Applied Animal Research* 45(1): 185-189. doi.org/10.1080/09712119.2016.1141778

Dok M (2014). The agricultural waste potential of the black sea region and exploit from the agricultural wastes as pellet fuel. *4th National Energy, Agriculture, and Biofuels Workshop* 211-222.

FAOSTAT 2020. *Food and Agriculture Data*.

Galindo J, González N, Marrero Y,Sosa A, Ruiz T, Febles G, Torres V, Aldana AI, Achang G, Moreira O, Sarduy L & Noda AC (2016). Effect of tropical plant foliage on the control of methane production and *in vitro* ruminal protozoa population. *Cuban Journal of Agricultural Science* 48(4): 359-364.

Haigh PM (1997). Silage dry matter content and predicted effluent production in England and Wales 1984–1994. *Journal of Agricultural Engineering* 66(1): 63-77. doi.org/10.1006/jaer.1996.0118

Jones R & Jones DIH (1996). The effect of in-silo effluent absorbents on effluent production and silage quality. *Journal of Agricultural Engineering* 64(3): 173-186. doi.org/10.1006/jaer.1996.0059

Khorvash M, Colombatto D, Beauchemin KA, Ghorbani GR & Samei A (2006). Use of absorbants and inoculants to enhance the quality of corn silage. *Canadian Journal of Animal Science* 86(1): 97-107. doi.org/10.4141/A05-029

Kung Jr L, Stokes MR & Lin CJ (2003). Silage additives. In: Buxton D R, Muck R E, Harrison J H (Eds.), Silage Science and Technology. *Agronomy Monographs* pp. 305-360.

Lahr DA, Otterby DE, Johnson DG, Linn JG & Lundquist RG (1983). Effects of moisture content of complete diets on feed intake and milk production by cows. *Journal of Dairy Science* 66(9): 1891-1900. doi.org/10.3168/jds.S0022-0302(83)82027-X

Makkar HP (2005). In vitro gas methods for evaluation of feeds containing phytochemicals. *Animal Feed Science and Technology* 123: 291-302. doi.org/10.1016/j.anifeedsci.2005.06.003

McAllister TA & Newbold CJ (2008). Redirecting rumen fermentation to reduce methanogenesis. *Australian Journal of Experimental Agriculture* 48(2): 7-13.

McDonald P, Henderson AR & Heron SJE (1991). The biochemistry of silage. 2nd ed. *Chalcombe Publications* doi.org/10.1017/S0014479700023115

Menke KH & Steingass H (1988). Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Animal Research and Development* 28: 7-55.

Menke KH, Raab L, Salewski A, Steingass H, Fritz D & Schneider W (1979). The estimation of the digestibility and metabolizable energy content of ruminant feeding stuffs from the gas production when they are ıncubated with rumen liquor *in vitro*. *Journal of Agricultural Sciences* 93(1): 217-222. doi.org/10.1017/S0021859600086305

Muck RE, Nadeau EMG, McAllister TA, Contreras-Govea FE, Santos MC & Kung L (2018). Silage review: Recent advances and future uses of silage additives. *Journal of Dairy Science* 101(5): 3980-4000. doi.org/10.3168/jds.2017-13839

Niderkorn V, Barbier E, Macheboeuf D, Torrent A, Mueller-Harvey I & Hoste H (2020). *In vitro* rumen fermentation of diets with different types of condensed tannins derived from sainfoin (*Onobrychis viciifolia Scop*.) pellets and hazelnut (Corylus avellana *L.*) pericarps. *Animal Feed Science and Technology* 259: 114357. doi.org/10.1016/j.anifeedsci.2019.114357

Orskov ER & McDonald I (1979). The estimation of protein degradability in the rumen from incubation measurement weighted according to rate of passage. *Journal of Agricultural Science and Technology* 92(2): 499-503. doi.org/10.1017/S0021859600063048

Ozcan U & Kılıc U (2018). Effect of additives on the forage quality of pelleted hazelnut husks. *Asian Journal of Animal and Veterinary Advances* 13(2): 189-196. doi.org/10.3923/ajava.2018.189.196

Özdüven ML, Koç F, Polat C & Coşkunt Şamlı L (2009). Effects of fermentation characteristics and feed value of some maize cultivars ensiled at different stages of maturity. *Journal of Tekirdag Agricultural Faculty* 6(2): 121-129.

Paydaş E, Savrunlu Z, Savrunlu M & Denek N (2019). Investigation of effect on silage quality and *in vitro* methane gas formation of pistachio (Pistacia Vera l.) outer shell with additional at different levels in corn silage. *Journal of Faculty of Veterinary Medicine* 16(1): 16-22. http://dergipark.gov.tr/download/article-file/667499

Piccinelli AL, Pagano I, Esposito T, Mencherini T, Porta A, Petrone AM, Gazzerro P, Picerno P, Sansone F, Rastrelli L & Aquino RP (2016). HRMS profile of a hazelnut skin proanthocyanidin-rich fraction with antioxidant and anti-candida albicans activities. *Journal of Agricultural and Food Chemistry* 64: 585-595. doi.org/10.1021/acs.jafc.5b05404

Porter MG & Murray RS (2001). The volatility of components of grass silage on oven drying and the inter-relationship between dry-matter content estimated by different analytical methods. *Grass and Forage Science* 56(4): 405-411. doi.org/10.1046/j.1365-2494.2001.00292.x

Ranjbari M, Ghorbani GR, Nikkhah A & Khorvash M (2007). Chemical composition, protein fractionation and ruminal degradation of maize silage produced in isfahan. *International Journal of Dairy Science* 2(1): 66-72. doi.org/10.3923/ijds.2007.66.72

Renna M, Lussiana C, Malfatto V, Gerbelle M, Turille G, Medana C, Daniela Ghirardello D, Antonio Mimosi A & Cornale P (2020). Evaluating the suitability of hazelnut skin as a feed ingredient in the diet of dairy cows. *Animals* 10(9): 1653. doi.org/10.3390/ani10091653

Rezaei J, Rouzbehan Y, Fazaeli H & Zahedifar M (2014). Effects of substituting amaranth silage for corn silage on intake, growth performance, diet digestibility, microbial protein, nitrogen retention and ruminal fermentation in fattening lambs. *Animal Feed Science and Technology* 192: 29-38. doi.org/10.1016/j.anifeedsci.2014.03.005

Salem AZM, Kholif AE, Elghandour MMY, Hernández J, Limas A & Santiago AMF (2013). Urea in sugarcane-based diets for dairy cows. *Revista Brasileira de Zootecnia* 42(6): 456-462.

Sariçiçek BZ & Kiliç Ü (2009). The effects of different additives on silage gas production, fermentation kinetics and silage quality. *Ozean Journal of Applied Sciences* 2(1): 11-18.

Solmaz M (2017). Kuruyemiş ansiklopedisi fındık. *Overteam* 155-158.

Stévigny C, Rolle L, Valentini N & Zeppa G (2007). Optimization of extraction of phenolic content from hazelnut shell using response surface methodology. Journal of the Science of Food and Agriculture 87(15): 2817-2822. doi.org/10.1002/jsfa.2994

Tabacco E, Borreani G, Crovetto GM, Galassi G, Colombo D & Cavallarin L (2006). Effect of chestnut tannin on fermentation quality, proteolysis, and protein rumen degradability of alfalfa silage. *Journal of Dairy Science* 89(12): 4736-4746. doi.org/10.3168/jds.S0022-0302(06)72523-1

Van Soest PJ, Robertson JD & Lewis BA (1991). Methods for dietary fibre, neutral detergent fibre and non-starch polysaccharides in relation to animals nutrition. *Journal of Dairy Science* 74: 3583-3597. doi.org/10.3168/jds.S0022-0302(91)78551-2

Williams AG & Coleman GS (1997). The rumen microbial ecosystem. *Blackıe Academıc & Professıonal* 73-139.

Woolford MK, Wilkinson JM & Cook JE (1983). Investigations on the effect of sodium bentonite and other moisture absorbants on the production of effluent from grass silage. *Animal Feed Science and Technology* 8(2): 107-118. doi.org/10.1016/0377-8401(83)90092-5

Xu Y, Sismour EN, Parry J, Hanna MA & Li H (2012). Nutritional composition and antioxidant activity in hazelnut shells from US-grown cultivars. *International Journal of Food Science and Technology* 47(5): 940-946. doi.org/10.1111/j.1365-2621.2011.02925.x

Zhang S, Cheng L, Guo X, Ma C, Guo A & Moonsan Y (2016). Effects of urea supplementation on rumen fermentation characteristics and protozoa population *in vitro*. *Journal of Applied Animal Research* 44(1): 1-4. doi.org/10.1080/09712119.2014.978779

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Fatty Acid Profiles of Fish Oil Derived by Different Techniques from By-products of Cultured Black Sea Salmon, *Oncorhynchus mykiss*

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ABSTRACT

This paper investigates the fatty acid profiles of fish oil extracted from by-products of cultured Black Sea salmon, *Oncorhynchus mykiss*, using conventional [conventional fish oil (CFO)] and dry freezing oil [dry freezer oil (DFO)] techniques. In the CFO and DFO groups, monounsaturated fatty acid + polyunsaturated fatty acid (PUFA) comprised 74.00% and 72.68% of the total fatty acids, respectively. The highest PUFA was linoleic acid (CFO=14.22%, DFO=13.15%) with Docosahexaenoic acid (DHA, C22:6n3) being the second most concentrated fatty acid for PUFA in the CFO (8.12%) and DFO (8.02%) groups, followed by eicosapentaenoic acid (EPA, C20:5n3) (CFO=4.39%, DFO=2.87%). Similarly, the difference between groups in omega-3 was statistically significant ($p<0.05$) and the CFO ratio was higher in the DFO. The PI, AI, TI, h/H, and UI percentages in the CFO group were 0.99, 0.37, 0.26, 2.98, and 1.73%, respectively, while in the DFO group they were 0.80, 0.35, 0.31, 2.83, and 1.61%, respectively. The findings of this study conclude that the oils obtained from Black Sea salmon by-products are rich in omega-3 fatty acids and have good lipid quality indexes.

Keywords: By-product, Conventional fish oil, Dry freezing, Fish oil quality, *Oncorhynchus mykiss*

1. Introduction

The global fish production from fishing is constrained by factors such as overfishing, climate change, and habitat destruction, which limit the amount of fish that can be sustainably caught. The increasing world population and the search for quality seafood support the development of aquaculture (Filogh et al. 2023; Bayrakli & Duyar 2021a,b; Sönmez et al. 2022). The use of fish meal and oil in feed rations is important for quality fish farming (Bayraklı & Duyar 2019a). Fish meal and oil production made from whole fish is also limited (Bayrakli et al. 2019).

The use of fish oil in the aquaculture sector to feed farmed fish is increasing, as well as in in the food industry to supplement foods with omega-3 fatty acids and in the pharmaceutical industry to produce omega-3 concentrates.

Fish oil is mainly derived from fish caught specifically for this purpose, such as anchovies, sardines, mackerel, and menhaden (Bayrakli et al. 2019). There are 85.4 million tons of aquaculture production in the world, with great potential for oil extraction from fish waste and by-products from the fish processing industry (TUIK 2021). According to TUIK (2021) data, Black Sea salmon production on land and in the sea in Turkey saw an increase from 123,089 tons in 2019 to 144,283 tons in 2020. Every year, the production of Black Sea salmon in Turkey continues to increase, and as a result, the amount of by-products generated also rises accordingly. By-products are fish pieces that have been removed before the fish reaches consumers to maintain fish quality, reduce shipping weight, or increase the value of the main fish product. Fish by-products contain significant amounts of fat and protein that can be used for human nutrition and animal feed, from a nutritional or food safety point of view (Ramirez 2007). The main components of aquaculture discards are the head, viscera, gills, bones, scales, fins, and sometimes skin. A significant amount of waste (20-80%) is generated depending on the fish

processing technique and fish species (Ghaly et al. 2013). Fish waste typically contains 58% crude protein, 22% ash, 19-25% ether extract and 1% crude fibre (Esteban et al. 2007).

A characterization of the lipid profile of oils found in fish is crucial to explore its potential application to new fish sources for omega-3 production. Recently, the potential use of these ingredients has attracted a great deal of attention. Fish oil is the main natural source of the healthiest omega-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Fish oil suitable for dietary supplementation is the relative amount of omega-3 and omega-6 fatty acids. To maintain the correct omega-3/omega-6 ratio in the diet, regular consumption of omega-3-rich and omega-6-poor fish is recommended, and the optimal omega-3/omega-6 dose or ratio ranges from 1/4 to 1/1. The demand for fish oil for direct human use as fish oil capsules and additives in the food industry continues to increase (Simopoulos 2002). The main fatty acid quality assessment indexes include the polyene index (PI), a ratio of polyunsaturated fatty acids to other fatty acids, as well as the atherogenicity index (AI), thrombogenicity index (TI), hypocholesterolemic/hypercholesterolemic ratio (h/H), fish lipid quality (FLQ), and unsaturation index (UI).

This study was carried out to characterize the fish oil profiles obtained from the non-consumed waste parts of Black Sea salmon reared in net cages in the Black Sea, by the conventional method and the freeze-drying method. Data obtained from this study can the basis for analyzing the possibility of using Black Sea salmon waste as a source of omega-3 rich oil.

2. Material and Methods

2.1. Fish by-products

Fish by-products were obtained from an industry-leading aquaculture processing facility headquartered in Samsun, Turkey. After Black Sea salmon is processed at the factory, about 40% is considered unsuitable for human consumption and subsequently leftover from industrial processing. Most of this leftover excess is the internal organs, as well as the head and gills. In order to conduct our study, we obtained 5 kg of Black Sea salmon by-products (Trim B waste obtained after fillet trimming in an aquaculture processing plant) and transported them to a fish meal oil factory in a Styrofoam box.

2.2. Extraction techniques and procedures

In this study, the fatty acid profiles of fish oil extracted from Black Sea salmon by-products through two different techniques were investigated.

1. Conventional fish oil (CFO): CFO was obtained from the company from which we provided our research material. This company applies the industrial conventional method. This wet reduction process involves fish cooking, pressing, solid and liquid separation (decanter), and centrifugation for extraction.

2. Dry freezer oil (DFO): Black Sea salmon wastes obtained from the same company were brought to the research laboratory and homogenized using a Mateka brand (GPS 300) food shredder (15 L and 5000 rpm). Then, the Black Sea salmon structure was obtained by drying in a lyophilizer. For this purpose, a Lyoquest-85 Plus Eco laboratory lyophilizer, made by Telstar, was used. The operating procedure of the device is provided in the Table 1.

Table 1- Method used in the lyophilizer device

Step no	***Process***	***Parameters***		
		Time (hh:mm)	***Pressure (mBar)***	***Temperature (°C)***
1	Freezing	01:00	0.2	-
2	Cool + vacuum	00:30	0.2	-
3	Heat shelves	24:00	0.2	20
4	Heat shelves	06:30	0.001	35
5	Stop	-	-	-

2.3. Fatty acid composition

Fatty acid methyl esters were prepared with hot alkali esterification according to IUPAC (1994) method. Basically, 150 mg of fish oil was weighed into an Erlenmeyer flask. Five mL of methanolic 0.5 N NaOH added, then boiled for 15 minutes by using a condenser. The mixture was cooled down to room the temperature. Further 5 mL of methanolic BF_3 (14%), was added into the mixture and boiled for 5 minutes. Methylated sample was cooled and a 5 mL of saturated NaCl solution was added into the solution. The solution was transferred into a screw capped tube and Fames were extracted with 2 mL of n heptane into an amber vial. Some anhydrous sodium sulphate (full spoon of microspatula) was added into a vial in order to hold moisture. The vials were kept at -20 °C till GC analysis.

The GC system consists of an FID detector (Flame Ionization Detector), gas chromatography (Shimadzu, GC2014, Japan), and autoinjector (AOC-20i, Shimadzu, Japan). The instrument is controlled by GC solution software (Version 2.41.00 su_1). FAME WAX (polyethylene glycol, 30 meter*0.25 mm I.D*0.2 µm, GC Columns Restek) was used as the chromatographic colon. The GC operation conditions were as follows; injection mode: split ratio, split: 1/10, injection and detector temperature: 260 and 280 °C, carrier gas and column flow: helium and 1.4 mL min^{-1}, temperature program: initial temperature 5 m 100 °C, 5 °C increase per minute from 100 °C to 150 °C, 15 m at 150 °C, 10 °C increase per minute to 210 °C, and 20 m at 210 °C.

Peaks were identified by comparing the retention time of the "Supelco 37 Component FAMEs Mix" standard. Results were expressed as percentage area of the identified individual fatty acids. The spectrum includes all commonly known fatty acid methyl esters.

According to this method, the fatty acid methyl esters were analysed using a PUE UNICAM 204 Gas Chromatography equipped with a flame ionization detector using a Degs capillary column (2 MX 1-8 inc) coated with 0.25 µl of Supelco GP% OV-275 on 100/120 PAW-PMCS.

2.4. Lipid quality indices

PI was used as a measure of PUFAs damage (Lubis & Buckle 2007) and calculated according to the formula below.

$$\mathrm{PI} = \frac{[\mathrm{EPA\ (C20{:}\ 5n3)} + \mathrm{DHA(22{:}\ 6n3)}]}{\mathrm{Palmitic\ acid\ (C16{:}\ 0)}}$$

AI, TI and h/H were calculated using the following equations (Abrami et al. 1992; Bayrakli 2021; Ulbricht & Southgate 1991), taking into account the different effects of different fatty acids on human health:

$$\mathrm{AI} = \frac{[12{:}\,0 + 4\,(14{:}\,0) + 16{:}\,0)]}{\mathrm{MUFA} + \mathrm{PUFA}}$$

$$\mathrm{TI} = \frac{(14{:}\,0 + 16{:}\,0 + 18{:}\,0)}{[0.5(\mathrm{MUFA}) + 0.5(\mathrm{n6PUFA}) + 3(\mathrm{n3PUFA}) + (\mathrm{n3PUFA/n6PUFA})]}$$

$$\mathrm{h/H} = \frac{(\mathrm{C18{:}\,1 + C18{:}\,2 + C18{:}\,3 + C20{:}\,3 + C20{:}\,4 + C20{:}\,5 + C22{:}\,4 + C22{:}\,5 + C22{:}\,6})}{(\mathrm{C14{:}\,0 + C16{:}\,0})}$$

$$\mathrm{UI} = [1 \times (\%\ \text{monoenoics}) + 2 \times (\%\ \text{dienoics}) + 3 \times (\%\ \text{trienoics}) + 4 \times (\%\ \text{tetraenoics}) + 5 \times (\%\ \text{pentaenoics}) + 6 \times (\%\ \text{hexaenoics})]/100$$

2.5. Statistical analysis

The data obtained from three different time periods were analysed by Student's t-test analysis using the SPSS statistical package program (Version 10, SPSS Inc., Chicago, IL, USA), and the differences among the means were compared using Duncan's multiple range test. A significance level of 0.05 was used and the results were shown as mean values ± standard deviation.

3. Results and Discussion

Although the aim of the research was not to optimize both techniques, the characterizations of the fish oil profiles obtained between these two techniques were compared and the analyses were performed with three replications. The fatty acid profiles of fish oil obtained from freeze-dried Black Sea salmon by-product are given in Table 2.

Table 2- Fatty acid composition (%) of Black Sea salmon fish oil processing by-product (CFO-DFO)

Fatty acid	*CFO*	*DFO*
C10:0 Capric	0.01±0.000	0.00±0.000
C12:0 Lauric	0.11±0.006	0.07±0.000
C13:0 Tridecanoic	0.03±0.000	0.02±0.000
C14:0 Myristic	3.69±0.101^{a}	2.96±0.052^{b}
C15:0 Pentadecanoic	0.61±0.026^{a}	0.44±0.012^{b}
C16:0 Palmitic	12.66±0.062^{b}	13.65±0.344^{a}
C17:0 Heptadecanoic	0.64±0.015^{a}	0.56±0.006^{b}
C18:0 Stearic	5.75±0.576^{b}	8.13±0.165^{a}
C20:0 Arachidic	0.95±0.006^{a}	0.83±0.046^{b}
C21:0 Henicosanoic	0.04±0.006	0.03±0.006
C22:0 Behenic	0.63±0.087	0.54±0.015
C23:0 Tricosanoic	0.09±0.010	0.07±0.006
C24:0 Lignoceric	0.79±0.026^{a}	0.02±0.006^{b}
Σ SFA	26.01±0.860^{a}	27.32±0.642^{a}
C14:1 Myristolec	0.25±0.006	0.15±0.006
C15:1	0.09±0.006	0.06±0.006
C16:1 Palmiteloic	0.55±0.010	0.41±0.012
C17:1 c Heptadecenoic	0.86±0.021^{a}	0.51±0.015^{b}
C18:1n9t Elaidic	1.58±0.667^{b}	2.82±0.044^{a}
C18:1n9c Oleic	23.28±1.020^{a}	23.66±0.814^{a}
C20:1n9 cEicossenoic	1.30±0.010^{b}	3.51±2.246^{a}
C22:1n9 Erucic	3.49±0.036^{a}	3.44±0.058^{a}
C24:1n9 Nervonic	1.33±0.035^{a}	1.20±0.025^{b}
Σ MUFA	32.74±1.236^{b}	35.76±1.285^{a}
C18:2n6t Linoleaidic	0.75±0.015^{a}	0.49±0.015^{b}
C18:2n6c Linoleic	14.22±0.119^{a}	13.15±0.179^{b}
C18:3n6 γ -Linolenic	0.87±0.006^{a}	0.60±0.092^{b}
C18:3n3a-Linolenic	5.72±0.070^{a}	5.15±0.078^{b}
C20:2 c Eicosadienoic	3.18±0.036^{a}	3.20±0.069^{a}
C20:3n3 α Eicosatrienoic	1.76±0.010^{a}	1.63±0.038^{b}
C20:3n6	0.57±0.026^{a}	0.30±0.006^{b}
C20:4n6 Arachidonic	1.60±0.065^{a}	1.45±0.040^{b}
C22:2 Docosadienoic	0.08±0.000	0.06±0.035
C20:5n3 EPA	4.39±0.165^{a}	2.87±0.046^{b}
C22:6n3 DHA	8.12±0.255^{a}	8.02±0.167^{a}
Σ PUFA	41.26±0.385^{a}	36.92±0.662^{b}

SFA: Saturated fatty acid, MUFA: Monounsaturated fatty acid, PUFA: Polyunsaturated fatty acid, Different letters (a,b,c) in the same row shows significant differences ($p<0.05$) among the freshness groups.

The SFA, MUFA, PUFA and MUFA+PUFA values were determined in fish oil obtained from Black Sea salmon by-products with 2 different techniques (CFO and DFO groups). SFA in CFO and DFO groups were calculated as 26.01% and 27.32%, respectively. MUFA was higher in the DFO group than the CFO group. PUFA values of CFO and DFO groups were found as 41.26% and 36.92%, respectively (Table 2). Compared to the DFO group, PUFA was higher in the CFO group. In the CFO and DFO groups, MUFA+PUFA constituted 74.00% and 72.68% of total fatty acids, respectively. The difference in SFA between CFO and DFO groups was insignificant ($p>0.05$), but the difference between the MUFA and PUFA groups were statistically significant ($p<0.05$). It was observed that the SFA results obtained in this study and the SFA values in fish oil obtained from Black Sea salmon, tilapia and carp processing by-products in other studies were similar (Table 3). The MUFA content of fish oil obtained from various fish by-products has been reported in several studies. Zhong et al. (2007), Crexi et al. (2010), Korkmaz & Tokur (2020), and Pateiro et al. (2020) reported lower MUFA content compared to our study. On the other hand, Bayrakli & Duyar (2019b), Khoddami et al. (2012), and Brelaz et al. (2019) reported higher MUFA content. The MUFA content reported by Nascimento et al. (2015) and Abiona et al. (2021) was found to be similar to our results. The PUFA obtained in this study was found to be higher than all the studies reported in Table 3.

Table 3- Literature information on fatty acids contents and Lipid Quality Indices determined for different fish oil products

Fatty Acids	*Abiona et al. (2021)*	*Inguglia et al. (2020)*	*Brelaz et al. (2019)*	*Khoddami et al. (2012)*	*Nascimento et al. (2015)*	*Pateiro et al. (2020)*	*Korkmaz and Tokur (2020)*	*Fiori et al. (2012)*	*Zhong et al. (2010)*	*Crexi et al. (2010)*	*Bayrakli & Duyar (2019b)*	*This Study*
	Scomber scombrus	*Salmo Salar*	*Fresh water fish*	*Euthynnus affinis*	*Fish of various species*	*Sparus aurata*	*Rainbow trouth*	*Rainbow trouth*	*Rainbow trouth*	*Cyprinus carpio*	*Anchovy**	*Rainbow trouth*
C14:0 Myristic	2.24-3.48	2.56		3.76-7.49	6.33	1.73-2.84	2.03-2.28	4.75-6.04	3.43	3.80	6.52	2.96-3.69
C16:0 Palmitic	27.49-34.16	9.57	29.01	27.63-32.74	19.67	13.27-14.13	14.27-15.47	15.7-17.8	15,7	16,20	19.80	12.66-13.65
C18:0 Stearic	8.17-10.20	0.54	9.62	8.82-13.62	4.91	2.70-4.51	4.44-4.88	3.59-3.88	4,50	3.13	3.60	5.75-8.13
C18:1n9c Oleic	24.31-31.99	39.47	18.48	9.16-11.95	12.65	32.99-35.91	37.14-39.47	17.9-19.0	28,6	25,84	13.59	23.28-23.66
C18:2n6c Linoleic	1.95-4.38	14.56	4.39	1.0-2.49	0.2	16.57-19.58	17.94-19.23	13.8-17.9	9,0	9.17	0.28	13.15-14.22
C18:3n3a-Linolenic		4.46	4.51		2	3.86-4.73	3.05-3.27	0.80	0,11	7.17	1.99	5.15-5.72
C22:6n3 DHA	1.62-9.31	2.78	4.20	14.18-15.70	9.12	3.51-5.20	4.37-5.11	6.02-7.30	7,98	1.20	18.64	8.02-8.12
C20:5n3 EPA					10.36	1.83-2.78	1.62-1.90	5.98-8.75	3,35	3.83	8.68	2.87-4.39
SFA	40-45.36		48.23	47.02-55.20	31.63	19.72-20.85	21.94-23.74	24.3-27.9	25	26.85	34.21	26.01-27.32
MUFA	33.85-42.32		28.92	20.82-24.20	30.59	44.05-46.08	44.05-46.46	72.1-75.7	40	41:90	21.51	32.74-35.76
PUFA	12.31-25.36		21.91	23.98-28.77	32.78	31.17-34	23.15-24.41		26	25.54	32.04	36.92-41.26
Omega 3		10.64	12.79	15.88-17.18		11.91-14.18	6.37-7.38	17.4-22.2		13.61	28.39	17.67-19.99
Omega 6		19.06	8.57	8.10-11.59		18.66-21.04	22.51-23.71	17.5-20.4		11.90	3.65	15.99-18.01
Omega 3/ Omega 6							0.27-0.33	0.85-1.18		1.14	7.78	1.10-1.11
UI								1.76-2.01				1.61-1:73

In terms of MUFA + PUFA values, the results of this study were similar to the literature on fish oil obtained from fish processing by-products of Black Sea salmon, *Cyprinus carpio*, *Spratus aurata*, *Oreochromis niloticus*, *Scomber scombrus* (Table 3). Fish oil made from whole anchovy fish and fish oil obtained from *Euthynus affinis* and some freshwater fish by-products were found to be higher. Fatty acid values differ according to the type of fish, whether it is wild or cultured fish, the processed part of the fish, and the processing method.

Palmitic acid has the highest amount among SFA in CFO and DFO groups (12.66% and 13.65%), followed by stearic acid (5.75% and 8.13%) and myristic acid (3.69% and 2.96%). The values of fish oil studies with Black Sea salmon by-product were lower than those made with similar different kinds of fish by-product. In addition, as seen in Table 3, the dominant SFA in all studies was palmitic acid, followed by stearic acid and myristic acid, as in this study.

Among MUFA, oleic acid had the highest percentage in CFO and DFO groups (23.28% and 23.66%, respectively) followed by erucic acid (3.49% and 3.44%, respectively). There was no statistically significant difference between the groups ($p>0.05$). Oleic acid was the highest MUFA as reported in other studies (Table 3). In the studies conducted by Korkmaz & Tokur (2020) and Zhong et al. (2007) with Black Sea salmon by-products, oleic acid was found to be lower compared to our findings, while it was higher than the value reported by Fiori et al. (2012). It was observed that the oleic acid values in fish oils made with the by-products of other fish species were lower than some studies and higher than others.

The PUFA results in this study were higher than the fish oil value obtained from all anchovy investigated by Bayraklı and Duyar (2019b). The highest concentration of PUFA in the CFO and DFO groups was found to be linoleic acid, similar to other fish by-products. In contrast, fish oil obtained from whole anchovy contained only 2% of this fatty acid (Bayraklı & Duyar 2019b). Among the PUFA, DHA (C22: 6n3) was the most abundant in both groups, with concentrations of 8.12% and 8.02%, followed by EPA (C20: 5n3) at 4.39% and 2.87%, respectively (Table 2). The difference between the two groups was statistically significant for omega-3 ($p<0.05$), with the CFO group having a higher concentration. The highest DHA/EPA ratio was observed in the DFO group (2.80) (Table 4). The omega-3 fatty acid value in this study was higher than in similar research by Fiori et al. (2012), while omega-6 fatty acid values were similar to those reported by the same study. It was also observed that the omega-3 and omega-6 values were lower or higher than some research results. According to the results, fish oil obtained from anchovy investigated by Bayraklı and Duyar (2019b) had a lower omega-3 value and a higher omega-6 value compared to CFO and DFO groups. The omega-3/omega-6 ratios were 1.11:1 and 1.10:1 in the CFO and DFO groups, respectively, which were higher than Korkmaz and Tokur's (2020) values, but similar to the results obtained from the Black Sea salmon fish processing by-products studied by Fiori et al. (2012) and Zhong et al. (2007). According to the results of this study, the fish oil value obtained from the whole anchovy was considerably lower (Bayraklı & Duyar 2019b). It was reported that the omega-3/omega-6 ratio in the fatty acid ratio of wild Black Sea salmon was 3.08, while it was 0.46 in culture (Oz & Dikel 2015). Plant-based raw materials are commonly used in feed rations for Black Sea salmon fish grown in aquaculture farms. For this reason, it was evaluated that omega-6 levels increased and omega-3 levels decreased, so this ratio may be lower than oils obtained from wild fish. The minimum recommended PUFA/SFA ratio value is 0.45 (HMSO 1994). This value is lower than the values obtained in both fish oil groups in this study.

Table 4- Fatty acid ratios and lipid quality indexes

Indexes	***CFO***	***DFO***
UNSFA/SFA	2.85±0.125	2.66±0.085
PUFA/SFA	1.58±0.144	1.35±0.103
n3	19.99±0.175	17.67±0.326
n6	18.01±0.187	15.99±0.294
n3/n6	1.11±0.003	1.10±0.008
n9	28.35±1.271	29.92±0.915
DHA/EPA	1.85±0.130	2.80±0.018
A_I	0.37±0.010	0.35±0.011
T_I	0.26±0.008	0.31±0.005
P_I	0.99±0.008	0.80±0.005
h/H	2.98±0.088	2.83±0.015
UI	1.73±0.004	1.61±0.011

SFA: Saturated fatty acid, MUFA: Monounsaturated fatty acid, PUFA: Polyunsaturated fatty acid, PI: Index of polyene, IA: Atherogenicity index, IT: Thrombogenicity index, FLQ: Fish lipid quality

Several studies have reported that individuals who consume omega-3 rich products are less likely to develop hypertension or other cardiovascular diseases. It has been emphasized that drugs used in the treatment of obesity and cardiovascular diseases can be reduced by decreasing the omega 6 ratio in diets and increasing the omega-3 ratios (Simopoulos et al. 2000). In the early studies on fatty acids, the omega-3/omega-6 ratio was initially reported to be 1:1, but over time, due to changing dietary habits in industrial societies, this ratio has increased to 30:1 to 50:1. The World Health Organization reported that the omega-3/omega-6 ratio should be between 5:1 and 10:1 (FAO/WHO 1994). However, this ratio should be between 1:1 and 1:4 in a healthy diet (Simopoulos et al. 2000). In the present study, omega-3/omega-6 ratio of 1.10:1 indicates that by-products of cultured Black Sea salmon appears to be a healthy food and can provide source of omega-3 fatty acids (Table 4).

The PI value (the coefficient of deterioration of PUFA) may be a useful tool for measuring the oxidative stability of fish oil. In general, a higher PI value is preferred. However, in our study, we found that the PI value was the lowest in the DFO group (0.80), and there were statistically significant differences when compared to the CFO group (0.96) (Table 4). Additionally, we found that fish oil obtained from whole anchovy (Bayraklı & Duyar 2019b) had a relatively low PI value (1.73). Based on this result, it can be concluded that the oxidation of PUFA was higher in oil obtained from fish processing by-products.

It is reported that atherogenic (AI) and thrombogenic (TI) indices that are higher than (>1.0) are harmful to human health (Ouraji et al. 2009). If this value gets lower, the risk of coronary heart disease decreases (Cutrignelli et al. 2008). The average AI values for the CFO and DFO groups were determined as 0.37, and 0.35, respectively. The average TI values for the CFO and DFO groups were determined as 0.26, and 0.31, respectively. The AI and TI values of fish oil made from whole anchovy were reported to be 0.86, and 0.28, respectively. Karsli (2021) reported AI values between 0.11-0.70 and TI between 0.01-0.36 fish oil supplement products. In conclusion, our study demonstrated that fish oil obtained from fish processing by-products had lower AI and TI compared to fish oil obtained from whole anchovy, and the consumption of fish oil supplements made from fish processing by-products does not pose significant risks to human health in terms of AI and TI.

The h/H ratio of fatty acids is the indicator of whether the fat in the product is nutritionally adequate (Caglak & Karsli 2017). The fact that this ratio is high indicates that the oil contained in the product is suitable for nutrition. In this study, the h/H ratio was determined as 2.98 for the CFO group and 2.83 for the DFO group, and these values were found to be higher than the data of the study (1.73) conducted by Bayraklı & Duyar (2019b). The high rate indicates that the oil in the product is suitable for nutrition. The fatty acid composition can be variable depending on fish species (Ozogul et al. 2013).

The UI shows the degree of unsaturation in lipids and is calculated as the sum of the percentage of each unsaturated fatty acids multiplied by the number of double bonds in that fatty acids (Logue et al. 2000). That this ratio is high indicates that the oil contained in the product is suitable for nutrition. In this study, the UI was found to be 1.73 and 1.61 in the CFO and DFO groups, respectively. The difference between the groups were found to be statistically significant. Fiori et al. (2012) reported that the UI value in Black Sea salmon processing by-producst ranged from 1.76 to 2.01, similar to the results of this research.

4. Conclusions

The study suggests that the use of vegetable oil in farmed fish feed may lead to an increase in the amount of omega-6 and a decrease in the amount of omega-3/omega-6 ratio as a cost-saving measure. In the Black Sea, aquaculture Black Sea salmon can be considered as a resource with good lipid quality indices, which can be used for the production of fish oil rich in omega-3.

An evaluation of Black Sea salmon by-products is especially important today when considering the ongoing global food shortages and it would be particularly beneficial to use this waste for human and animal consumption.

Fish by-products are organs where microbial degradation rapidly takes place. Considering the environmental factors, these fish by-products must be processed quickly. There are various difficulties in the traditional fishmeal and oil production processing of fish by-products. Future studies should consider economical, simple and feasible extraction of fish oil from fish residues processed by using different processing techniques or new technologies in small-capacity aquaculture processing plants.

Data availability: Data are available on request due to privacy or other restrictions.

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References

Abiona O O, Awojide S H, Anifowose A J, Adegunwa A O, Agbaje W B & Tayo A S (2021). Quality characteristics of extracted oil from the head and gills of Catfish and Titus fish. *Bulletin of the National Research Centre* 45(1): 101. doi.org/10.1186/s42269-021-00557-3

Abrami G, Natiello F, Bronzi P, McKenzie D, Bolis L & Agradi E (1992). A comparison of highly unsaturated fatty acid levels in wild and farmed eels (Anguilla Anguilla). *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* 101(1-2): 79-81. doi.org/10.1016/0305-0491(92)90161-J

Bayrakli B (2021). Monthly Variations in Proximate Composition Fatty Acid Quality and Amino Acid Score of Warty Crab, Eriphia verrucosa (Forsskal 1775) from the Southern Coast of Black Sea, Turkey. *Pakistan Journal of Zoology* 53(5): 1729-1741. doi.org/10.17582/journal.pjz/20210318090304

Bayrakli B & Duyar H A (2021a). Effect of Freshness on Fish Meal Quality; Anchovy Meal. *Journal of Anatolian Environmental and Animal Sciences* 6(1) 57-65. doi.org/10.35229/jaes.824885

Bayrakli B, Özdemir S & Duyar H A (2019). A Study on Fishing and Fish Meal-oil Processing Technology of Anchovy (Engraulis encrasicolus) and European Sprat (Sprattus sprattus) in the Black Sea. *Menba Journal of Fisheries Faculty* 5(2): 9-16.

Bayraklı B & Duyar H A (2019a). Nutritional Composition of Fishmeal Obtained from Different Raw Materials in the Black Sea. *Journal of Anatolian Environmental and Animal Sciences* 4(3): 545-550. doi.org/10.35229/jaes.636806

Bayraklı B & Duyar H A (2019b). The Effect of Raw Material Freshness on Fish Oil Quality Produced in Fish Meal & Oil Plant. *Journal of Anatolian Environmental and Animal Sciences* 4(3): 473-479. doi.org/10.35229/jaes.636002

Bayraklı B & Duyar H A (2021b). The Effect On Fishmeal Element Quality Of Different Processing Methods: Evaporator System. *Marine Science and Technology Bulletin* 10: 251-257. doi.org/10.33714/masteb.922865

Brelaz K C B T R, Cruz F G G, Brasil R J M, Silva A F, Rufino J P F, Costa V R & Viana Filho G B (2019). Fish waste oil in laying hens* Diets. *Revista Brasileira de Ciencia Avicola* 21(4). doi.org/10.1590/1806-9061-2019-1069

Caglak E & Karsli B (2017). Investigations on mineral content in warty crab Eriphia verrucosa (Forskal 1775) from the Rize Coast of Black Sea, Turkey. *Indian Journal of Fisheries* 64(4): 80-86. doi.org/10.21077/ijf.2017.64.4.55342-11

Crexi V T, Monte M L, Soares L A, de S & Pinto L A A (2010). Production and refinement of oil from carp (Cyprinus carpio) viscera. *Food Chemistry* 119(3): 945-950. doi.org/10.1016/j.foodchem.2009.07.050

Cutrignelli M I, Calabrò S, Bovera F, Tudisco R D, Urso S, Marchiello M, Piccolo V & Infascelli F (2008). Effects of two protein sources and energy level of diet on the performance of young Marchigiana bulls. 2. Meat quality. *Italian Journal of Animal Science* 7(3): 271-285. doi.org/10.4081/ijas.2008.271

Esteban M B, Garcia A J, Ramos P & Márquez M C (2007). Evaluation of fruit–vegetable and fish wastes as alternative feedstuffs in pig diets. Waste Management 27(2): 193-200. doi.org/10.1016/j.wasman.2006.01.004

FAO/WHO (Food and Agriculture Organization of the United Nations/ World Health Organization) (1994). Fats and oils in human nutrition. Report of a joint expert consultation. Food and Agriculture Organization of the United Nations Rome Italy.

Filogh A, Bilen S, Sönmez A Y & Elp M (2023). Growth, Blood Parameters, Immune Response and Antioxidant Enzyme Activities in Rainbow Trout (Oncorhynchus mykiss Walbaum, 1792) Fed Diets Supplemented with Fumitory (Fumaria officinalis). *Journal of Agricultural Sciences* 29(1): 47-59. doi.org/10.15832/ankutbd.982032

Fiori L, Solana M, Tosi P, Manfrini M, Strim C & Guella G (2012). Lipid profiles of oil from trout (Oncorhynchus mykiss) heads spines and viscera: Trout by-products as a possible source of omega-3 lipids? *Food Chemistry* 134(2): 1088-1095. doi.org/10.1016/j.foodchem.2012.03.022

Ghaly A E, Ramakrishnan V V, Brooks M S, Budge S M & Dave D (2013). Fish processing wastes as a potential source of proteins, amino acids and oils: A critical review. *Journal of Microbial and Biochemical Technology* 5(4): 107-129. doi.org/10.4172/1948-5948.1000110

HMSO, UK (1994). Nutritional aspects of cardiovascular disease. 46: 1-186.

Inguglia L, Chiaramonte M, Di Stefano V, Schillaci D, Cammilleri G, Pantano L, Mauro M, Vazzana M, Ferrantelli V, Nicolosi R & Arizza V (2020). Salmo salar fish waste oil: Fatty acids composition and antibacterial activity. *PeerJ* 8: e9299 doi.org/10.7717/peerj.9299

IUPAC (1994). Protocol For The Design, Conduct And Interpretation Of Method-Performance Studies. *Pure and Applied Chemistry* 67(2): 331-343.

Karsli B (2021). Comparative analysis of the fatty acid composition of commercially available fish oil supplements in Turkey: Public health risks and benefits. *Journal of Food Composition and Analysis* 103: 104105. doi.org/10.1016/J.JFCA.2021.104105

Khoddami A, Ariffin A A, Bakar J & Ghazali H M (2012). Quality and fatty acid profile of the oil extracted from fish waste (head, intestine and liver) (Euthynnus affinis). *African Journal of Biotechnology* 11(7): 1683-1689. doi.org/10.5897/ajb10.1699

Korkmaz K & Tokur B (2020). Effect of different time and temperature on fatty acid composition of trout waste hydrolized by Alkali Protease enzyme. *Turkish Journal of Maritime and Marine Sciences* 6(2): 239-251.

Logue J A, de Vries A L, Fodor E & Cossins A R (2000). Lipid compositional correlates of temperature-adaptive interspecific differences in membrane physical structure. *Journal of Experimental Biology* 203(14): 2105-2115. doi.org/10.1242/jeb.203.14.2105

Lubis Z & Buckle K A (2007). Rancidity and lipid oxidation of dried-salted sardines. *International Journal of Food Science & Technology* 25(3): 295-303. doi.org/10.1111/j.1365-2621.1990.tb01085.x

Nascimento V L V, do Bermúdez V M S, Oliveira A L L, de Kleinberg M N, Ribeiro R de T M, Abreu R F A de & Carioca J O B (2015). Characterization of a hydrolyzed oil obtained from fish waste for nutraceutical application. *Food Science and Technology (Campinas)*, 35(2): 321-325. doi.org/10.1590/1678-457x.6583

Ouraji H, Shabanpour B, Kenari A A, Shabani A, Nezami S, Sudagar M & Faghani S (2009). Total lipid fatty acid composition and lipid oxidation of Indian white shrimp (Fenneropenaeus indicus) fed diets containing different lipid sources. *Journal of the Science of Food and Agriculture* 89(6): 993-997. doi.org/10.1002/jsfa.3545

Oz M & Dikel S (2015). Comparison of Body Compositions and Fatty Acid Profiles of Farmed and Wild Rainbow Trout (Oncorhynchus mykiss). *Food Science and Technology* 3(4) 56-60. doi.org/10.13189/fst.2015.030402

Ozogul Y, Aydin M, Durmus M, Karadurmus U, Öz M, Yuvka I, Uçar Y, Ayas D, Kuley E & Kösger A (2013). *The Effects of Season and Gender on the Proximate and Fatty Acid Profile of Male and Female Warty Crab (Eriphia Verrucosa) From Black Sea*. *Rapport Commission International Mer Mediterranee* 40: 634.

Pateiro M, Munekata P E, Domínguez R, Wang M, Barba F J, Bermúdez R, & Lorenzo J M (2020). Nutritional profiling and the value of processing by-products from gilthead sea bream (Sparus aurata). *Marine Drugs* 18(2): 101. doi.org/10.3390/md18020101

Ramirez A (2007). Salmon by-product proteins. *FAO Fisheries Circular* 1027: 30.

Simopoulos A (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedicine & Pharmacotherapy* 56(8): 365-379. doi.org/10.1016/S0753-3322(02)00253-6

Simopoulos A P, Leaf A & Salem N (2000). Workshop statement on the essentiality of and recommended dietary intakes for omega-6 and omega-3 fatty acids. *Prostaglandins Leukotrienes and Essential Fatty Acids* 63(3): 119-121. doi.org/10.1054/plef.2000.0176

Sönmez A Y, Bilen S, Özdemir K Y, Alagöz K & Özçelik H (2022). Effect of aqueous methanolic extract of pomegranate peel (Punica granatum) and Veratrum (Veratrum album) on oxidative status, immunity and digestive enzyme activity in rainbow trout (Oncorhynchus mykiss). *Journal of Agricultural Sciences* 28(2): 159-170. doi.org/ 10.15832/ankutbd.870923

TUIK (2021). Fishery Statistics.Turkish Statistical Institute.

Ulbricht T L V & Southgate D A T (1991). Coronary heart disease: seven dietary factors. *The Lancet* 338(8773): 985-992. doi.org/10.1016/0140-6736(91)91846-M

Zhong Y, Madhujith T, Mahfouz N & Shahidi F (2007). Compositional characteristics of muscle and visceral oil from steelhead trout and their oxidative stability. *Food Chemistry* 104(2): 602-608. doi.org/10.1016/j.foodchem.2006.12.036

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In Vitro Evaluation of Apricot Cultivars Response to *Pseudomonas syringae* Pathovars: Image Processing as an Alternative Method

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ABSTRACT

Türkiye, with an apricot (*Prunus armeniaca* L.) production amount of 833,398 tons per year, ranks first in fresh apricot production and dried apricot export in the world. Malatya, Iğdır, and Elazığ constitute the main apricot production centers in Türkiye. Many table and dried apricot cultivars have been grown in Türkiye. Local apricot cultivars such as cv. Şalak (or Aprikoz), cv. Tebereze, cv. Ordubat, cv. Ağcanabat, and cv. Ağerik are widely grown in the Aras Valley, including Iğdır and Kağızman. In this study, DNA barcoding of local cultivars based on the internal transcribed spacer region was performed and their distribution demonstrated in the Aras Valley. The reactions of these apricot cultivars to the causal agents of bacterial canker, which negatively affect the yield and quality of apricot cultivation, were also determined. Alternative methods such as image-processing technology and CHAID analysis have also been successfully used for cultivar reaction tests. It was determined that "Şalak" is economically important and the most common apricot cultivar in the Aras Valley. In addition, the Ağcanabat cultivar was sensitive to the causal agents of disease, while other local apricot cultivars were tolerant to it.

Keywords: Stone fruits, Local cultivars, Bacterial canker, DNA barcoding, CHAID

1. Introduction

Apricot (*Prunus armeniaca* L.) has been cultivated for more than 5000 years across a wide area of the world, including Türkiye, Iran, Turkestan, Afghanistan, Central Asia, and western China (Ercisli 2009; Bakır et al. 2019). Apricots consumed fresh and dried are among the agricultural products with high nutritional value (USDA 2022). With an apricot production of 833,398 tons/year, Türkiye is ranked first in fresh apricot production and dried apricot exports worldwide (FAO 2020). The most economically important apricot-growing area in Türkiye is the Eastern Anatolia region (Akın et al. 2017; Abacı & Asma 2010) where 65-70% of the world's dried apricots are produced in this region (Ercisli 2009). The main apricot production areas in this region are Malatya (389,396 tons/year), Iğdır (42,989 tons/year), and Elazığ (31,179 tons/year) (TÜİK 2021). The Aras Valley, which includes Iğdır and Kağızman, has approximately 6.37% of Türkiye's apricot production (800,000 tons/year), with a production total of 51,033 tons/year (TÜİK 2021). Previous studies have determined the phenological and pomological characteristics of local apricot cultivars (cv. Şalak or Aprikoz, cv. Tebereze, cv. Ordubat, cv. Ağcanabat, and cv. Ağerik) widely grown in the Aras Valley (Özyörük & Güleryüz 1992; Ercisli 2009; Muradoğlu et al. 2011). Referring to the different names of local cultivars or varieties among geographic regions, countries, and continents is a global problem and this issue is particularly important for apricot cultivars in Türkiye. Therefore, to generate a standardized database, it is necessary to resolve the local cultivars of apricots based on DNA barcoding of the universal barcode region internal transcribed spacer (ITS). By doing this it is possible to identify apricot samples by matching them with the generated database that includes known samples as positive controls. DNA barcoding has been successfully applied to some apricot cultivars such as cv. Şalak (Hürkan 2020), Tunusian cultivars (Batnini et al. 2019), and Egyptian cultivars (Sayed et al. 2022).

Due to the small difference in altitude among the apricot orchards, all apricot plantations are exposed to frost in the spring of some years in the Aras Valley, especially in Iğdır (Ercisli 2009). Canker formation on trees is induced by stress factors, such as frost exposure, which makes trees susceptible to infection and injury from frost damage. Pathogen inoculation trials in some studies have consistently shown that increased infection rate and canker length are associated with frost damage (Sobiczewski & Jones 1992; Weaver 1978). *Pseudomonas syringae* van Hall, which has ice nucleation activity, makes some plants susceptible to frost damage at -5 °C (Lindow et al. 1982). Bacterial canker diseases in stone fruits are characterized by the systemic movement of bacteria in all aboveground parts of the plant, especially in the vascular tissues (Lamichhane et al. 2014). It has been reported that disease agents including *P. syringae* pv. *syringae* (*Pss*), *P. syringae* pv. *morsprunorum* race 1 (*Psm* R1), *P. syringae* pv. *morsprunorum* race 2 (*Psm* R2), and *P. syringae* pv. *cerasicola* (*Psc*) are pathogenic in apricot plants and may cause bacterial canker symptoms (Hulin et al. 2020). Disease-causing bacteria infect all aboveground plant organs throughout the season, causing symptoms such as fruit spots, necrosis, dead buds, flower blight, cankers on stems and branches, and bacterial oozing (Kennelly et al. 2007; Hulin et al. 2020). Apricots (Kavak & Çitir 1995; Kotan & Sahin 2002), peaches (Özaktan et al. 2008), cherries (Bülbül & Mirik 2014; Erkek 2017; Akbaba & Özaktan 2021), and citrus (Mirik et al. 2005) have been reported to be causative agents of bacterial canker in Türkiye. The control of diseases caused by *P. syringae* pathogens is almost impossible because of the lack of effective chemical or biocontrol agents, the low number of resistant cultivars, and the endophytic nature of the pathogen (Kennelly et al. 2007). Different pathogenic strains of *P. syringae* have a large genetic variability that is adaptable to different hosts, cultivars, and pedoclimatic conditions, making it difficult to control bacterial canker (Morris et al. 2009). Gilbert et al. (2010) suggested the need for alternative methods for the management of bacterial diseases in orchards, including approaches based on biological control and plant resistance. For this reason, one of the most viable and economical methods for the management of bacterial cancer in apricots is to use resistant cultivars. Only a few apricot cultivars in previous studies worldwide have reported resistance to *P. syringae* pv. *syringae* (Scortichini et al. 1999; Singh et al. 2005; Giovanardi et al. 2018).

Improving the conditions for the control of bacterial canker requires determining the diversity, epidemiology, and distribution of the causal agents of the disease. In addition, the distribution, diversity, and susceptibility of host cultivars are important in the epidemic risk of the disease. The present study aims to generate a DNA barcode database for the local apricot cultivars (cv. Ağcanabat, cv. Ağerik, cv. Aprikoz, cv. Ordubat, and cv. Tebereze) resolve phylogenetic relationships among the cultivars, identify samples with the generated database, and determine the resistance reactions of these cultivars against *P. syringae* pathovars, which are the causal agents of bacterial canker in apricots. Image processing technology and MATLAB software were used to determine the responses of the cultivars as an alternative method, in contrast to previous studies.

2. Material and Methods

2.1. Collection of plant samples

In the field survey, a simple random sampling method was applied to represent the apricot production areas in the Aras Valley (Türkiye) (Bora & Karaca 1970). According to the method; for 1-5 da (1 piece), 5-10 da (2 pieces), 10-50 da (3 pieces), 50-100 da (4 pieces), 100-500 da (5 pieces), and 500-1000 da (6 pieces) were collected as plant samples from apricot orchards. The samples were made by increasing the sampling rate by 1 for every 500 decares after 1000 da area. Leaf samples from the apricot cultivars (Table 1) were collected for DNA Barcoding. "Şalak" is known as "Aprikoz" cultivar in local public in Kağızman.

Table 1- Characteristics of local apricot cultivars in the Aras Valley (Reviewed in Ercisli 2009)

Cultivars	*Fruit weight (g)*	*Fruit shape*	*Skin colour*	*Freedom of pit*	*Type of consumption*	*Flesh firmness*	*TSS (%)*	*Location*
Şalak	63	Cylindiric	Yellow	Free	Table	Soft	14	Iğdır
Ordubat	25	Cylindiric	Orange	Semi-cling	Dried	Medium	18	Iğdır
Tebereze	38	Round	Orange	Free	Dried	Medium	17	Iğdır
Agcanabat	51	Round	Cream	Free	Table	Medium	14	Iğdır
Agerik	45	Round	White	Free	Dried	Soft	14	Iğdır
Şekerpare	22	Round	Cream	Free	Table	Soft	20	Malatya

2.2. DNA extraction and amplification of ITS region for DNA Barcoding

We used 100 mg of fresh leaf tissues from each cultivar as the starting material for DNA extraction, which was performed according to the literature (Aydin et al. 2018). The extracted DNAs were checked for integrity on 1.5% agarose gel electrophoresis, and the concentration was measured using NanoDrop (Maestrogen, Taiwan) spectrophotometer. The DNA extracts were stored at -45 ºC until polymerase chain reaction (PCR). We amplified the partial ITS region using the primers ITS1A (5´-GACGTCGCGAGAAGTCCA-3´), and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) described by Gulyás et al. (2005) and White et al. (1990), respectively. Twenty-five microliter of PCR reaction mix was prepared with a 2X Reaction Buffer (Thermo Scientific, Cat. No. EP0401), 0.1 mM dNTPs, 0.2 µM both primers, 1 U Taq DNA polymerase (Thermo Scientific, Cat. No. EP0401), 1 mM Mg^{+2}, 20 ng DNA and nuclease-free water. Thermal cycling was performed in a SimpliAmp™ instrument (Thermo Scientific, USA) using conditions first denaturation at 95 °C for 1 min and followed by 35 cycles of 95 °C for 30 s denaturation, 54 °C for 30 s annealing, 72 °C for 1 min elongation. PCR was finalised with 72 °C for 10 min final elongation step. All the PCR products were checked for specific amplicons (~800 bp) on 2% agarose gel electrophoresis. After ensuring for specific amplicons, we directly sent the PCR products to Macrogen Inc. (The Netherland) for purification and Sanger sequencing for both directions using the same primers on the PCR.

2.3. Bioinformatics and phylogenetics

The obtained DNA sequences were imported to Geneiour R8 (Dotmatics, Australia) bioinformatics platform and checked for sequencing quality. The primer binding regions and low quality ending parts of the sequences were trimmed, and the sequences for both directions were aligned to generate consensus sequences for each apricot cultivar. The consensus sequences of the positive controls (*P. armeniaca* cv. Ağcanabat, *P. armeniaca* cv. Ağerik, *P. armeniaca* cv. Şalak (Aprikoz), *P. armeniaca* cv. Ordubat, and *P. armeniaca* cv. Tebereze) were submitted to the National Center for Biotechnology Information GenBank and deposited with the accession numbers OP804469, OP804470, OP804471, OP804472, and OP804473, respectively. The ITS sequence of *P. armeniaca* cv. Şalak were obtained from Genbank (MT072696). Then, to identify the 67 apricot samples collected in this study, we compared their sequences with positive controls by aligning all the sequences and constructing a phylogenetics tree using Neighbor-Joining approach with 1,000 bootstrap replicates. *Prunus persica* (DQ006273) was selected as the outgroup.

2.4. Determination of response of cultivars against P. syringae pathovars

Five different local apricot cultivars and cv. Şekerpare in Table 1, commonly cultivated in Türkiye, were used in this study (Ercisli 2009). According to Görmez et al. (2013), cv. Şekerpare is a tolerant cultivar of *P. syringae* pv. *syringae*. *Pseudomonas syringae* strains (*P. syringae* pv. *morsprunorum* R1 strain 25B and *P. syringae* pv. *morsprunorum* R2 strain 732 from Poland and *Pseudomonas syringae* pv. *syringae* strain BAY3 from Türkiye) in bacteriology laboratory stocks were also used as reference pathogenic bacteria.

The methods specified in previous studies by Sobiczewski & Jones (1992) and Li et al. (2015) were optimized and used for the shoot inoculation tests in this study. One-year dormant shoots of apricot cultivars were randomly collected from orchards in and around Iğdır in 2021 (December) and used for cultivar reaction tests. Each shoot was cut into equal lengths of 10 cm. For surface sterilization, the cut shoots were kept in 0.5% sodium hypochlorite for 5 min, rinsed with sterile distilled water, and left to dry on blotting paper for a while.

For shoot inoculation, the strains of pathogenic bacteria were grown in King's B medium (Schaad et al. 2001) for 24-48 h at 24±2 °C. Growing bacterial cultures were suspended in 20 mL of sterile water and prepared for all strains at the same concentration (OD_{600}: 0.1) using a spectrophotometer. Sterile distilled water was used as the negative control. The upper 5 mm of each 10 cm shoot tip was removed with a scalpel and immersed in the bacterial suspension for 5 min. The tips of the shoots immersed in the bacterial suspension were covered with Parafilm. The untreated tips of the shoots were cut (approximately 10 mm) and placed in transparent polyethylene bags containing sterile cotton and sterile distilled water (0.1% sodium hypochlorite dropped) to a depth of 20 mm. To prevent cross-contamination, separate polyethylene bags were used for each treatment. Cultivar × strain treatment was performed with 5 repetitions. The shoots were incubated in closed polyethylene bags at 15 °C for 1 week under a photoperiod of 16 h in light and 8 h in darkness. To show the effect of frost damage on the infection process in the experiment, the shoots were incubated for 1 week at -2 °C. Finally, the 10 mm untreated part of each shoot was discarded, and the shoots were placed in sterile polyethylene bags according to a completely randomized plot experiment design. It was incubated at 15 °C for four weeks under the same light conditions as previously mentioned, maintaining high humidity.

At the end of the incubation period, the necrotic areas on the shoots were evaluated for the severity of the bacterial canker. First, the uppermost layer of the bark was stripped from the top to reveal symptoms. The shoots were then cut from the bottom to approximately 8 cm long and digitally photographed to detect the necrotic areas. According to the classical method used by Sobiczewski & Jones (1992), the length of the necrosis was measured using a ruler, and the percentage of disease severity was determined by proportioning it to the entire length of the shoot. As an alternative to this evaluation method, a new method based on image processing using MATLAB was used in this study. This new method allows for the detection of the entire necrotic area compared with the widely used subjective evaluation method based on the measurement of necrosis length specified in previous studies.

In the detection of disease by image processing method, the (red, green, and blue) values of the diseased parts of the apricot shoot were examined at the first stage, and the black-and-white (binary) image obtained by following the instructions given in Supplemantary data 1 and the processing steps specified in Figure 1 was used in calculating the area of the apricot area and the diseased area in the apricot. Perimeter and area measurements are meaningful only for binary images, for this purpose the percentage of diseased area was calculated using the "bwarea" command (Pratt 1991) in MATLAB.

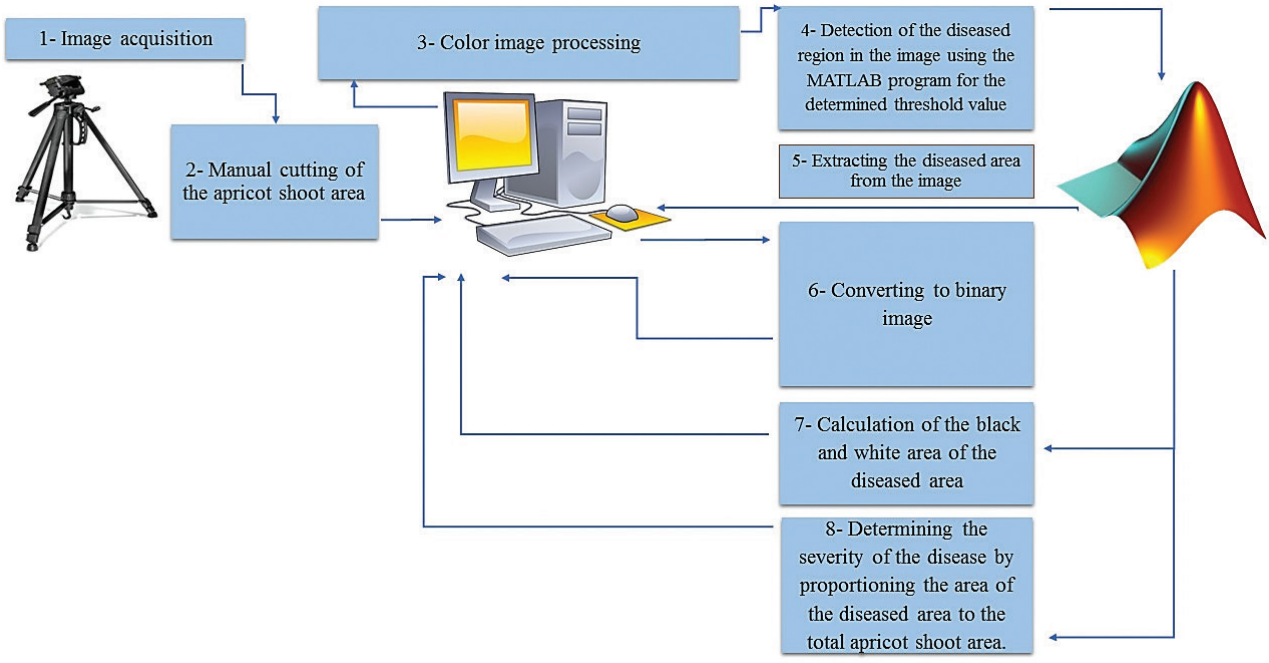

Figure 1- Image processing steps using MATLAB

2.5. Statistical analysis

In the evaluation of the cultivar x treatment reaction was used CHAID analysis which is a non-parametric analysis, it is a statistical method that is independent of the preconditions and assumptions required by parametric tests and does not require any transformation in the data. In the chaid analysis, the parent and child node ratio is taken as 10:5 and tree depth 3.

3. Results

3.1. Collecting of apricot samples

Based on the results published by Özyörük and Güleryüz (1992), local cultivars cultivated in the Aras Valley were determined, as shown in Figure 2. Leaf samples of local apricot cultivars were collected from 71 points representing the apricot production areas in the Aras Valley (Supplementary data 2).

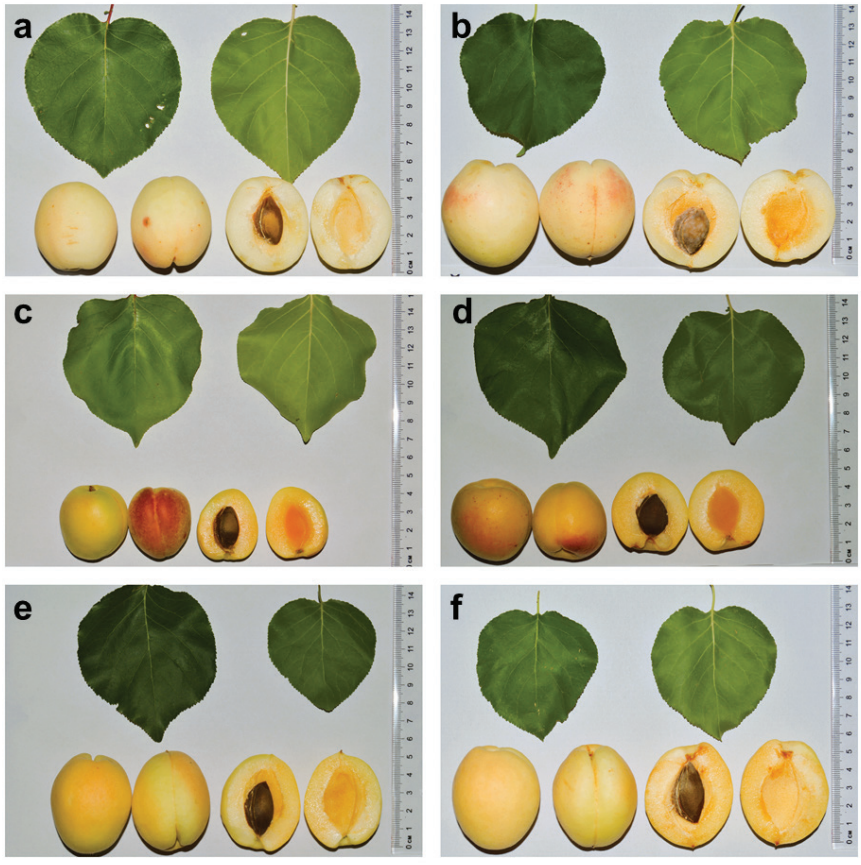

Figure 2- Local cultivars cultivated in the Aras Valley; Ağerik (a), Ağcanabat (b), Ordubat (c), Tebereze (d), Şalak (e), Aprikoz (f)

3.2. DNA barcoding of the apricot samples

We successfully extracted DNA from the samples with the concentrations ranging from 18.39-84.42 ng μL^{-1}, the A260/A230 ratio was 1.30-1.69, and the A260/A280 ratio was 1.83-2.30. The nanodrop results confirmed that the extracted DNAs were ready for PCR reaction. Agarose gel electrophoresis validated ITS barcode region were amplified successfully for all the samples (data not shown). Nucleotide sequences for ITS region were obtained with sequencing quality scores ranging from 80.6-96.7. After trimming and aligning the sequences, the aligned length was 716 bp.

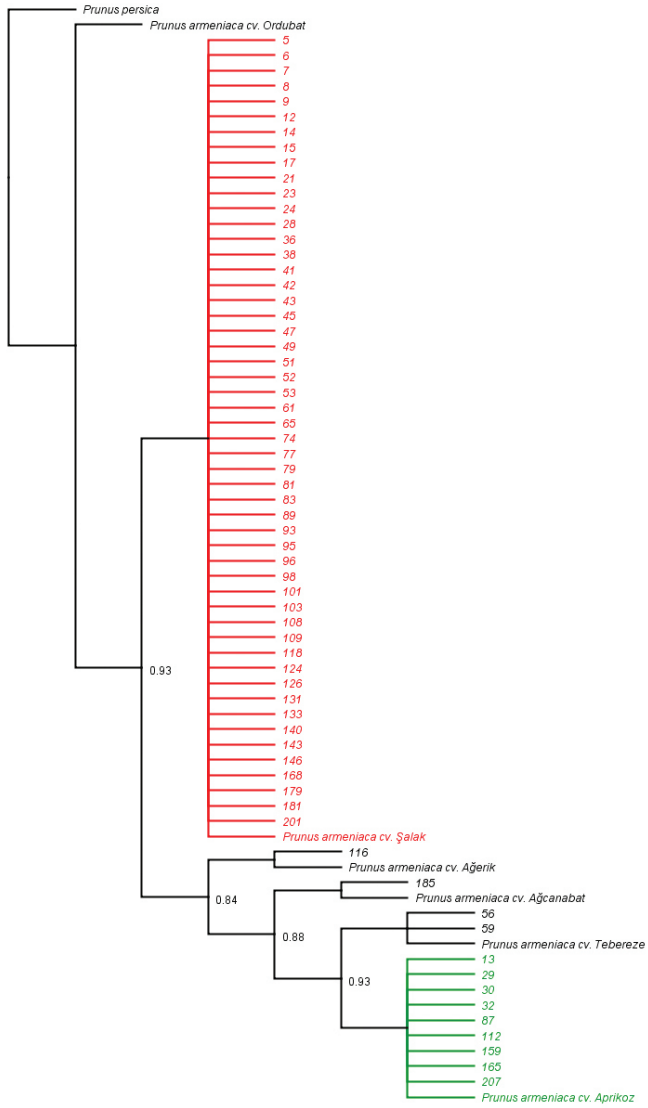

Figure 3- Phylogram reconstructed with internal transcribed spacer sequence of 71 apricot *(Prunus armeniaca L.)* samples. The Aprikoz cultivar groups were coloured as green, the Şalak cultivar group were coloured as red. Bootstrap support values were placed on the nodes

The phylogram reconstructed with ITS sequences identified the sampled by grouping the samples with closely related positive controls (Figure 3). The outgroup was placed as the natural outgroup on the tree. The branchings were highly supported by bootstrap values (85 to 93%). According to the tree, most of the (52 samples) samples matched with *P. armeniaca* cv. Şalak, 9 samples were matched with *P. armeniaca* cv. Aprikoz, two samples (56 and 59) matched with *P. armeniaca* cv. Tebereze, the sample 185 matched with *P. armeniaca* cv. Ağcanabat, and the sample 116 matched with *P. armeniaca* cv. Ağerik. There was no matching with *P. armeniaca* cv. Ordubat. According to the DNA barcoding results in these 65 locations, 80% cv. Şalak, 14% cv. Şalak (Aprikoz), and 3% cv. Tebereze cultivars were identified, and the remaining 3% belonged to the Ağcanabat, Ordubat, and Ağerik cultivars (Figure 4).

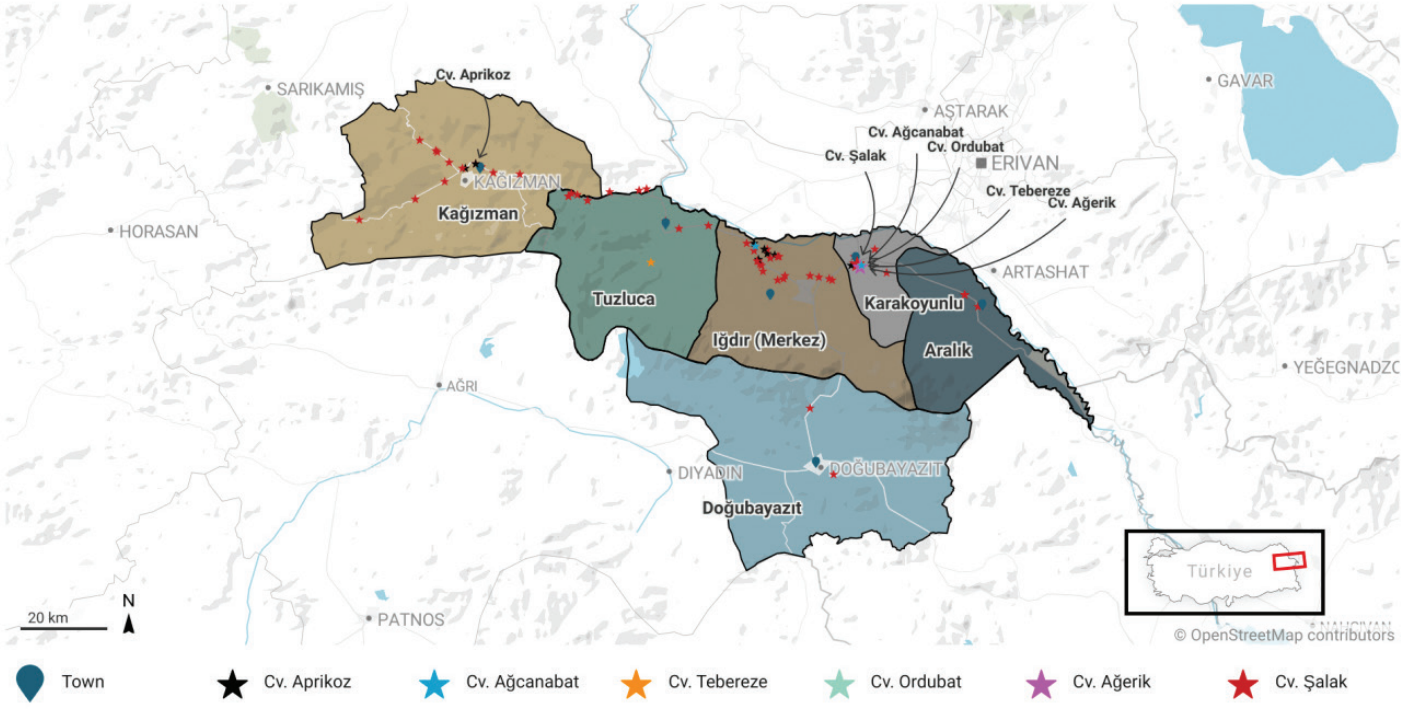

Figure 4- Distribution map of apricot cultivars in Aras Valley based on GPS data

3.3. An alternative method to detect the reactions of apricot cultivars to pathogens

In this study, results were obtained regarding the reactions of six different apricot cultivars against three different *P. syringae* pathovars, which are causal agents of bacterial canker in apricot (Giovanardi et al. 2018). An alternative new method based on image processing technology instead of the classical method has been attempted to obtain results (Figure 1). Data on disease severity obtained using MATLAB were statistically compared with the results obtained using the classical method (Figure 5). A correlation of 0.7587 was found between the disease severity data determined using these two methods.

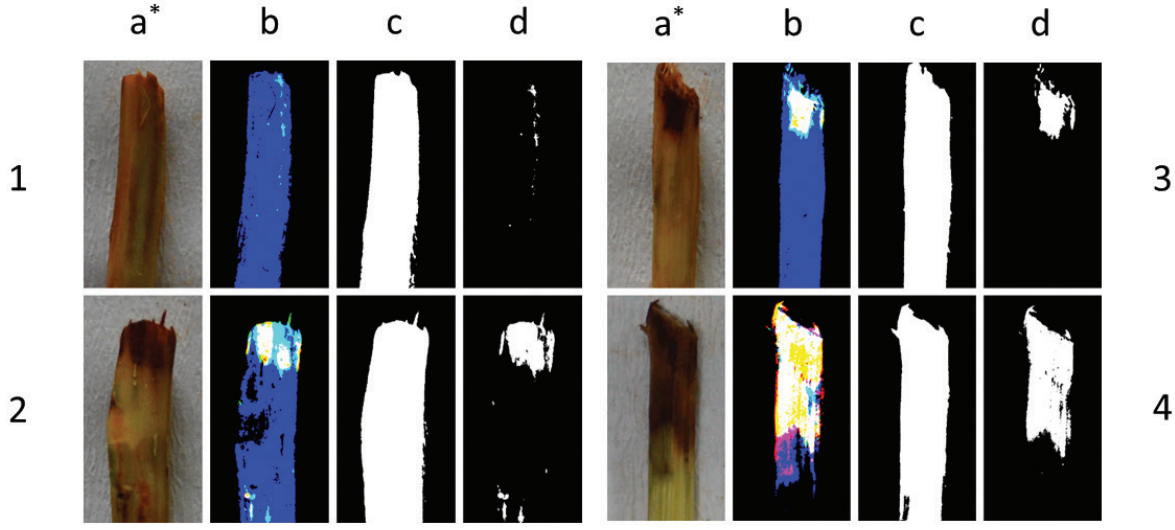

Figure 5- Stages of image processing (IP) at cultivar reactions to bacterial pathogens; Apricot cultivar (cv. Ağcanabat*); treatments (NC[1], strain BAY3[2], strain 25B[3], strain 7324); Image processing (Original[a], RGBcolour[b], RGBblack_white[c], RGBdiseased_area[d])

In the classical method, different researchers can detect the disease severity at different rates. However, the image-processing method provides the same results as an objective tool for researchers who use the same command and threshold values. Therefore, in the next step of the study, the data obtained from the image processing method using MATLAB was used to determine the average disease severity (%) values showing the response of the cultivars to the pathogens.

Table 2- Reactions of cultivars with *P. syringae* pathovars (%)

Cultivars	*NC*	*Strain BAY3 (Pss)*	*Strain 25B (Psm R1)*	*Strain 732 (Psm R2)*
Ağcanabat	0.75	9.13	1.69	15.72
Şalak	0.08	0.80	1.24	2.22
Tebereze	0.00	1.68	0.45	0.85
Ordubat	0.01	3.73	1.29	1.32
Ağerik	0.10	5.52	3.43	2.73
Şekerpare	0.39	1.74	2.82	4.33

The results were evaluated with reference to cv. Şekerpare, which has been reported to be tolerant to *Pss* in previous studies. According to the results of the CHAID analysis shown in Figure 6, disease severity was determined at an average rate of 7.032% in 120 apricot shoots (node-0). Pathogens (treatments) affected the disease severity more than the cultivar factor; therefore, the first node (node-0) was attached to the node of the treatments. Disease severity was divided into three groups based on the pathogens. In the negative control group, an average of 0.033% disease severity was determined (node-1). Strains BAY3 and 732 were included in the same group in terms of their effect on disease severity and caused 11.075% of disease on average (node-2). The disease severity rate caused by strain 25B was determined to be 5.943% and was included in a different group from the others (node-3). Strains BAY3 (*Pss*) and 732 (*Psm* R2) were in the same statistical group according to the disease severity values created by pathogen application on the cultivars. The Ağcanabat cultivar was in the sensitive group with a disease severity rate of 17.810% (node-4) for these two pathogens, whereas other cultivars were in the same statistical group with the more tolerant cv. Şekerpare with an average disease severity of 9.728% (node-5). According to the results, the Ağcanabat cultivar was more sensitive than the cv. Şekerpare (Table 2). The reaction differences between cultivars other than the Ağcanabat cultivar against these two pathogens were statistically insignificant. There was no statistical difference between the cultivars with respect to strain 25B (*Psm* R1). All cultivars for this strain were in the same statistical group as that of the tolerant reference cultivar. When the image processing method was used to evaluate the results, an average of 0.033% disease severity values were measured in the negative control. According to expert opinions, the error of the method is a tolerable level for plant samples (Figure 5, Table 2, node-1). The coefficient of determination (R^2) for the model was 0.599.

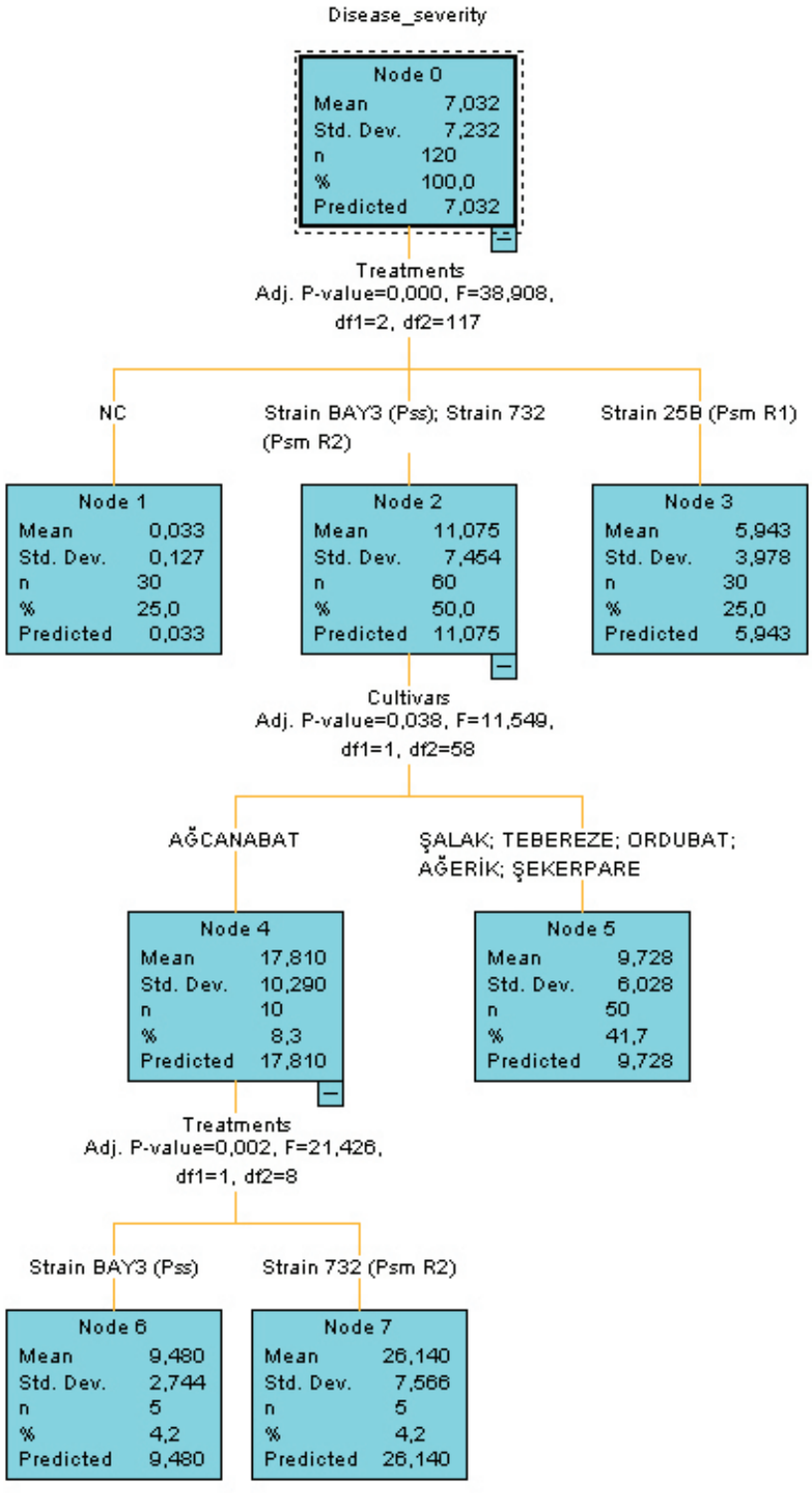

Figure 6- CHAID diagram: Effect of cultivar and pathogen treatments on disease severity

4. Discussion

Many table [Hasanbey, Şalak (Aprikoz), Şekerpare, Şam, Turfanda İzmir, Tokaloğlu, Alyanak, Ethembey, Karacabey, Mahmudun Eriği, Adilcevaz 5, İri Bitirgen, Precoce de Tyrinthe, Precoce de Colomer, Canino, Luizet, Roxana, Ninfa, Aurora ect.] and dried apricot cultivars (Hacıhaliloğlu, Kabaaşı, Soğancı, Çataloğlu) are grown in Türkiye, placing the country first in world apricot production (Paydaş Kargı et al. 2015). The Aras Valley, which includes Iğdır and Kağızman, accounts for approximately 6.37% of this production (TÜİK 2021). Economically important apricot cultivars such as Şalak (Aprikoz), Tebereze, Ordubat, Ağcanabat, and Ağerik are widely grown in the Aras Valley, and their phenological and pomological characteristics have been determined in previous studies (Özyörük & Güleryüz 1992; Ercisli 2009; Muradoğlu et al. 2011). Therefore, it is crucial to identify the apricot cultivars for breeding and commercial use. Bourguiba et al. (2010) reported sequence-tagged microsatellites (SSR) and amplified fragment length polymorphism (AFLP) markers could be used to identify apricot cultivars and discover synonyms and homonyms. Genotyping studies were limited to AFLP, SSR, and random-amplified fragment length polymorphism (RAPD) techniques for Turkish apricot genotyping studies. The next level genotyping method based on DNA sequencing was also applied to identify and discriminate apricot cultivars from closely related apricot types. In a study performed by Hürkan (2020), the DNA sequences of barcoding regions ITS, LEAFY, matK, rbcL, and ycf1 for *P. armeniaca* cv. Şalak were obtained and tested for their discrimination performance. The results of the study showed that ITS and LEAFY DNA sequences successfully discriminated the Şalak cultivar from other cultivars. Previous studies have mentioned that "Aprikoz" and "Şalak" are the same cultivars (Asma 2000, Alim & Kaya 2005; Asma & Ozturk 2005). However, these cultivars differed according to the DNA sequencing results based on the ITS gene region. In the present study's DNA sequencing based analysis, the cultivars distributed as Şalak (80%), Aprikoz (14%), and Tebereze (3%) in the Aras Valley. The remaining 3% included the Ağcanabat, Ağerik, and Ordubat cultivars. The results confirmed the previous studies identification of apricot cultivars distributed in the Aras Valley. Alim & Kaya (2005) reported Şalak (Aprikoz) was the most cultivated cultivar with 85%, and Tebereze, Ağerik and Ordubat cultivars was 15%. *P. syringae* pathovars have high genetic diversity, making bacterial canker difficult to control (Morris et al. 2009). One of the most important methods for controlling the causal agents of bacterial canker is the use of resistant apricot cultivars (Donmez et al. 2010). However, only a few apricot cultivars have been reported to be resistant to *Pss* until now (Scortichini et al. 1999; Singh et al. 2005). In a study conducted on apricot cultivars in Türkiye, Hacıhaliloğlu was reported to be very sensitive to *Pss*, while the Roxana, Hasanbey, and Şekerpare cultivars were tolerant to *Pss* (Görmez et al. 2013). Apricot cultivars that show resistance or sensitivity to the causal agents of bacterial canker in Türkiye and the world have been determined by many similar studies (Donmez et al. 2010; Bibi et al. 2022; Cetinkaya et al. 2022). However, the local cultivars in the Aras Valley were not included in these studies. Therefore, in this study, the reactions of apricot cultivars in the Aras Valley against *P. syringae* pathovars caused bacterial canker on apricot were detected for the first time using the image processing method.

The determination coefficients of the disease severity values obtained by image processing and classical methods were calculated as R^2=0.5756. The most important reason for not obtaining a high level of determination coefficient is that the region of the disease is in the form of a line in some cases, whereas in some cases, it is spread over the entire shoot (Figure 5). In the classical method, disease severity is determined by measuring the length of necrosis and dividing it by the shoot length. For this reason, even if the disease symptom was a thin line, the entire shoot area with symptoms was considered to be diseased. However, in the image-processing method, disease severity is only obtained by the ratio of the specified necrotized area to the area of the shoot, which eliminates this disadvantage (Figure 5). In this study, an image processing method was developed using the MATLAB package program to obtain faster and more objective results than the classical method (Sobiczewski & Jones 1992) in the evaluation of disease severity. In a similar study, a general, rapid and reliable approach was used for lesion measurement using image recognition and an artificial neural network model, and it was suggested that it produced more objective results than visual scoring (Li et al. 2015).

In this study, CHAID analysis, which allows for easier interpretation of the data, was used as a statistical method to evaluate the reactions of local cultivars to *P. syringae* pathovars. In most scientific studies, the findings obtained cannot provide the assumptions of the ANOVA test (normality, homogeneity of variances, etc.). In this situation, applying one of the transformation methods (angle, log, ln, etc.) to the data, the dataset was transformed to be suitable for a normal distribution, an ANOVA test was applied to these new data, and the results obtained were evaluated. The CHAID analysis, which has become increasingly popular in recent years, was used as an alternative to the classical method in this study. The CHAID analysis is a decision tree method in which the relationship between the dependent and independent variables in the dataset and the relationships among the independent variables are examined (Akın et al. 2017). CHAID is a nonparametric analysis, is independent of the preconditions and assumptions required by parametric tests, and does not require any transformation of the data (Koc et al. 2019).

5. Conclusion

According to our results of DNA Barcoding based on ITS, "Şalak" is the most common apricot cultivar in Aras Valley. Alternative methods such as image-processing technology and CHAID analysis have also been successfully used for cultivar reaction tests in this study. It was also determined that the Ağcanabat cultivar, which is a local apricot cultivar in the Aras Valley, is sensitive to strains BAY3 (*Pss*) and 732 (*Psm* R2), whereas the other local cultivars were found to be tolerant. All cultivars were in the tolerant group of strain 25 B (*Psm* R1), and the Ağcanabat apricot cultivar was highly sensitive to strain 732 (*Psm* R2).

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: M.A., K.H., A.E.K., Design: M.A., K.H., A.E.K., Data Collection or Processing: M.A., K.H., A.E.K., Analysis or Interpretation: M.A., K.H., A.E.K., Literature Search: M.A., K.H., A.E.K., Writing: M.A., K.H., A.E.K.

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References

Abacı Z T & Asma B M (2010). Bazı kayısı çeşitlerinin farklı ekolojik alanlardaki biyolojik özelliklerinin analizi. *Research Journal of Biological Sciences* 3(1):165-168.

Akbaba M & Özaktan H (2021). Evaluation of bacteriophages in the biocontrol of *Pseudomonas syringae* pv. *syringae* isolated from cankers on sweet cherry (Prunus avium L.) in Turkey. *Egyptian Journal of Biological Pest Control* 31(1):1-11. doi.org/10.1186/s41938-021-00385-7

Akın M, Eyduran E & Reed B M (2017). Use of RSM and CHAID data mining algorithm for predicting mineral nutrition of hazelnut. *Plant Cell, Tissue and Organ Culture* 128(2):303-316. doi.org/10.1007/s11240-016-1110-6

Alim M & Kaya G (2005). Iğdır'da kayısı tarımı ve başlıca sorunları. *Doğu Coğrafya Dergisi* 10(14): 47-65.

Asma B M (2000). Kayısı Yetiştiriciliği. Evin Ofset.

Asma B M & Ozturk K (2005). Analysis of morphological, pomological and yield characteristics of some apricot germplasm in Turkey. *Genetic Resources and Crop Evolution* 52: 305-313. doi.org/10.1007/s10722-003-1384-5

Aydin A, Ince A G, Uygur Gocer E & Karaca M (2018). Single cotton seed DNA extraction without the use of enzymes and liquid nitrogen. *Fresenius Environmental Bulletin* 27(10): 6722-6726.

Bakır M, Dumanoğlu H, Erdoğan, V, Ernim C & Macit T (2019). Characterization of wild apricot (Prunus armeniaca L.) genotypes selected from Cappadocia Region (Nevşehir-Turkey) by SSR markers. *Journal of Agricultural Sciences* 25(4): 498-507. doi.org/10.15832/ankutbd.457850

Batnini M A, Bourguiba H, Trifi-Farah N & Krichen L (2019). Molecular diversity and phylogeny of Tunisian *Prunus* armeniaca L. by evaluating three candidate barcodes of the chloroplast genome. *Scientia Horticulturae* 245: 99-106. doi.org/10.1016/j.scienta.2018.09.071

Bibi S, Inam-ul-Haq M, Riaz A & Malik S I (2022). Identification of Host Resistance against bacterial canker disease on apricot, plum and peach grown in Punjab and KPK. *International Journal of Plant Pathology* 11(1): 43-48. doi.org/10.33687/phytopath.011.01.4130

Bora T & Karaca İ (1970). Kültür bitkilerinde hastalığın ve zararın ölçülmesi, *Ege Üniversitesi Ziraat Fakültesi Yardımcı Ders Kitabı* 167: 43.

Bourguiba H, Krichen L, Audergon J M & et al. (2010). Impact of mapped SSR markers on the genetic diversity of apricot (Prunus armeniaca L.) in Tunisia. *Plant Molecular Biology Reporter* 28: 578-587. doi.org/10.1007/s11105-010-0189-x

Bülbül M & Mirik M (2014). Tekirdağ'da kiraz ağaçlarında bakteriyel kanser etmenlerinin yaygınlığı, izolasyonu ve tanımlanması üzerinde araştırmalar. *Journal of Turkish Phytopathology*, 43(1-3): 15-24.

Cetinkaya Yildiz R, Karatas A, Horuz S & et al. (2022). Determination of susceptibility of citrus and stone fruit cultivars against Pseudomonas syringae pv. syringae in Turkey. *Gesunde Pflanzen* 74: 639-646. doi.org/10.1007/s10343-022-00638-x

Donmez MF, Karlidag H & Esitken A (2010). Identification of resistance to bacterial canker (Pseudomonas syringae pv. syringae) disease on apricot genotypes grown in Turkey. *European Journal of Plant Pathology* 126: 241-247. doi.org/10.1007/s10658-009-9536-x

Ercisli S (2009). Apricot culture in Turkey. *Scientific Research and Essays* 4(8): 715-719.

Erkek E (2017). Determination of Epiphytic and Pathogenic Bacterial Agents in Cherry Trees in Amasya Province. Master Thesis.

FAO (2020). Production of Apricots: top 10 producers. Accessed 13 October 2022 https://www.fao.org/faostat/en/#data/QCL/visualize

Gilbert V, Planchon V, Legros F, Maraite H & Bultreys A (2010). Pathogenicity and aggressiveness in populations of *Pseudomonas syringae* from Belgian fruit orchards. *European Journal of Plant Pathology* 126: 263-277. doi.org/10.1007/s10658-009-9538-8

Giovanardi D, Ferrante P, Scortichini M & Stefani E (2018). Characterisation of *Pseudomonas* syringae isolates from apricot orchards in north-eastern Italy. *European Journal of Plant Pathology* 151(4): 901-917. doi.org/10.1007/s10658-018-1424-9

Görmez A, Sahin F, Gulluce M & Aslan, I (2013). Identification and characterization of Pseudomonas syringae isolated from apricot trees in the Erzurum province of Turkey and evaluation of cultivar reaction. *Journal of Plant Pathology* 95(3): 525-532.

Gulyás G, Sramkó G, Molnár V A, Rudnóy S, Illyés Z, Balázs T & Bratek Z (2005). Nuclear ribosomal DNA ITS paralogs as evidence of recent interspecific hybridization in the genus Ophrys (Orchidaceae). *Acta Biologica Cracoviensia Series Botanica* 47(2):61-67.

Hulin M T, Jackson R W, Harrison R J & Mansfield J W (2020). Cherry picking by pseudomonads: after a century of research on canker, genomics provides insights into the evolution of pathogenicity towards stone fruits. *Plant Pathology* 69(6): 962-978. doi.org/10.1111/ppa.13189

Hürkan K (2020). Analysis of various DNA barcodes on the turkish protected designation of origin apricot "Iğdır Kayısısı" (*Prunus armeniaca* cv. Şalak). *Turkish Journal of Agriculture - Food Science and Technology* 8(9): 1982-1987. doi.org/10.24925/turjaf.v8i9.1982-1987.3594

Kavak H & Çitir A (1995). Malatya ili merkez ilçede kayisilarda görülen hastaliklarin tanilari ve yayginlik oranlari üzerine araştırmalar. *VII. Türkiye Fitopatoloji Bildirileri* 531-534.

Kennelly M M, Cazorla F M, de Vicente A, Ramos C & Sundin G W (2007). Pseudomonas syringae diseases of fruit trees: progress toward understanding and control. *Plant Disease* 91(1): 4-17. doi.org/10.1094/PD-91-0004

Koc A, Karahan A E & Bingül M (2019). Determination of relationship between land surface temperature and different land use by chaid analysis. *Applied Ecology and Environmental Research* 17(3): 6051-6067. doi.org/10.15666/aeer/1703_60516067

Kotan R & Sahin F (2002). First record of bacterial canker caused by *Pseudomonas syringae* pv. *syringae* on apricot trees in Turkey. *Plant Pathology* 51: 798. doi.org/10.1046/j.1365-3059.2002.00768.x

Lamichhane J R, Varvaro L, Parisi L, Audergon J M & Morris C E (2014). Disease and frost damage of woody plants caused by *Pseudomonas syringae*: seeing the forest for the trees. *Advances in Agronomy* 126: 235-295. doi.org/10.1016/B978-0-12-800132-5.00004-3

Li B, Hulin M T, Brain P, Mansfield J W, Jackson R W & Harrison R J (2015). Rapid, automated detection of stem canker symptoms in woody perennials using artificial neural network analysis. *Plant Methods* 11(1): 57. doi.org/10.1186/s13007-015-0100-8

Lindow S E, Arny D C & Upper C D (1982). Bacterial ice nucleation: a factor in frost injury to plants. *Plant Physiology* 70(4): 1084-1089. doi.org/10.1104/pp.70.4.1084

Mirik M, Baloglu S, Aysan Y, Cetinkaya-Yildiz R, Kusek M & Sahin F (2005). First outbreak and occurrence of citrus blast disease, caused by Pseudomonas syringae pv. syringae, on orange and mandarin trees in Türkiye. *Plant Pathology* 54(2): 238. doi.org/10.1111/j.1365-3059.2005.01134.x

Morris C E, Bardin M, Kinkel L L, Moury B, Nicot P C & Sands D C (2009). Expanding the paradigms of plant pathogen life history and evolution of parasitic fitness beyond agricultural boundaries. *PLoS Pathogens* 5(12): e1000693. doi.org/10.1371/journal.ppat.1000693

Muradoğlu F, Pehluvan M, Gündoğdu M & Kaya T (2011). Iğdır yöresinde yetiştirilen bazı kayısı (Prunus armeniaca L.) genotiplerin fizikokimyasal özellikleri ile mineral içerikleri. *Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi* 1(1): 17-22. dergipark.org.tr/en/pub/jist/issue/7924/104203

Özaktan H, Akkopru A, Bozkurt A & Erdal M (2008). Information on peach bacterial canker in Aegean Region of Turkey. In Proceedings of STF Meeting on "Determination of the incidence of the different pathovars of Pseudomonas syringae in stone fruits"COST Action 873. *Bacterial diseases of stone fruits and nuts* 8.

Özyörük C & Güleryüz M (1992). Iğdir ovasinda yetişen kayisi çeşitleri üzerinde pomolojik biyolojik ve fenolojik araştırmalar. *Atatürk Üniversitesi Ziraat Fakültesi Dergisi* 23(1): 16-28.

Paydaş Kargı S, Kafkas E & Dikkaya Y R (2015). Düşük soğuklamalı bazı kayısı çeşitlerinin KKTC ekolojik koşullarına adaptasyonları (TAGEP PROJESİ: TAE-P2000014). Çukurova Üniversitesi Ziraat Fakültesi - KKTC Tarım ve Doğal Kaynaklar Bakanlığı (in turkish)

Pratt WK (1991). *Digital Image Processing*. New York, John Wiley & Sons 634.

Sayed H A, Mostafa S, Haggag I M & Hassan N A (2022). DNA barcoding of prunus species collection conserved in the national gene bank of Egypt. *Molecular Biotechnology* 65: 410-418. doi.org/10.1007/s12033-022-00530-z

Schaad N W, Jones J B & Chun W (2001). Laboratory guide for identification of plant pathogenic bacteria. American Phytopathological Society (APS Press) St Paul USA p.373.

Scortichini M, Pellegrino S & Berra L (1999). Susceptibility of apricot germplasm to natural infection by Pseudomonas syringae pv. *syringae* in Piedmont. *Rivista di Frutticoltura e di Ortofloricoltura* 61: 83-86

Singh H V, Mir M S & Wani M A (2005). Effect of host genotypes and age of plant on resistance to gummosis disease in apricot of Kargil. *Annals of Plant Protection Sciences* 13: 152-155.

Sobiczewski P & Jones A L (1992). Effect of exposure to freezing temperatures on necrosis in sweet cherry shoots inoculated with Pseudomonas syringae pv. *syringae* or *P s* morsprunorum. *Plant Disease* 76: 447-451. doi.org/10.1094/pd-76-0447

TÜİK (2021). Bitkisel Üretim İstatistikleri (Kayısı)

United States Department of Agriculture (USDA) (2022). Apricots, raw.

Weaver D J (1978). Interaction of Pseudomonas syringae and freezing in bacterial canker on excised peach twigs. *Phytopathology* 68: 1460-1463. doi.org/10.1094/phyto-68-1460

White T J, Bruns T, Lee S J W T & Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In PCR protocols: a guide to methods and applications* 18(1): 315-322. doi.org/10.1016/b978-0-12-372180-8.50042-1

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Removal of Zinc Pollution by Using Some Hyperaccumulator Plants in Sewage Sludge Treated and Untreated Soils

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ABSTRACT

Soil pollution caused by heavy metals has emerged as one of the most significant environmental problems in the world. In soils, specific plant species are able to grow, adapt and absorb heavy metals. Phytoremediation is an emerging technology in which higher plants are used to reclaim the contaminated environment. In this study, the possibilities of removing the pollution caused by Zn, which is applied to the loamy soil together with and without sewage sludge at increasing levels (0, 75, 150, 300, 600 and 1200 µg g^{-1}), has been researched with certain hyperaccumulator plants such as *Brassica juncea*, *Raphanus sativus* and *Silene vulgaris* grown in Bafra ecological conditions.

In order to clean Zn added to the soil at increasing levels with or without sewage sludge by using phytoremediation technology, *Silene vulgaris* was found to remove the highest amount of Zn in the soil by producing the greatest amount of biomass in the ecological conditions of the region compared to *Brassica juncea* and *Raphanus sativus,* and other hyperaccumulator plants grown in the plots. Significant differences were determined in the development of plants and Zn removal between the sludge treated and untreated plots.

Water-soluble Zn, which was found at high levels in the cultivation of plants at 600 and 1200 µg g^{-1} Zn application doses in the sewage sludge treated plots, was determined at lower levels at the end of the harvest of the plants. In the application of increasing levels of Zn with sewage sludge, the lowest organic bound Zn was determined in the plots where *Silene vulgaris* was grown. The highest exchangeable Zn concentration was determined in soil samples taken after the harvest of the *Raphanus sativus* plant among the hyperaccumulator plants grown at all Zn application doses in the trials with and without sewage sludge application.

Keywords: Phytoremediation, *Brassica juncea, Raphanus sativus*, *Silene vulgaris,* Labile Zn fractions

1. Introduction

In this age of rapid industrialization, exposure to toxic chemicals and metals is unavoidable. In particular, heavy metal pollution has become a serious threat to the environment and food security due to the rapid growth in industry and agriculture and the rapid increase in the world population, which has a detrimental effect on the natural ecosystem. Unlike organic pollutants, heavy metals do not biodegrade but constantly accumulate in the environment. The accumulation of these heavy metals in agricultural lands and water resources poses a major threat to human health since they run the risk of entering the food chain. Zinc is an essential trace element in plant nutrition, but it becomes toxic at high doses and acts like a heavy metal (Chakroun et al. 2010; Fässler et al. 2010; Demim et al. 2013; Küçükyumuk & Erdal 2014). The bulk of research on the atmospheric accumulation of heavy metals in various European countries suggests that Zn is the most accumulated element in the soil (Shi et al. 2018). Zinc accumulation resulting from human activities comes from three activity groups; 1- mining and industrial resources, 2- agricultural activities (fertilizers and pesticides) and 3- other activities such as road traffic and incineration of waste.

Many physical, chemical and biological techniques are used to improve heavy metal contaminated soil. However, among these various methods, phytoremediation is considered to be the most economical and environmentally friendly method. (Prasad 2003; Padmavathiamma & Li 2007). This procedure is relatively inexpensive compared to other remediation techniques (Wan et al. 2016) and also leads to less environmental degradation since it produces less secondary sewage (Cunningham & Berti 2000).

Since the plant is the most important material in this technology, hyperaccumulator plants must have certain properties as follows: the ability to deposit metals in above-ground organs, the tolerance of metal accumulation, the ability to produce effective biomass, being easy to grow and harvest, developing rapidly throughout the whole season and being continuous for other seasons. In addition, the distribution and depth of the plant root must be appropriate. Proper plant selection is crucial for the success of phytoremediation. The best starting point for the selection of appropriate plant species is the use of vegetation that grows naturally in polluted areas.

Silene vulgaris is a plant commonly found in many metal-rich soils in Europe. This plant is tolerant to high heavy metal concentrations and is capable of accumulating heavy metals. It can also produce a vast amount of biomass fast, and the root system is quite large (Nadgórska-Socha & Ciepal 2009). *Raphanus sativus* is used as a model plant in laboratory toxicology studies for various pollutants and is preferred in phytoremediation due to its rapid growth, large biomass, and sensitivity to heavy metals (Hamadouche 2012). *Brassica juncea* is considered one of the most promising species for plant breeding. It is an oilseed plant with a root system known to excessively accumulate certain heavy metals (Goswami & Das 2015). *Silene vulgaris* and *Raphanus sativus* are commonly found in Black Sea region (Mumcu & Korkmaz 2018; Ozbucak et al. 2006). *Brassica juncea*, on the other hand, can spread in the ecological conditions of Turkey (Güner et al. 2012). These three plants may be preferrable for field applications due to their easy availability.

The effectiveness of phytoremediation is affected by the growth of plants, which is slow for practical applications. For this reason, the use of soil conditioners accelerates and increases the reclamation of the soil. For this purpose, many inorganic and organic materials are used. Organic materials can increase the metal uptake of plants by promoting the root growth of the plant by improving some physical and chemical properties of soils such as the soil ion exchange capacity, soil structure, water holding capacity, drainage and aeration conditions, and soil salinity. In addition, the solid form of organic matter attracts zinc to surface functional groups, reducing the solubility of zinc (Boguta & Sokolowska 2016), while complexing of zinc with dissolved organic compounds increases its solubility and mobility (Weng et al. 2002; Houben & Sonnet 2012). For this reason, it is important to reveal the effects of sewage sludge, which is an organic material, on phytoremediation. Some sewage sludges contain high concentrations of hazardous pollutants. However, sewage sludges are very rich in organic matter and contain significant amounts of N, P, K and micronutrients, so they are effective on plant growth. They also play an important role in the chelation of metals due to their various organic chelates. For these reasons, sewage sludge can be used as a suitable source of organic material in phytoremediation technology.

Heavy metal accumulation in plants is not only related to total metal concentrations in the soil but also highly dependent on other factors such as plant uptake mechanisms, soil physicochemical properties, chemical behaviour of metals, soil texture, quality and amount of nutrients, climate, and geological material (Antoniadis et al. 2017). Many researchers also note that the ecological and toxicological effects of heavy metals depend on the distribution of their fractions rather than the total concentration in the soil (Tuzen 2003; Mousavi et al. 2018). The identification of main binding sites and phase associations of trace elements in soils leads to a better understanding of geochemical processes in order to evaluate the remobilization potential and the induced risks. Speciation of the metals can help assess how strongly they are retained in soil, how easily they may be released into soil solution, and finally how they can affect environmental and human health. Therefore, knowing the metal speciation and mobility of heavy metals is crucial in order to evaluate the phytoremediation efficiency (García et al. 2005).

This study investigates the possibilities of removing the pollution caused by increasing levels of Zn, which is applied to loamy soil together with and without sewage sludge in a year with the hyperaccumulator plants *Brassica juncea, Raphanus sativus* and *Silene vulgaris* grown in Bafra (Turkey) ecological conditions.

2. Material and Methods

2.1. Materials

The research has been carried out on land belonging to the Bafra Agriculture District Directorate in Bafra District of Samsun Province, Turkey (41°34'34"N 35°53'53"E). Since the trial area soils show little pedogenetic horizon development and are located on the flood plains on the alluvials brought by Kızılırmak, they are defined as “Typic udifluvent” (Yüksel & Dengiz 1996). In the Bafra Plain, summers are generally hot, and winters are warm and rainy. The plain has a warm and temperate climate. In winter, the Bafra Plain

receives more precipitation than summer. According to the Köppen-Geiger climate classification, it can be referred to as a Csa climate (Mediterranean climate). The annual average temperature is 13.6 °C, and the annual average rainfall is 730 mm (https://tr.climate-data.org/asya/tuerkiye/samsun/bafra-8522/).

The sewage sludge used in the study was obtained from Bafra Municipality Sewage Water Treatment Facility. The solid matter ratio of the cake coming out of the facility is 41.39%. The zinc required to ensure Zn pollution in the experiment was obtained from Ekmekçioğulları Incorporated Company in the form of $ZnSO_4.7H_2O$ (22% Zn). *Brassica juncea, Silene vulgaris,* and *Rapanus sativus* were used as phytoremediation plants. These plants are winter plants and are part of the natural ecology of the region. However, the seeds of *Brassica juncea* and *Silene vulgaris* were obtained from abroad (www.herbiseed.com) as certified, and for *Rapanus sativus* was obtained domestically.

2.2. Methods

2.2.1. Establishment of trials

The trials were set up with 3 replications based on the randomized block experimental design in the form of 2 trials side by side in the field on 18.10.2004. One of the experiments was created with a constant level of sewage sludge and increasing levels of Zn application while the other by only increasing the levels of Zn application. The organic matter contents of the trial area (1.53% organic matter) and the sewage sludge (52.86% organic matter) used as the material in the experiment were determined, and the amount of sewage sludge required to increase the organic matter content of the soil up to 3% was calculated on dry weight and applied equally to each plot and mixed with soil. In order to determine the Zn levels in the application, the Zinc-buffering capacity of the soil was determined by adding Zn at increasing levels under laboratory conditions to the soil samples taken from the experimental area. This level was determined to be 650 µg g^{-1} in the soil sample. Based on this level, Zn pollution levels in the experiment were determined as 0-75-150-300-600-1200 µg g^{-1}, respectively. During the trials, the application doses determined in Zn applications were performed to cover 0-75-150-300-600-1200 µg g^{-1} Zn by preparing 15 Lt aqueous solutions of zinc sulfate. After these applications, the area was fallowed for one year. In the first year of the experiment, no cultural practices were undertaken (irrigation, fertilization, spraying, etc.) after the addition of the trial materials (sewage sludge and Zn) to the plots, and the weeds grown in the plots were cleaned by hand at the beginning of their development.

At the end of the first year of the experiment (18.10.2005), the plots belonging to each application were divided into 3 sub-plots of 1 m^2 and the second year trials were established. The second year trials consisted of 108 plots. Certified seeds of *Brassica juncea, Silene vulgaris,* and *Rapanus sativus* determined as hyperaccumulator plants were planted in these sub-plots. Plants were thinned immediately after emergence so that an equal number of plants were found in each plot (25 plants/plot). In the second year of the experiment, no other cultural practices (irrigation, fertilization, spraying, etc.) but weeds grown in the plots were cleaned by hand at the beginning of their development.

Soil sampling was carried out in the last month of the first year of the trial, before planting (18.10.2005) and at the harvest (26.06.2006). In these samples, labile Zn forms (water soluble, changeable and organic bound) were determined. In addition, the underground and aboveground biomass and Zn contents of the hyperaccumulator plants grown were identified.

2.2.2. Soil and plant analysis

Water-soluble Zn was shaken in 1:10 (w/v) soil: pure water extract for 30 minutes and filtered (Kacar 1995); exchangeable Zn was shaken in 1:4 (w/v) soil: 1 M $Mg(NO_3)_2$ extract and filtered; and organic bound Zn was shaken in 1:2 (w/v) soil: 0.7 M NaOCl (pH 8.5) extract, boiled in a hot water bath for 30 minutes and filtered (Shuman 1985). The Zn contents in the soil extracts were determined using an Atomic Absorption Spectrophotometer (Perkin Elmer, Analyst 300).

At the harvest time, all hyperaccumulator plants were harvested and their dry weights were determined. The Zn contents of the underground and aboveground parts of the plants were identified individually according to Kacar (1972), and the amount of Zn that each hyperaccumulator plant removed from the soil was calculated based on the dry matter weigh of the plant and the Zn concentration in the dry matter according to Kacar (1972) and Ryan et al. (2001).

2.2.3. Statistical analysis

The statistical evaluations were made using the TARIST package program based on the predictions by Yurtsever (1984).

3. Results and Discussion

Properties of soils and sewage sludge

The soil used in the experiment was loamy in texture (17.40% clay, 34.29% silt, 48.31% sand), medium calcareous (11.08%), unsalty (0.32 dS m^{-1} EC), had low organic matter content (1.53%) and a slightly alkaline reaction (8.25 pH). Its N content was 0.10% and total Zn was 88.49 µg g^{-1}.

The Total N, organic carbon, C/N ratio and pH of the sewage sludge were found as 2.20%, 28.7%, 13.1, and 6.65, respectively. It contained 647 µg g^{-1} Zn, 45 µg g^{-1} Pb, 121 µg g^{-1} Cu, 53 µg g^{-1} Cr, 2.1 µg g^{-1} Cd and 58 µg g^{-1} Ni.

3.1. Effects of sewage sludge and zinc applications on zinc fractions of the soil

As a result of the statistical evaluations, changes caused by the application of sewage sludge and increasing levels of zinc in the water-soluble, organic bound and exchangeable Zn fractions in the soil samples taken during the harvest, and their interactions ($p<0.01$) were found to be significant (Table 1). The exchangeable Zn content was 0.1-4.7 µg g^{-1}, organic bound Zn content was 0.3-2.0 µg g^{-1} and exchangeable Zn content was 0.3-160 µg g^{-1} in post-harvest soils in both sludge treated and untreated plots (Figures 1-3). As seen from the results, the highest labile Zn fraction was changeable Zn, followed by water-soluble Zn, and the lowest was organic bound Zn. In other words, Zn is not predominantly fixed organically and similar results have been reported in other studies (Huang et al. 2020).

Table 1- Effects on Soil Labile Zn fractions (water soluble, organic bound and exchangeable)

Source of variation	*SD*	*LSD (%1) Water soluble Zn*	*LSD (%1) Organic bound Zn*	*LSD (%1) Exchangable Zn*
Sewage sludge (A)	1	0.130***	0.056***	3.484***
Plant (B)	2	0.159***	0.068***	4.267***
A×B	2	0.225***	0.097***	6.035***
Dose (C)	5	0.225***	0.097***	6.035***
A×C	5	0.318***	0.137***	8.535***
B×C	10	0.390***	0.168***	10.453***
A×B×C	10	0.551***	0.237***	14.783***

*p<0.05, **p<0.01, ***p<0.001

At all hyperaccumulator plants and Zn application doses, the labile Zn fractions (water soluble, organic bound, exchangeable) of the plots treated with sewage sludge were found to be higher than those without sewage sludge ($p<0.001$). This may be the result of the Zn content (650 µg g^{-1}) in the applied sewage sludge. In fact, Zn has been reported to be the most important pollutant in sewage sludge in other notable studies (Chen et al. 2003; Qiao et al. 2003; Liang et al. 2011). In addition, organic compounds released during the decomposition of various organic materials help the retention of various nutrients, including Zn, in the soil and their uptake by plants by preventing certain processes such as fixation, oxidation, precipitation and washing (Scheid et al. 2009; Houben & Sonnet 2012; Boguta & Sokolowska 2016). Zinati et al. (2001), Udom et al. (2004) and Nagar et al. (2006) reported that the total Zn content of the soils increased with the sewage sludge application, and as a result of this, there was a significant increase in labile Zn forms such as water-soluble. As being in the water soluble Zn, organic bound Zn levels also increased with sewage sludge application (Figure 2). Parat et al. (2005), Hseu (2006), and He et al. (2007) determined that the organic bound Zn concentrations of the soil increased with the increasing amount of sewage sludge applied to the soil. Jakubus & Czekala (2001) determined that 1-9.3% of Zn in the structure of sewage sludge is in organic bound form. In addition, organic C compounds, which are 28.7% in the structure of the sewage sludge used in the experiment, and the increasing level of Zn added to the soil merge to form organically bound Zn complexes. Thus, the organic bound Zn content of soils can increase. Similarly, higher Zn values were also found in the exchangeable Zn fraction in the plots treated with sewage sludge (Figure 3). Nogueira et al. (2010) stated that the exchangeable Zn fraction increased with the sewage sludge application at increasing doses.

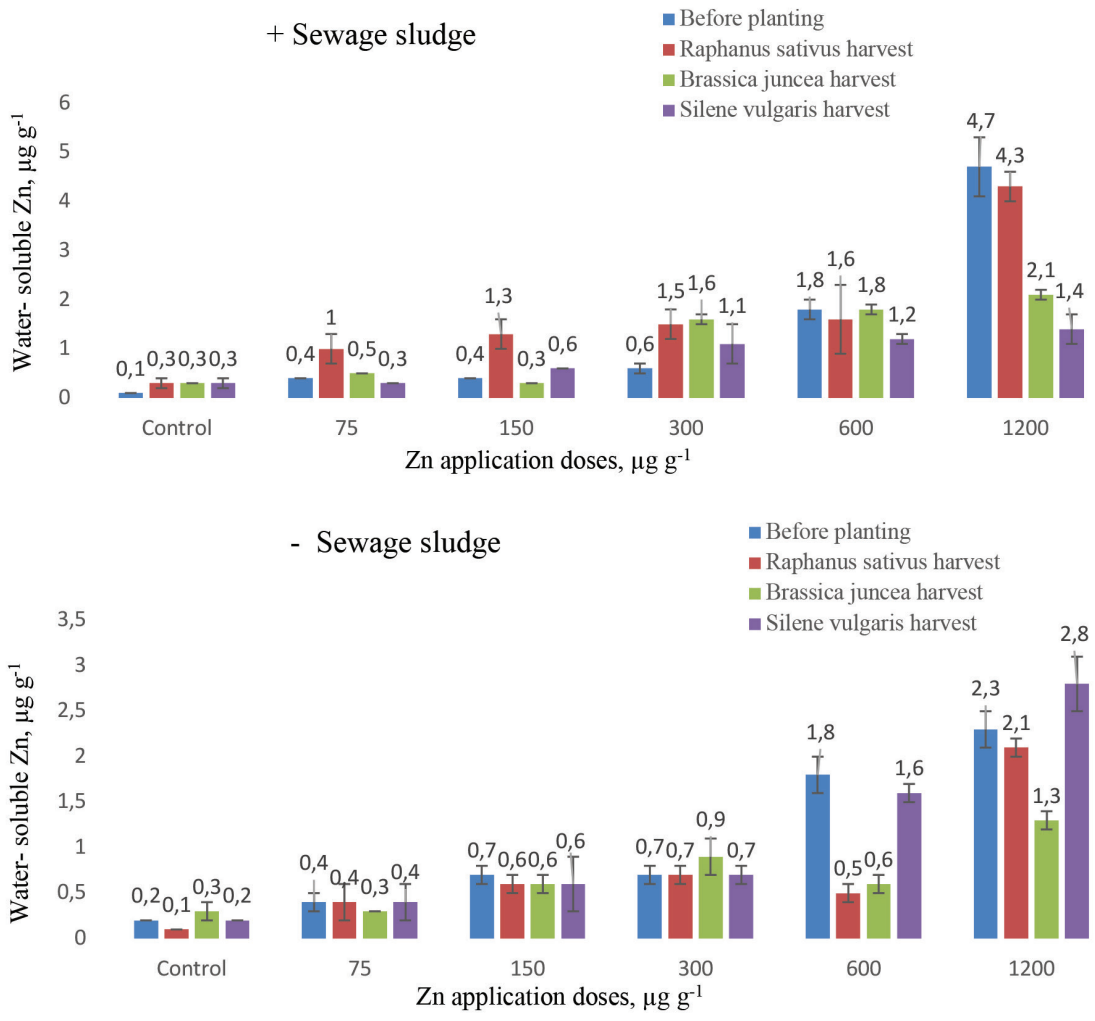

Figure 1- Changes in water soluble Zn with increasing levels of Zn application

As shown in Table 1, there were significant increases in labile Zn fractions (water soluble, organic bound, exchangeable) as the Zn application dose increased both in the plots with sewage sludge and without sewage sludge ($p<0.001$). Yadav et al. (2013) and Almendros et al. (2015) underlined that various Zn fractions in the surface soil increased significantly with increasing levels of Zn applications.

Water-soluble Zn, which was found at high levels in the planting of plants at 600 and 1200 µg g^{-1} Zn application doses treated with sewage sludge, was determined at lower levels at harvest (Figure 1). The lowest values were seen in the soil samples from the *Silene vulgaris* harvest. On the other hand, the results of the experiment without sewage sludge application showed that water soluble Zn values in the soil samples taken after harvest at all Zn application doses in the plots where *Raphanus sativus* was grown were lower than the soil samples taken before planting. As a result of the experiment where Zn was applied with increasing levels of sewage sludge, among the hyperacumulator plants grown the lowest organic bound Zn was found in the plots where *Silene vulgaris* was grown. Similar results were obtained in the trial where sewage sludge was not applied.

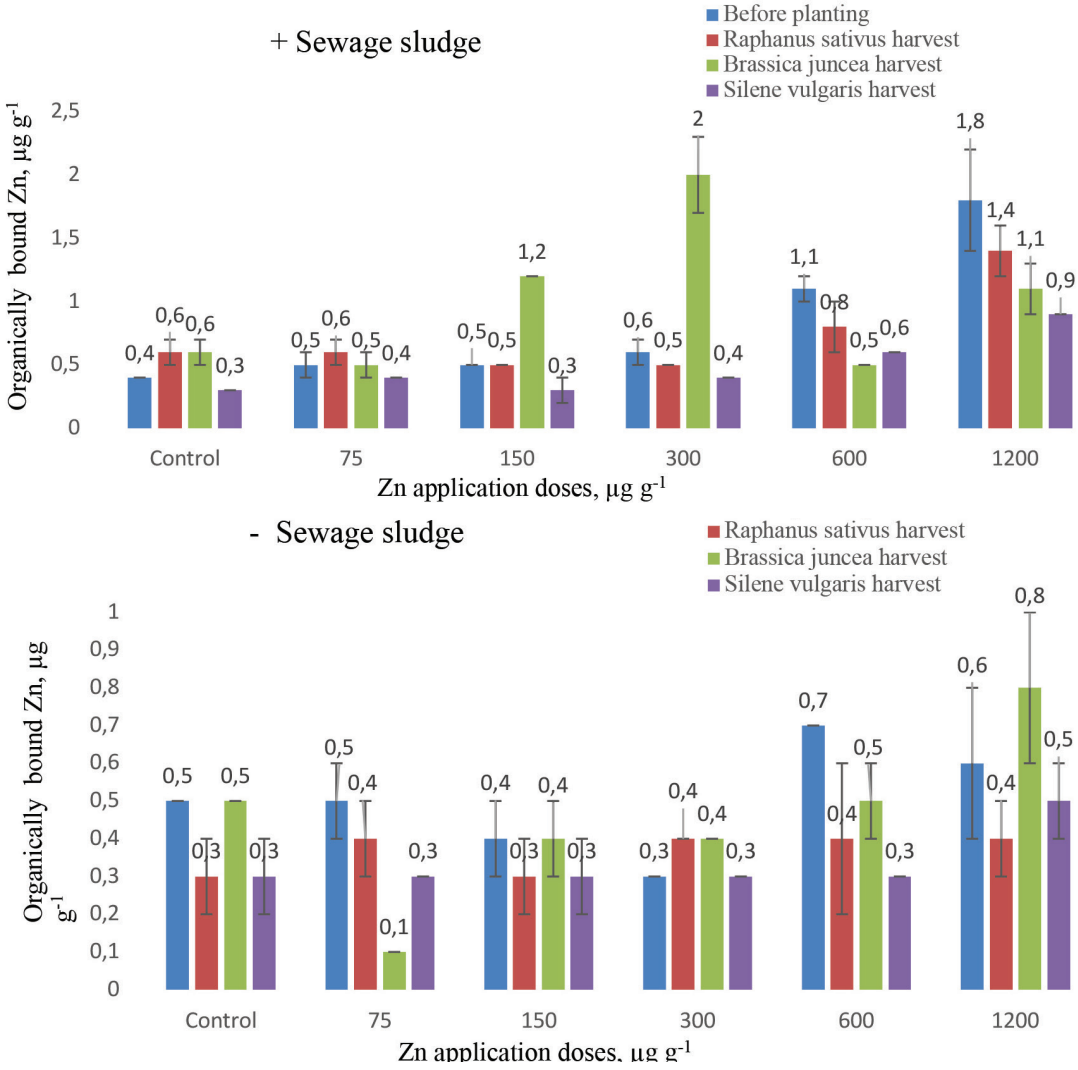

Figure 2- Changes in organic bound Zn with increasing levels of Zn application

It was found that there were significant increases in the exchangeable Zn fraction as the Zn application dose increased in the experiments with and without sewage sludge ($p<0.001$). These increases were clearer at the 600 and 1200 µg g^{-1} doses. The results of the tests, with and without sewage sludge, also showed that the highest exchangeable Zn concentration at all Zn application doses was determined in soil samples taken after the harvest of *Raphanus sativus* among the hyperaccumulator plants.

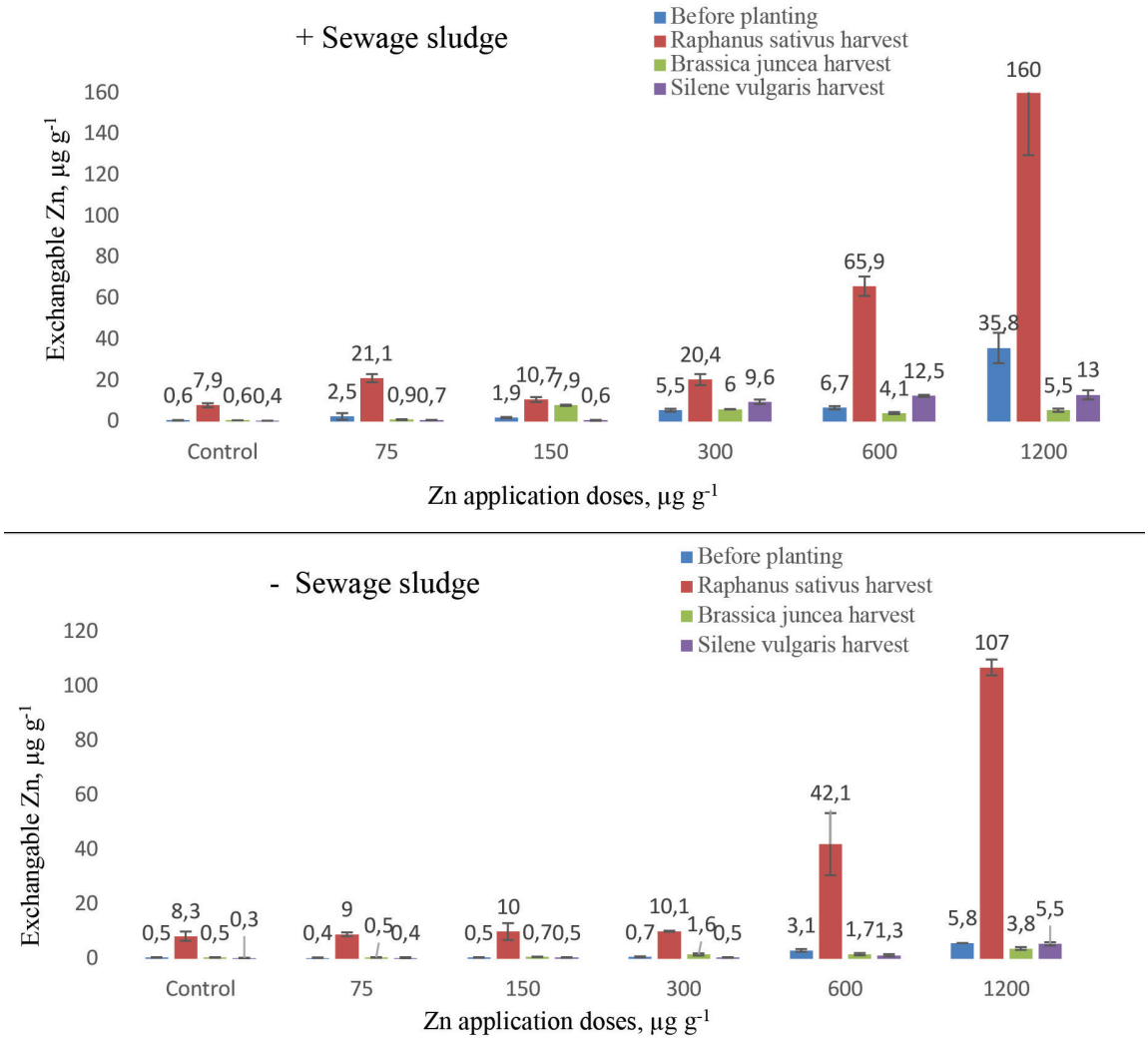

Figure 3- Changes in exchangeable Zn with increasing levels of Zn application

Although, increases in Zn doses were identical at the end of the trials, it was found that there were increases in the labile Zn contents of the soils and significant differences between the hyperaccumulator plants grown with the sewage sludge application. This may be the result of biotic factors such as the root system and the secretions secreted from the root as well as organic and inorganic compounds in the structure of the sewage sludge. McGill et al. (1986), and Huang and Schoenau (1997) found that organic carbon-containing compounds such as pollysaccharides, mucilagels and carbohydrates were synthesized from plant roots into the soil, although their amounts and types varied by plant roots. However, it was determined that the rhizosphere region was more acidic than the outside and as a result, the solubility of micro elements such as Zn in the rhizosphere region was higher. Indeed, root activity induces several modifications in the rhizospheric soil properties, like the pH, microbial activity, chemical equilibrium, mobility, and bioavailability (Clemente et al. 2010; Seshadri et al. 2015).

3.3. Zinc content of the hyperaccumulator plants and zinc amounts they removed from the soil

3.3.1. Zinc content of the hyperaccumulator plants

As a result of the statistical evaluations, with increasing levels of Zinc and sewage sludge applications, changes in the Zn content of the above-ground and underground parts of the plants and their interactions were found to be significant (Table 2). Also, a statistically significant difference was found between the plants in terms of underground and above-ground Zn contents ($p<0.001$). The Zn contents of the above-ground parts of the plants were determined to be between 7-197 µg g^{-1} and the Zn contents of the underground parts were determined to be between 45-2687 µg g^{-1} in the plots where the sewage sludge was applied. However, these values were found to be

2-186 µg g^{-1} and 41-967 µg g^{-1}, respectively, in the plots where sewage sludge was not applied (Figure 4). Zinc is an essential trace element for the mineral nutrition of plants, but at high doses it becomes toxic for these plants and behaves like a heavy metal. For most plants, 15-20 mg kg^{-1} Zn as dry matter is required for proper growth. At levels above this, it can be toxic (Tripathi et al. 2015). The toxicity of Zn in plants depends on different factors that affect the availability of the element, such as soil pH, root exudate, microbial communities and soil organic matter (Balafrej et al. 2020).

Table 2- LSD (1%) values of the underground and aboveground parts of the plants

Source of variation	***SD***	***LSD (%1) Aboveground Zn***	***LSD (%1) Underground Zn***
Sewage sludge (A)	1	10.007**	5.373***
Plant (B)	2	12.257***	6.580***
A×B	2	17.333**	9.306***
Dose (C)	5	17.333**	9.306***
A×C	5	24.513***	13.160***
B×C	10	30.022***	16.118***
A×B×C	10	42.458***	22.794***

*p<0.05 **p<0.01 ***p<0.001

As shown in Figure 4, as the dose of Zn content increased in the treatment, significant increases (p<0.001) took place in the Zn content in the underground and aboveground parts of the plants, both in the trials with and without sewage sludge. Calace et al. (2002) found that the Zn content of the plants cultivated in the soils with different quantities of Zinc differed significantly from each other, and the Zn content in the underground and aboveground parts of the plants increased depending on the increase in the Zn content in the soil. Moreover, *Silene vulgaris, Raphanus sativus,* and *Brassica juncea* cultivated in the trials, were hyperacummulator plants, and are able to accumulate Zn, which is found at high levels in the soil (Ebbs & Kochian 1998; Máthé-Gáspár & Anton 2002; Salinitro et al. 2021).

It was determined that the Zn contents of the hyperacumulator plants grown in the plots containing Zn added to the soil together with the sewage sludge and without sewage sludge differed significantly from each other, and the plant that accumulated the most Zn in their leaves was *Brassica juncea*; this was followed by *Raphanus sativus*, and the plant with the least amount of Zn in its leaves was *Silene vulgaris*. However, the highest amount of Zn was detected in the underground part of *Silene vulgaris*. When *Silene vulgaris* plant is used at a phytoremediation technology, the roots must be removed from the soil system. Muszyńska and Labudda (2020) stated that *Silene vulgaris* can survive by developing various mechanisms when grown in environments rich in heavy metal. Hanus-Fajerska et al. (2019) pointed out that *Silene vulgaris* plant is well-adapted to high metallic element levels in the rhizosphere and arid and nutrient-poor habitats; however, the translocation factor (stem Zn/root Zn) for both Zn and Cd elements is too low for phyto extraction. Ernst et al. (2000) found that the Zn concentration in different parts of *Silene vulgaris* grown on soils contaminated by heavy metals increased significantly due to the increase in Zn in the soil, but Zn removal by the roots is not possible in soils containing high Zn (100 µmol g).

The highest Zn content was obtained at the Zn application dose of 300 µg g^{-1} in the Zn doses of *Silene vulgaris* applied with sewage sludge, and at the Zn application dose of 1200 µg g^{-1}in the experiment without sewage sludge.

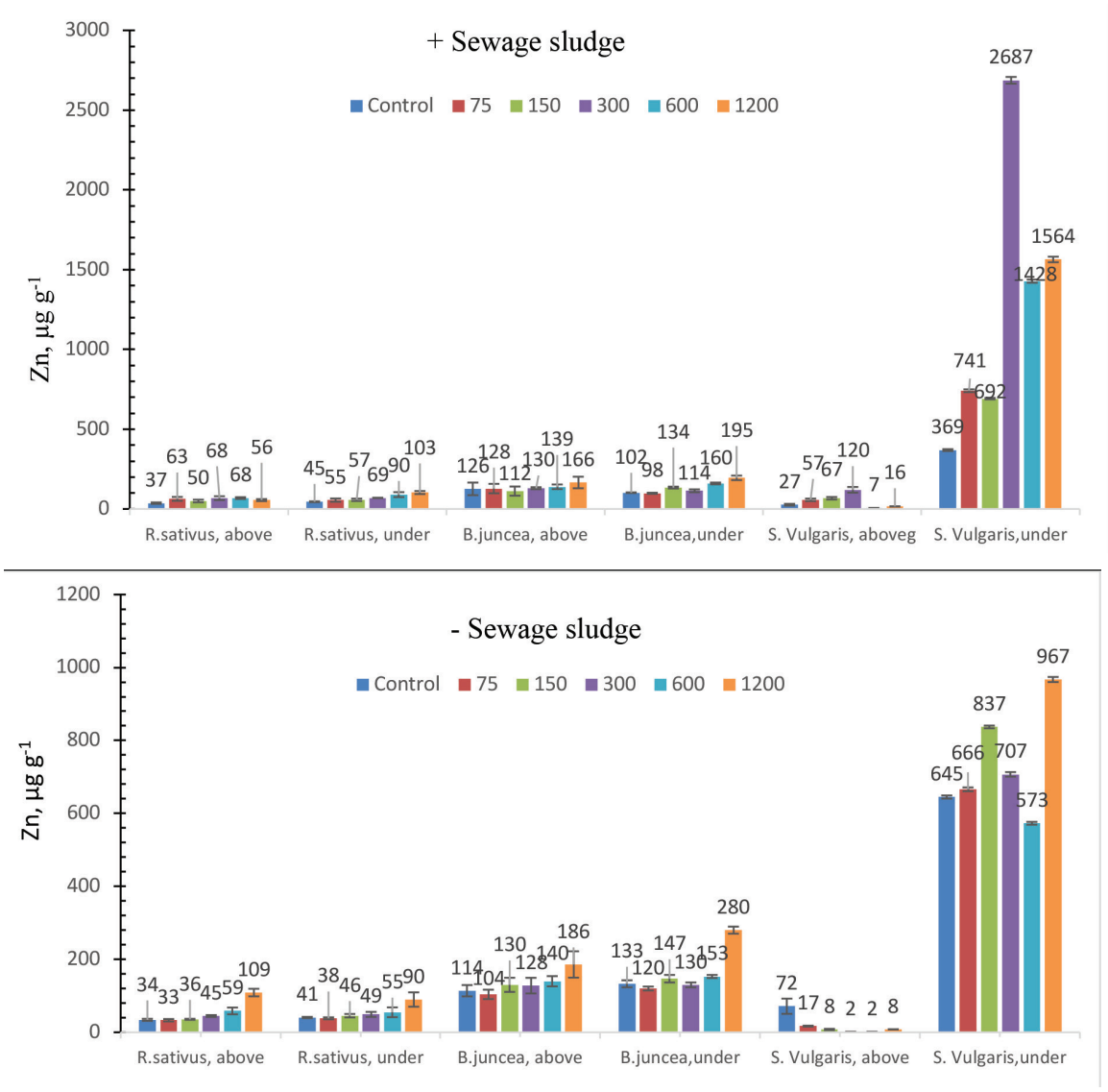

Figure 4- Zn contents of underground and aboveground parts of the hyperacumulative plants

3.3.2. Amounts of zinc removed by hyperaccumulator plants from the soil

While determining the amount of Zn removed by the hyperaccumulator plants, the Zn (88.49 µg g^{-1}) existing in the soil under natural conditions and the Zn input from the sewage sludge (647 µg g^{-1}) were also taken into consideration. The Zn removed by the above-ground part of the plants where sewage sludge was applied was determined as 0.59-5.81 g m^{-2} and the Zn removed by the underground part was determined as 0.2-31.9 g m^{-2}. The Zn removed by the above-ground and underground part of the plants was determined between 0.05-1.6 g m^{-2} and 0.1-12.3 g m^{-2}, respectively, in the plots where sludge was not applied. According to the results, it has been determined that *Silene vulgaris* was the plant removed most Zn from the soil in the ecological conditions of the region among the hyperaccumulator plants (Figure 5).

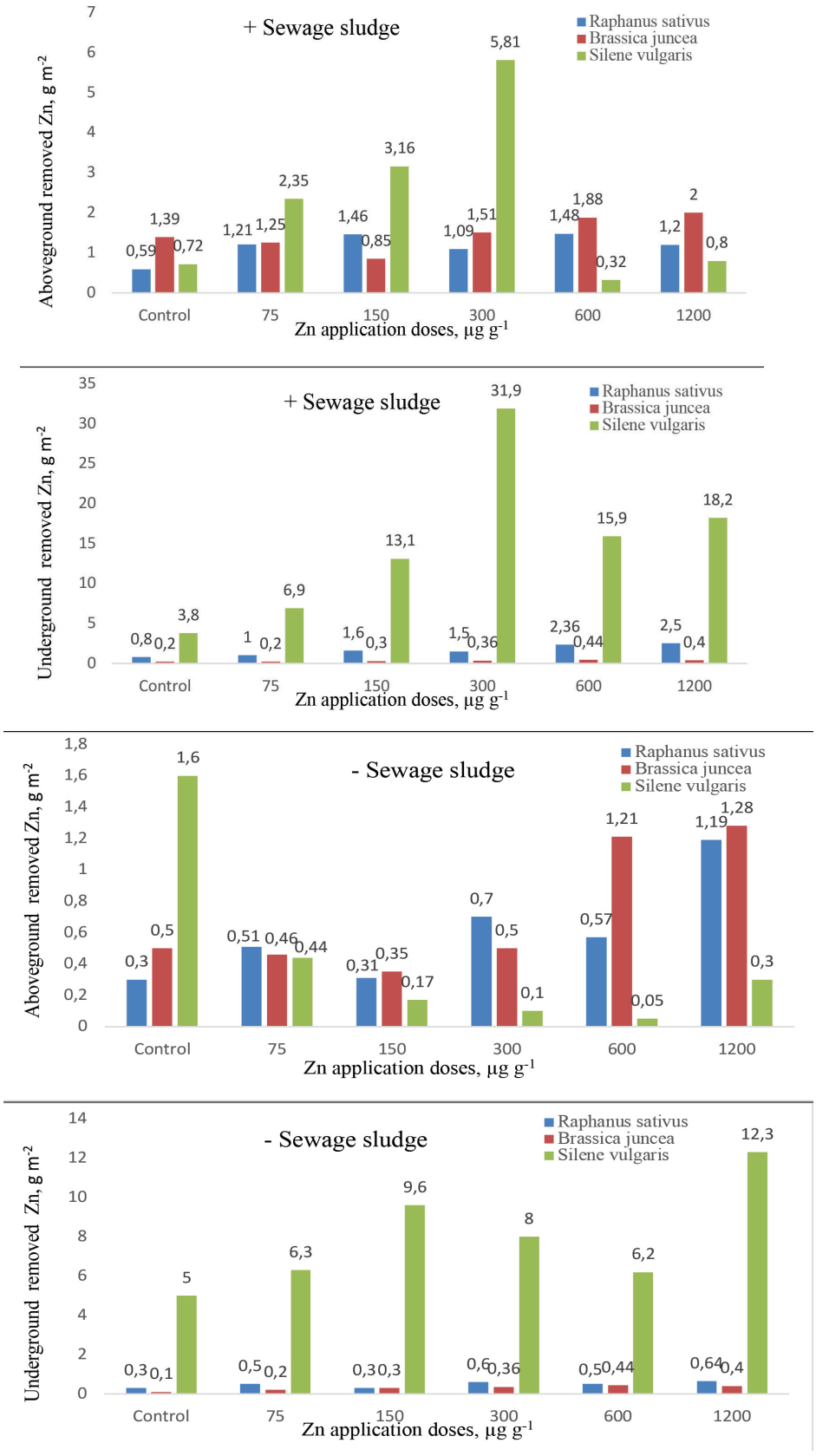

Figure 5- Zn removed from the soil by hyperaccumulator plants with their underground and aboveground parts

At the end of the trial, Zn, which was added in increasing doses in the sewage sludge treated plots, was removed from the soil by *Raphanus sativus*, *Brassica juncea* and *Silene vulgaris* at a rate of 1.18-4.82% (at the maximum Zn application dose of 75 µg g^{-1}), 1.14-3.23% (at the maximum in the control plot) and 5.51-32.23% (at the maximum Zn application dose of 300 µg g^{-1}), respectively. On the other hand, as a result of increasing Zn application without sewage sludge, *Raphanus sativus, Brassica juncea* and *Silene vulgaris* removed 0.83-3.15%, 0.74-3.08% and 3.84-30.34% of the total Zn in soils, respectively. This finding also presents that the Zn added to the soil with the sewage sludge is removed by the hyperaccumulator plants at higher rates. This may be due to the fact that the nutrients found in the sewage sludge, and necessary for the plants, are taken by the plants for a better development and more biomass, and at the same time, various organic compounds in the sewage sludge chelate with the Zn and thus facilitate the uptake of Zn by the plants. In addition, the transport of zinc applied with the sewage sludge to the plant and its concentration in the plant may be higher due to the effect of the high organic matter in the sewage sludge regulating the physico-chemical properties of the soils. Researches have shown that sewage sludge added to soils can increase vegetative production due to the nutrients it contains (Garvanska 2000; Antonkiewicz et al. 2020; Abdoli 2022) and that metals chelated with various organic chelates are taken by plants in larger amounts and accumulate more metals from the soil environment (Lombi et al. 2001; Boye 2002; Praburaman et al. 2020; Pandey et al. 2022). However, Hamlin & Barker (2002) found that N fertilizers applied to hyperaccumulator plants increased the phytoextraction potential of Zn. The 2.2% total N content that exists in the sewage sludge used in the experiment, may shown this effect. Similarly, Panwar et al. (2011) and Revathi et al. (2011) revealed that hyperaccumulator plants increased the amount of heavy metals removed from the soil by increasing their biomass with the application of farmyard manure and vermicompost. Phytoremediation can be assisted by certain practices, including organic sources (such as farmyard manure composts, biosolids, and municipal sewage, etc.) that will increase soil organic matter and fertility, as well as various inorganic substances (Park et al. 2011; Mousavi et al. 2018; Wang et al. 2019; Shaheen et al. 2019; Huang et al. 2020).

4. Conclusions

In this study, the removal possibilities of Zn added as $ZnSO_4.7H_2O$ at increasing doses (0, 75, 150, 300, 600 and 1200 µg g^{-1}) were investigated by using *Brassica juncea*, *Raphanus sativus* and *Silene vulgaris* hyperaccumulator plants in sewage sludge treated and untreated loamy soil. It was determined that among the hyperaccumulator plants, *Silene vulgaris* was the plant that removed the most Zn in the soil by producing the most plant biomass in the ecological conditions of the region. It was determined that the underground parts of all hyperaccumulator plants, including *Silene vulgaris*, accumulated more Zn than the above ground parts. This suggests that if these plants are used as phytoremediation technology in regional conditions, their roots should be removed from the soil environment. *Brassica juncea* and *Raphanus sativus* grown at the Zn doses applied with the sewage sludge produced more plant biomass and removed more Zn from the soil. Similarly, Zn application with sewage sludge in *Silene vulgaris* accumulated more Zn compared to the experiment with increasing levels of Zn in soils without sewage sludge. This is due to the fact that various organic compounds in the structure of the sewage sludge are chelated with Zn and increased uptake by the plants, and the nutrients in the sewage sludge, such as N, increase the plant biomass more.

Based on the obtained results, *Silene vulgaris* can be suggested to be the most suitable hyperaccumulator plant in the ecological conditions of the region. In addition, when there is 300 µg g^{-1} Zn in the soil, approximately 3 vegetation periods are required to clean the land by using phytoremediation technology with *Silene vulgaris*, and in case of sewage sludge application, 12 vegetation periods are required without sewage sludge application.

In the experiments, sewage sludge and increased Zn applications were considered as factors; however, no nutrients were added to the soils by fertilization. In future studies, the hyperaccumulator performance of these plants should be tested by applying a fertilization program.

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Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: B.B., R.K., Design: B.B., R.K., Data Collection or Processing: B.B., R.K., Analysis or Interpretation: B.B., R.K., Literature Search: B.B., R.K., Writing: B.B., R.K.

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References

Abdoli M (2022). Sustainable Use of Sewage Sludge in Soil Fertility and Crop Production. In *Sustainable Management and Utilization of Sewage Sludge* 297-333. doi.org/10.1007/978-3-030-85226-9_15

Almendros P, Obrador A, Gonzalez D & Alvarez J M (2015). Biofortification of zinc in onions (Allium cepa L.) and soil Zn status by the application of different organic Zn complexes. *Scientia Horticulturae* 186: 254-265. doi.org/10.1016/j.scienta.2015.02.023

Antoniadis V, Levizou E, Shaheen S M, Ok Y S, Sebastian A, Baum C, Prasad M N V, Wenzel W W & Rinklebe J (2017). Trace elements in the soil-plant interface: Phytoavailability, translocation, and phytoremediation. A review. *Earth-Sci. Rev* 171: 621-645. doi.org/10.1016/j.earscirev.2017.06.005

Antonkiewicz J, Popławska A, Kołodziej B, Ciarkowska K, Gambuś F, Bryk M & Babula J (2020). Application of ash and municipal sewage sludge as macronutrient sources in sustainable plant biomass production. *Journal of environmental management 264*: 110450. doi.org/10.1016/j.jenvman.2020.110450

Balafrej H, Bogusz D, Triqui Z E A, Guedira A, Bendaou N, Smouni A & Fahr M (2020). Zinc hyperaccumulation in plants: A review. *Plants* 9(5): 562.

Boguta P, Sokolowska Z (2016). Interactions of Zn (II) Ions with humic acids isolated from various type of soils. *PLoS One* 11(4): e0153626. doi.org/10.1371/journal.pone.0153626

Boye K (2002). Phytoextraction of Cu, Pb and Zn: A greenhouse study (Doctoral dissertation, Sveriges lantbruksuniv).

Calace N, Petronio B M, Picciolo M & Pietroletti M (2002). Heavy metal uptake by barley growing in polluted soils: relationship with heavy metal speciation in soils. *Communications in soil science and plant analysis* 33(1-2): 103-115. doi.org/10.1081/CSS-120002381

Chakroun H K, Souissi F, Bouchardon J L, Souissi R, Moutte J, Faure O & Abdeljaoued S (2010). Transfer and accumulation of lead, zinc, cadmium and copper in plants growing in abandoned mining-district area. *African Journal of Environmental Science and Technology* 4(10): 651-659.

Chen T B, Huang Q F& Gao D (2003) Heavy metal concentrations and their decreasing trends in sewage sludges of China. *Acta Scientiae Circumstantiae* 23: 561-569.

Clemente R, Hartley W, Riby P, Dickinson N M & Lepp N W (2010). Trace element mobility in a contaminated soil two years after field-amendment with a greenwaste compost mulch. *Environmental pollution* 158(5): 1644-1651. doi.org/10.1016/j.envpol.2009.12.006

Cunningham S D & Berti W R (2000). Phytoextraction and phytostabilization: technical, economic and regulatory considerations of the soil-lead issue. *Phytoremediation of contaminated soil and water* 359-376.

Demim S, Drouiche N, Aouabed A & Semsari S (2013). CCD study on the ecophysiological effects of heavy metals on Lemna gibba. *Ecological engineering* 57: 302-313. doi.org/10.1016/j.ecoleng.2013.04.041

Ebbs S D & Kochian L V (1998). Phytoextraction of zinc by oat (Avena sativa), barley (Hordeum vulgare), and Indian mustard (Brassica juncea). *Environmental science & technology 32*(6): 802-806. doi.org/10.1021/es970698p

Ernst W H, Nelissen H J & Ten Bookum W M (2000). Combination toxicology of metal-enriched soils: physiological responses of a Zn-and Cd-resistant ecotype of Silene vulgaris on polymetallic soils. *Environmental and Experimental Botany* 43(1): 55-71. doi.org/10.1016/S0098-8472(99)00048-9

Fässler E, Robinson B H, Stauffer W, Gupta S K, Papritz A & Schulin R (2010). Phytomanagement of metal-contaminated agricultural land using sunflower, maize and tobacco. *Agriculture, ecosystems & environment* 136(1-2): 49-58. doi.org/10.1016/j.agee.2009.11.007

García G, Zanuzzi A L & Faz Á (2005). Evaluation of heavy metal availability prior to an in situ soil phytoremediation program. *Biodegradation* 16: 187-194. doi.org/10.1007/s10532-004-4880-1

Garvanska S M (2000). Determination of the effect of the sludge from waste water treatment station near sofia-city fertilizer. *Proceedings of Intrnational Symposium on Desertifisation* 13-17.

Goswami S & Das S (2015). A study on cadmium phytoremediation potential of Indian mustard, Brassica juncea. *International journal of phytoremediation* 17(6): 583-588. doi.org/10.1080/15226514.2014.935289

Güner A, Aslan S, Ekim T, Vural M, Babaç M T & Yıldırım H (2012). Turkey plant list (Vascular plants). *Publication of Nezahat Gökyiğit Botanical Garden and Flora Research Association, Istanbul.*

Hamadouche N A (2012). Phytoremediation potential of Raphanus sativus L. for lead contaminated soil. *Acta Biologica Szegediensis* 56(1): 43-49.

Hamlin R L & Barker A V (2002). Phytoekstraction potential of indian mustard as influenced by range of zinc concentration in solution culture.

Hanus-Fajerska E, Ciarkowska K & Muszyńska E (2019). Long-term field study on stabilization of contaminated wastes by growing clonally reproduced Silene vulgaris calamine ecotype. *Plant and Soil* 439(1): 431-445. doi.org/10.1007/s11104-019-04043-8

He M M, Tian G M, Liang X Q, Yu Y T, Wu J Y & Zhou G D (2007). Effects of two sludge application on fractionation and phytotoxicity of zinc and copper in soil. *Journal of Environmental Sciences* 19(12): 1482-1490. doi.org/10.1016/S1001-0742(07)60241-1

Houben D, Sonnet P (2012). Zinc mineral weathering as affected by plant roots. *Appl. Geochem* 27(8): 1587-1592. doi.org/10.1016/j.apgeochem.2012.05.004

Hseu Z Y (2006). Extractability and bioavailability of zinc over time in three tropical soils incubated with biosolids. *Chemosphere* 63(5): 762-771. doi.org/10.1016/j.chemosphere.2005.08.014

Huang H, Luo L, Huang L, Zhang J, Gikas P & Zhou Y (2020). Effect of manure compost on distribution of Cu and Zn in rhizosphere soil and heavy metal accumulation by Brassica juncea. *Water, Air, & Soil Pollution* 231(5): 1-10. doi.org/10.1007/s11270-020-04572-4

Huang W Z & Schoenau J J (1997). Seasonal and spatial variations in soil nitrogen and phosphorus supply rates in a boreal aspen forest. *Canadian journal of soil science* 77(4): 597-612. doi.org/10.4141/S97-002

Jakubus M & Czekala J (2001). Heavy metal speciation in sewage sludge. *Polish Journal of Environmental Studies* 10(4): 245-250

Kacar B (1972). Bitki ve Toprağın Kimyasal Analizleri. I. Bitki Analizleri. *Ankara Üniversitesi Ziraat Fakültesi Yayınları* 453.

Kacar B (1995). Bitki ve Toprağın Kimyacal analizleri. *Ankara Ünv. Ziraat. Fak. Eğitim, Araştırma, Geliştirme Vakf* 3: 705.

Küçükyumuk Z & Erdal İ (2014). Yapraktan Çinko Sülfat Uygulamasının Granny Smith Elma Çeşidine Olumsuz Etkisi. *Yüzüncü Yıl Üniversitesi Tarım Bilimleri Dergisi* 24(2): 140-147.

Liang Z, Peng X & Luan Z (2011). Immobilization of Cd, Zn and Pb in sewage sludge using red mud. *Environmental Earth Sciences* 66(5): 1321-1328. doi.org/10.1007/s12665-011-1341-0

Lombi E, Zhao F J, Dunham S J & McGrath S P (2001). Phytoremediation of heavy metal–contaminated soils: Natural hyperaccumulation versus chemically enhanced phytoextraction. *Journal of Environmental Quality* 30(6): 1919-1926. doi.org/10.2134/jeq2001.1919

Máthé-Gáspár G & Anton A (2002). Heavy metal uptake by two radish varieties. *Acta Biologica Szegediensis* 46(3-4): 113-114.

McGill W B, Cannon K R, Robertson J A & Cook F D (1986). Dynamics of soil microbial biomass and water-soluble organic C in Breton L after 50 years of cropping to two rotations. *Canadian journal of soil science* 66(1): 1-19. doi.org/10.4141/cjss86-001

Mousavi S M, Motesharezadeh B, Hosseini H M, Alikhani H & Zolfaghari A A (2018). Geochemical fractions and phytoavailability of zinc in a contaminated calcareous soil affected by biotic and abiotic amendments. *Environmental geochemistry and health* 40(4): 1221-1235. doi.org/10.1007/s10653-017-0038-z

Mumcu Ü & Korkmaz H (2018). Vascular flora of the central and western parts of Yeşilırmak Delta (Çarşamba/Samsun/Turkey). *Phytologia Balcanica: International Journal of Balkan Flora and Vegetation* 24(1): 87-98.

Muszyńska E & Labudda M (2020). Effects of lead, cadmium and zinc on protein changes in Silene vulgaris shoots cultured in vitro. *Ecotoxicology and Environmental Safety* 204: 111086. doi.org/10.1016/j.ecoenv.2020.111086

Nadgórska-Socha A & Ciepał R (2009). Phytoextraction of zinc, lead and cadmium with Silene vulgaris Moench (Garcke) in the Postindustrial Area. *Ecological Chemistry and Engineering* 16(7): 831-837.

Nagar R, Sarkar D & Datta R (2006). Effect of sewage sludge addition on soil quality in terms of metal concentrations. *Bulletin of environmental contamination and toxicology* 76(5): 823-830. doi.org/10.1007/s00128-006-0993-z

Nogueira T A R, Melo W J, Fonseca I M, Marcussi S A, Melo G M P & Marques M (2010). Fractionation of Zn, Cd and Pb in a tropical soil after nine-year sewage sludge applications. *Pedosphere 20*(5): 545-556. doi.org/10.1016/S1002-0160(10)60044-6

Ozbucak T B, Kutbay H G & Akcın O E (2006). The Contributıon of Wıld Edible Plants to Human Nutrıtion in the Black Sea Regıon of Turkey. *Ethnobotanical Leaflets* 2006(1): 10.

Padmavathiamma P K & Li L Y (2007). Phytoremediation technology: hyper-accumulation metals in plants. *Water, Air, and Soil Pollution* 184(1): 105-126. doi.org/10.1007/s11270-007-9401-5

Pandey J, Sarkar S & Pandey V C (2022). Compost-assisted phytoremediation. In *Assisted Phytoremediation* 243-264. doi.org/10.1016/B978-0-12-822893-7.00001-X

Panwar B, Kádár I, Bíró B, Rajkai-Vegh K, Ragályi P, Rékási M & Márton L (2011). Phytoremediation: Enhanced cadmium (Cd) accumulation by organic manuring, EDTA and microbial inoculants (Azotobacter sp., Pseudomonas sp.) in Indian mustard (Brassica juncea L.). *Acta Agronomica Hungarica* 59(2): 117-123. doi.org/10.1556/AAgr.59.2011.2.2.

Parat C, Chaussod R, Lévêque J & Andreux F (2005). Long-term effects of metal-containing farmyard manure and sewage sludge on soil organic matter in a fluvisol. *Soil Biology and Biochemistry* 37(4): 673-679. doi.org/10.1016/j.soilbio.2004.08.025

Park J H, Lamb D, Peneerselvam P, Choppala G, Bolan N & Chung J W (2011). Role of organic amendments on enhanced bioremediation of heavy metal(loid) contaminated soils. *J. Hazardous Materials* 185(2-3): 549-574. doi.org/10.1016/j.jhazmat.2010.09.082

Praburaman L, Park J H, Park Y J, He Z, Kamala-Kannan S & Oh B T (2020). Effect of panchakavya (organic formulation) on phytoremediation of lead and zinc using Zea mays. *Chemosphere* 246: 125810. doi.org/10.1016/j.chemosphere.2019.125810

Prasad M N V (2003). Phytoremediation of metal-polluted ecosystems: hype for commercialization. *Russian Journal of Plant Physiology* 50(5): 686-701. doi.org/10.1023/A:1025604627496

Qiao X L, Luo Y M, Christie P& Wong M H (2003). Chemical speciation and extractability of Zn, Cu and Cd in two contrasting biosolidsamended clay soils. *Chemosphere* 50: 823-829. doi.org/10.1016/S0045-6535(02)00226-6

Revathi K, Haribabu T E & Sudha P N (2011). Phytoremediation of chromium contaminated soil using sorghum plant. *International Journal of Environmental Sciences* 2(2): 417-428.

Ryan J, Estefan G & Rashid A (2001). Soil and plant analysis laboratory manual. *International Center for Agricultural Recearch in the Dry Areas (ICARDA)*.

Salinitro M, Montanari S, Simoni A, Ciavatta C & Tassoni A (2021). Trace metal accumulation and phytoremediation potential of four crop plants cultivated on pure sewage sludge. *Agronomy* 11(12): 2456. doi.org/10.3390/agronomy11122456

Scheid S, Günthardt-Goerg M S, Schulin R & Nowack B (2009). Accumulation and solubility of metals during leaf litter decomposition in non-polluted and polluted soil. *Eur. J. Soil Sci* 60(4): 613-621. doi.org/10.1111/j.1365-2389.2009.01153.x

Seshadri B, Bolan N S & Naidu R (2015). Rhizosphere-induced heavy metal (loid) transformation in relation to bioavailability and remediation. *Journal of soil science and plant nutrition* 15(2): 524-548. http://dx.doi.org/10.4067/S0718-95162015005000043

Shaheen S M, Wang J X, Ann-Christin S, Feng X B, Nanthi B & Rinklebe J (2019). Enhancing phytoextraction of potentially toxic elements in a polluted floodplain soil using sulfur-impregnated organoclay. *Environmental Pollution* 248: 1059-1066. doi.org/10.1016/j.envpol.2019.02.073

Shi T, Ma J, Wu X, Ju T, Lin X, Zhang Y & Wu F. (2018). Inventories of heavy metal inputs and outputs to and from agricultural soils: A review. *Ecotoxicology and environmental safety* 164: 118-124. doi.org/10.1016/j.ecoenv.2018.08.016

Shuman L M (1985). Fractionation method for soil microelements. *Soil science* 140(1): 11-22. doi.org/10.1097/00010694-198507000-00003

Tripathi D K, Singh S, Singh S, Mishra S, Chauhan D K & Dubey N K (2015). Micronutrients and their diverse role in agricultural crops: advances and future prospective. *Acta Physiologiae Plantarum* 37: 1-14

Tuzen M (2003) Determination of trace metals in the River Yesilirmak sediments in Tokat, Turkey using sequential extraction procedure. *Microchem. Journal* 74(1): 105-110. doi.org/10.1016/S0026-265X(02)00174-1

Udom B E, Mbagwu J S C, Adesodun J K & Agbim N N (2004). Distributions of zinc, copper, cadmium and lead in a tropical ultisol after long-term disposal of sewage sludge. *Environment International* 30(4): 467-470. doi.org/10.1016/j.envint.2003.09.004

Wan X, Lei M & Chen T (2016). Cost–benefit calculation of phytoremediation technology for heavy-metal-contaminated soil. *Science of the Total Environment* 563-564: 796-802. doi.org/10.1016/j.scitotenv.2015.12.080

Wang L, Hou D, Shen Z, Zhu J, Jia X, Ok Y S, Tack F M G & Rinklebe J (2019). Field trials of phytomining and phytoremediation: a critical review of influencing factors and effects of additives. *Critical Rev. Environ. Sci. Technology* 50: 2724-2774. doi.org/10.1080/10643389.2019.1705724

Weng L, Temminghoff E J M, Lofts S, Tipping E & Van Riemsdijk W H (2002). Complexation with dissolved organic matter and solubility control of heavy metals in a sandy soil. *Environ. Sci. Technology* 36(22): 4804-4810. doi.org/10.1021/es0200084

Yadav B, Khamparia R S & Kumar R (2013). Effect of zinc and organic matter application on various zinc fractions under direct-seeded rice in vertisols. *Journal of the Indian Society of Soil Science* 61(2): 128-134.

Yüksel M & Dengiz O (1996). Bafra ovası sağ sahil topraklarının sınıflandırılması. *Ankara Üniversitesi Ziraat Fakültesi Tarım Bilimleri Dergisi* 2(2): 95-101.

Yurtsever N (1984). Deneysel İstatistik Metodları. Tarım, Orman ve Köyişleri Bakanlığı. *Köy Hizmetleri Genel Müdürlüğü Yayınları* 623.

Zinati G M, Li Y & Bryan H H (2001). Accumulation and fractionation of copper, iron, manganese, and zinc in calcareous soils amended with composts. *Journal of Environmental Science and Health, Part B* 36(2): 229-243. doi.org/10.1081/PFC-100103746

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Is the Nutritional Composition of Safflower Oilseed Meal Sufficient for Alternative or Complementary Aqua Feeds-raw Material?

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ABSTRACT

Safflower (*Carthamus tinctorius* L.) is grown in many countries, even in arid regions. Due to its important nutrients, safflower has the potential to be used as raw material for the nutrition of many animals and aquaculture species. For this reason, the objective of this study is to determine crude protein, crude oil, ash, and nitrogen-free extract (NFE) values, as well as amino acid, fatty acid, and mineral values in safflower seed meal. In addition, fish meal, soybean meal, wheat, and canola meal values are compared with safflower seed. On a dry matter basis, the crude protein, crude oil, crude ash, and NFE values were found at 19.42% ±0.32, 8.76% ±0.21, 2.82% ±0.1, and 62.68% ±0.88 respectively. Safflower meal contains significant arginine, histidine, and phenylalanine levels, with C18:2 n-6 being one of the most abundant fatty acids. According to the fatty acid values examined, the total saturated fatty acid values were to be 9.79%; the total monounsaturated fatty acid values are 27.58%; total n-6 PUFA values are 61.49%; total n-3 fatty acid values are 0.55% and total n-3 HUFA values are 0.22% in safflower oilseed meal. The potassium and magnesium content in safflower seed meal is similar to that of soybean meal and fishmeal. Safflower oilseed meal or oil can be used as complementary feedstuff in both marine and freshwater fish feeds. In future studies, observing the balance of essential amino acids and polyunsaturated fatty acids and conducting detailed studies will be effective in closing the gap in this area.

Keywords: Safflower meal and oil, Nutritional composition, Model for amino acids, Cultured fish nutrition, Complementary feedstuff

1. Introduction

The aquaculture industry ensures that cultured fish receive the food and nutrients they need, both in terms of quality and quantity (FAO 1996). However, in aquaculture, while the fish must be fed to survive, a nutritionally balanced level is required (Tecnovit 2014). Feed costs are the major portion (about 40-50%) of aquaculture expenditure. The aquaculture feed industry has expanded rapidly in recent years, and this trend is expected to continue. Fish meal is the preferred raw material in fish feeds due to its high protein and essential amino acid content. According to the FAO (2011), the reliance on fishmeal for aquaculture jeopardizes both marine biodiversity and human food security. Because aquaculture feeds' over-reliance on fishmeal may be unsustainable, the aquafeed industry's costs require the discovery of alternative ingredients (Kiron et al. 2016; Sarker et al. 2016). In 2020, the world produced 4.9 million tons of fish meal and 1.1 million tons of fish oil (OECD/FAO 2022). Fish meal and oil (FM and FO) are the most used raw materials in the aquafeed industry. However, despite the occasional difficulties in obtaining sufficient quantities of these raw materials, the search for alternative raw materials is continuous. For the most part, it is predicted that including different raw material sources in the ratios as a protein source would be more beneficial both economically and nutritionally for aquafeeds (Turchini et al. 2019).

Research continues on fish meal and oil, which is one of the most important raw materials in fish diets, and on marine and terrestrial raw materials that can be partially substituted for these two critical raw materials. On the other hand, Turchini et al. (2019) examined 7390 articles/documents in their research with the keywords "alternatives and aquafeeds". Raw materials must be consistent and economical,

available in sufficient quantities, have essential nutrients, be free from contaminants and other undesirable factors, and be capable of withstanding a range of processing constraints. In addition, consideration should be given to how new raw materials may interact with others. Knowing the positive and negative effects of raw materials on each other during feed production creates very useful information.

Glencross (2020), recommends that seven steps (characterization, palatability, digestibility, utilization, immunological, processing effects, and product quality influence) should be considered when deciding whether or not a feed raw material will be used.

With its high protein percentage, balanced amino acid profile, low price, high digestibility, and easy accessibility, soybean meal is an important raw material that can be used in the feed of many aquaculture species (Storebakken et al. 2000). Because of supply issues and the environmental effects of fish meal, the use of soybean meal in aquafeed has increased in recent years, and it has emerged as a dietary protein source (Murashita et al. 2015). In 2019, the world's total soybean production was 333 million tons (FAOSTAT 2020).

Despite soybean meal being the most commonly used vegetable raw material in aquafeeds, the following studies have attempted to summarize the fact that many vegetables raw materials are used in various fish species.

In European seabass feeds, Tibaldi & Kaushik (2005), used corn gluten meal, wheat gluten, wheat meal, soybean meal, and linseed meal. It was determined that there were no negative effects on somatic growth or nitrogen absorption. Suárez et al. (2009), in their 2009 study, used a mixture of soybean and canola meal was used in white-leg shrimp feeds and found that growth was also not affected. Köprücü and Sertel (2012), fed grass carp linseed meal, sunflower meal, and corn meal and observed no negative effect on somatic growth or nitrogen utilization. Rubber meal was used in carp feeds by Suprayudi et al. (2015), who found in growth and feed evaluation no observable negative effects.

The safflower (*Carthamus tinctorius* L.) oilseed plant is a tall and yearly plant with a length can that can range from 0.3 to 1.5 meters and roots that can grow to be 2-3 meters long. The first root's contact with the soil occurs at a depth of 30 cm. (Oyen & Umali 2007; Ecoport 2010) When compared to soybean and sunflower, it requires less water when growing in arid conditions. It is a very sustainable plant because it is an oilseed and easy to extract (Gilbert 2008).

Safflower is grown in over 60 countries (Ekin 2005; Kinupp & Lorenzi 2014). It is grown in tropical regions (1400 m in Ethiopia, 1800 m in Kenya) between latitudes of 20 °S and 40 °N, from sea level to 900 m and above, due to its tolerance to cold and drought (Ecoport 2010).

It is known that there are 25 wild safflower species in the world, and some of these species C*arthamus lanatus, C. tinctorius* and *C. dentatus* are also found in Türkiye. Safflower plants have been grown around the world for many years, particularly in China, Japan, India, Egypt, and Iran. It is known that it was cultivated in the Medieval Era in Italy, France, and Spain and that after the discovery of America, it was first taken to Mexico by the Spanish, and then to Venezuela and Colombia. The safflower plant arrived in the United States in 1925 via the Mediterranean countries.

In Türkiye, the genus *Carthamus*, which has 25 taxons worldwide, is *C. oxyacantha* (2n=24), and *C. tinctorius* (2n=24) is cultivated (Meshram et al. 2011; Çulha Erdal et al. 2021). Between 1929 and 1930, the Eskişehir-Sazova breeding station began cultivating and breeding safflower in Türkiye (Babaoğlu 2017).

Although there are many companies producing safflower oilseed meal in Türkiye, the RIPSA company (Kayseri), safflower oilseed meal supplier, is one of the leading companies in this field and has been producing the "Balcı" seed, which is the most widely used seed type in Türkiye recently. Therefore, the "Balcı" seed used in this study was thought to reflect the safflower profile in Türkiye (RİPSA 2021).

Safflower (*Carthamus tinctorius* L.) is an oleaginous plant that was once widely known throughout the world (Mansouri et al. 2018). Safflower is a member of the Asteraceae family and is grown in many parts of the world due to its adaptability to a variety of conditions, including areas with low rainfall (Baümler et al. 2006). Currently, it is grown in nearly 60 countries around the world (FAO 2017), with Kazakhstan, India, Mexico, and the United States being the main producers of safflower.

In 2019, the total global safflower production was 627,653 tons. Kazakhstan (214,149), the United States (107,200), Mexico (58,675), India (55,000), and Türkiye (35,000) were the world's top manufacturers (FAO 2020). According to FAOSTAT (2017), the average global cycle of safflower cultivation for seed production varies from 200 to 230 days in the autumn and winter seasons and from 120 to 160 days in the spring and summer seasons, when including the emergence periods and harvest of seeds/grains.

Safflower whole seed is mainly composed of 33-60% coat and 40-67% food stores (Dajue & Mündel 1996; Pahlavani 2005). The oil content of the seed ranges from 15% to 45% depending on the variety and environment in which it grows (Emongor 2010).

Safflower oilseed plant meal is found in the feed of many farm animals and some cultured fish species. Studies have revealed that safflower meal can be used as a protein source up to 20% in rainbow trout and shrimp feeds (Galicia-González et al. 2010; Ustaoglu Tiril & Kerim 2015; Ustaoğlu Tiril et al. 2016; Çantaş & Yıldırım 2020).

Safflower is used in pharmaceuticals, infant formulas, cosmetics, and biodiesel production (GRDC 2010) and does not require much nitrogen fertilizer (which may be deleterious to livestock and oil quality). Due to its taproot, safflower uses NO_3 leachates left in groundwater and for this reason is considered environment friendly (Yau & Ryan 2010).

There are some deficiencies in the literature in studies evaluating nutrients as a whole (protein, lipid, NFE, amino acids, fatty acids, and minerals). As a result, in this study, we determined the nutritional properties of safflower oilseed meal, which is an alternative or complementary source of raw materials in terms of all nutrients, and we revealed which limits should be considered as a vegetable protein source in aquatic feeds.

Due to the rapid development of the aquaculture industry, the demand for fishmeal/fish oil in aquafeed has increased, but its availability and increase in price have also raised concerns. For this reason, it is vital to search for high-quality, economical, and environmentally friendly feed raw materials (Hekmatpour & Mozanzadeh 2021). Efforts are being made to reduce the use of fishmeal and oil in fish nutrition. In aquaculture, feeding studies have been conducted to reduce fishmeal oil to 10% or less in the diet without adversely affecting fish growth performance (Apper-Bossard et al. 2013). The purpose of this research is to evaluate safflower oilseed plant meal, its history, production conditions, and its current status in Türkiye and around the world. In addition, it is aimed to compare the nutrients of safflower oilseed meal with other raw materials (soybean, wheat, and canola meal) and to reveal to what extent it can meet the nutritional needs of species of importance in aquaculture (rainbow trout, carp, and Nile tilapia).

2. Material and Methods

The safflower oilseed meal used in this study was obtained from a private company (RİPSA) and analyzes were carried out in the laboratory with 3 replications. The safflower meal known as “Dinçer” is the oldest registered type of safflower. In recent years, the “Balcı” seed variety has been widely used in Türkiye and the safflower variety we examined in this study is this oilseed.

Crude protein, crude lipid, crude ash, and moisture of safflower meal were determined using standard methods (AOAC 1990; AOAC 1995; AOAC 2002; AOAC 2006).In this study, the cold-press extraction method was used to extract the safflower oilseed. The oil extraction process obtains two types of safflower meal (Knowles & Ashri 1995), indicating a significant variation in composition. One is the hull and labelled as a hulled safflower meal, while the other is de-hulled (totally or partially) and labelled as a de-hulled safflower meal. The safflower oilseed meal in which coat is not separated is commonly used in the safflower variety we analyzed in this study.

2.1. Amino acid content

The LCMS/MS system was used to measure amino acid concentrations to determine the amino acid profiles of the samples. The method in question is described further below. The Jasem LC-MS / MS amino acid analysis kit was used, which included a calibrator set containing standards at five different concentrations, a stable isotope-labeled internal standard mix, mobile phases, reagents, chromatographic separation and mass detection method parameters, and a modified sample preparation process that included acidic hydrolysis. The concentration of target amino acids was determined using the multiple reaction monitoring mode based on electrospray ionization (Bilgin et al. 2018).

2.2. Fatty acid content

Lipids were extracted using the method described by Folch et al. (1957). Following lipid extraction, fatty acid methyl esters (FAME) were prepared as described by Metcalfe and Schmitz (1961), and analyzed as previously described (Czesny & Dabrowski 1998) with some modifications. Briefly, the FAME obtained was separated using gas chromatography (Agilent 6820 A), equipped with a flame ionization detector and fitted with a DB 23 capillary column (60 m, 0.25 mm i.d., and 0.25 µm). The injector temperature program was set to 190 °C for 35 minutes, and then increased at a rate of 30 °C per minute until it reached 220 °C, where it was held for 5 minutes. The carrier gas was hydrogen (2 mL min^{-1} with a split ratio of 30:1). Individual fatty acids were identified by comparing their retention times to a standard fatty acid mixture (Supelco 37 component FAME mix).

2.3. Mineral analysis

Analyses were performed on samples that had been dried at 70 °C. Boron (B), Calcium (Ca), Copper (Cu), Iron (Fe) Potassium (K) with Total Nitrogen (N) Kjeldahl device, Magnesium (Mg), Manganese (Mn), Sodium (Na), Phosphorus (P) and Zinc (Zn) were determined by reading the solution obtained by wet burning with acid in ICP-OES by reading the solution obtained by wet burning with acid (USEPA 2007). All analyses were done in triplicate, and the mean values were used for data analysis.

2.4. Comparison of nutritional compositions of feed raw materials (simple comparison model for amino acids)

The data obtained in the analysis of the safflower seed meal were compared with the nutritional composition data of fish meal, soybean meal, wheat meal, and canola meal in the literature. The essential amino needs of three important cultured species were also compared with data from NRC (2011). In this study, fishmeal was used as a basic feed ingredient and compared with other ingredients. Furthermore, considering that fishmeal covers all essential amino acid needs, we have taken this value 100% and developed a simple and useful comparative model with other raw materials (safflower, soybean, wheat, rapeseed meal). This is a simple way to model the availability of other ingredients in feed compared to fishmeal.

3. Results

Analyses were performed to determine the amino acid content, fatty acid content, and mineral content, which were then compared in Tables 1-4.

3.1. Nutritional values of feed raw materials

The safflower oilseed meal contains relatively moderate levels of protein 19.42%; besides the crude lipid 8.76%; the ash 2.82% and the nitrogen-free extract 62.68%. The biochemical and essential amino acid values of fish meal, safflower oilseed meal, soy meal, wheat meal, and canola meal are given comparatively in Table 1.

Table 1- Nutritional values of feed raw materials

Proximate analysis	***Marine meals***	***Terrestrial plant meals***			
	Fish meal[1]	***Safflower oilseed meal[2*]***	***Soybean meal[3**]***	***Wheat meal[4]***	***Canola meal[5**]***
Dry matter (%)	91.80	96.24	88.00	88.30	92.10
Protein (%)	67.00	19.42	55.20	13.35	36.90
Lipid (%)	10.90	8.76	1.70	1.63	33.20
Fibre (%)	-	-	4.40	12.05	14.90
Ash (%)	13.90	2.82	7.60	1.66	3.70
NFE (%)	-	62.68	18.40	59.6	10.20
EAA (g $100g^{-1}$ DM)					
Arginine	3.90	1.68	3.43	0.72	2.28
Histidine	2.50	0.81	1.22	0.35	1.38
Isoleucine	3.00	0.51	2.19	0.55	1.21
Leucine	5.00	1.21	3.97	0.99	2.43
Lysine	5.30	0.81	2.67	0.41	2.02
Methionine	2.00	0.13	0.49	0.58	0.68
Phenylalanine	2.70	0.93	2.51	0.68	1.40
Threonine	2.50	0.64	2.41	0.43	1.62
Valine	3.70	0.94	2.50	0.72	1.66

[1]Data on proximate composition and amino acid contents of fishmeal are from Glencross (2020); [2]Amino acid contents of safflower meal obtained are from this study; [3]Data on proximate composition of soybean meal are from Heuzé et al. (2020) and Zheng et al. (2022); [4]Data on proximate composition and amino acid contents of wheat meal are from Thacker and Widyaratne (2012); [5]Data on proximate composition and EAA of canola meal are from Mejicanos et. al. (2016) & MacIntosh et al. (2021); *Cold press extraction process; **Solvent extraction process

3.2. Amino acid content

The amount of essential and non-essential amino acids is determined in safflower oil seed meal using standard methods. Arginine, leucine, valine, and phenylalanine were the most abundant amino acids in the safflower oilseed meal. The methionine level of the safflower oilseed meal was low and limited. The amino acid contents are shown in Table 2. The essential amino acid comparison between raw materials is shown in Figure 1.

Table 2- Safflower oilseed meal amino acid contents

Essential amino acid (g/100g)		***Non-essential amino acid (g/100g)***	
Arginine	1.68	Aspartic acid	1.96
Histidine	0.81	Glutamic acid	4.09
Isoleucine	0.51	Ornithine	0.19
Leucine	1.21	Proline	1.02
Lysine	0.81	Taurine	0.00
Methionine	0.13	Serine	1.11
Phenylalanine	0.93	Alanine	0.88
Threonine	0.64	Cystine	0.26
Valine	0.94	Glycine	1.13

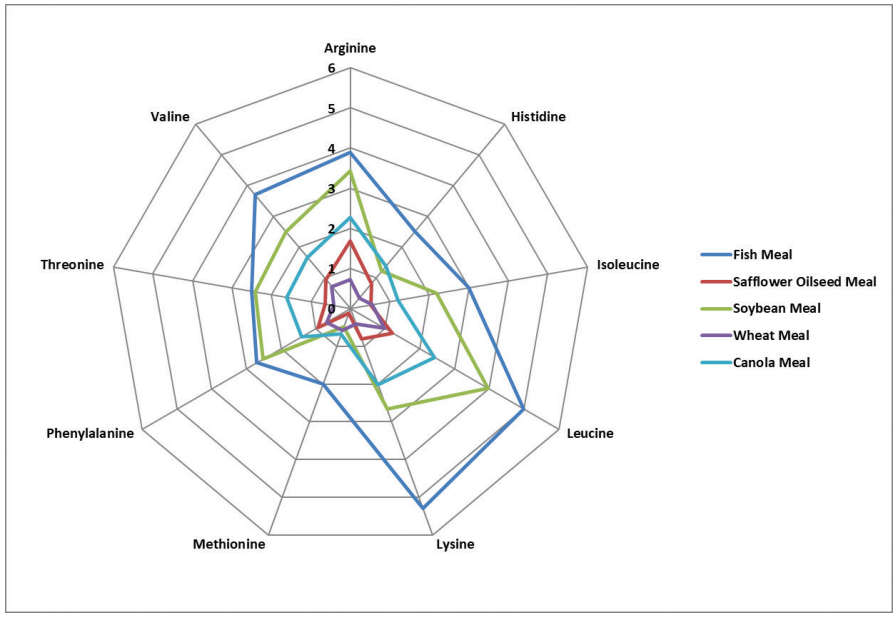

Figure 1- Comparison of essential amino acid content in raw materials

3.3. Fatty acid content of safflower oilseed meal

According to the fatty acid analysis, linoleic acid made up 60.57% while oleic acid made up 26.03%. The total saturated fatty acid values were 9.79; total monounsaturated fatty acid values were 27.58; total n-6 PUFA values were 61.49; total n-3 fatty acid values were 0.55 and total n-3 HUFA values were 0.22 in safflower oilseed plant meal. When compared with other raw materials, it is clear that they are present in significant quantities. A comparison of the fatty acid contents of fish meal, soybean meal, wheat meal, and canola meal with safflower meal is shown in Table 3.

Table 3- Fatty acid content of safflower oilseed meal

Fatty acid	*Marine meals (g/100g)*	*Terrestrial plant meals (g/100g)*			
	Fish meal[1]	*Safflower seed meal*[**2]	*Soybean meal*[**3]	*Wheat meal*[4]	*Canola meal*[**5]
C14:0	3.40	0.13	0.08	-	0.08
C15:0	0.30	0.02	0.01	-	-
C16:0	13.10	6.94	11.13	19.56	4.31
C18:0	18.90	2.69	4.08	1.37	2.21
C20:0	0.20	0.00	0.31	-	0.48
C22:0	0.10	-	0.36	0.26	0.19
C24:0	0.20	0.01	0.13	-	0.10
Total saturates	36.20	9.79	16.10	1.63	7.37
C16:1n-9	0.10	-	0.05	-	-
C16:1n-7 (PA)	3.80	0.04	0.13	-	0.27
C18:1n-9 (OA)	6.00	26.03	23.18	20.28	57.07
C18:1n-7	1.80	0.70	-	-	-
C20:1n-11	0.10	0.24	-	-	-
C20:1n-9	0.60	0.01	0.26	-	0.95
C20:1n-7	0.10	-	-	-	-
C22:1n-11	0.40	0.24	-	-	-
C22:1n-9	0.10	0.29	-	-	-
C24:1n-9	0.60	0.03	-	-	-
Total monounsaturated	13.40	27.58	23.57	20.28	58.29
C18:2n-6 (LA)	0.60	60.57	52.91	57.67	19.34
C18:3n-6	0.10	0.49	0.02	-	9.20
C20:2n-6	0.10	0.02	-	-	0.06
C20:3n-6	0.10	0.37	-	-	-
C20:4n-6 (ARA)	0.60	0.04	-	-	-
C22:4n-6	0.00	-		-	-
C22:5n-6	0.30	-	-	-	-
Total n-6 PUFA	1.80	61.49	52.93	57.67	28.60
C18:3n-3 (LNA)	0.50	0.22	5.92	-	11.18
C18:4n-3	1.40	-	-	-	0.03
C20:3n-3	0.10	0.07	-	-	0.03
C20:4n-3	0.50	-	-	-	0.07
C20:5n-3 (EPA)	9.50	0.07	-	-	0.05
C21:5n-3	0.40	-	-	-	-
C22:5n-3 (DPA)	1.40	0.04	-	-	0.07
C22:6n-3 (DHA)	14.60	0.15	-	-	0.15
Total n-3 PUFA	28.40	0.55	5.92	0.00	11.58
Total n-3 HUFA	24.10	0.22	0.00	0.00	0.20

[1]Data on proximate composition and fatty acid contents of fishmeal is from Glencross (2020); [2]Fatty acid contents of safflower oilseed meal obtained are from this study; [3]Fatty acid contents of soybean meal is from Oliveira et al. (2021); [4]Wheat meal fatty acid content is from Nikolić et al. (2008);
[5]Canola meal fatty acid content is from MacIntosh et al. (2021). *Cold press extraction process, **Solvent extraction process

3.4. Mineral composition of safflower meal

A comparison of the mineral content of a fish meal, soybean meal, wheat meal, and canola meal with the mineral content of a safflower meal is shown in Table 4. A Safflower oilseed meal is a better source of phosphorus, potassium, calcium, and iron than a wheat meal. As for magnesium, it is relatively low.

Table 4- Safflower oilseed meal, fish meal, soybean meal, wheat meal, and mineral contents

	Marine meals	***Terrestrial plant meals***			
Mineral name	***Fish meal[1]***	***Safflower meal[*2]***	***Soybean meal[**3]***	***Wheat meal[3]***	***Canola meal[**3]***
Phosphorus (P)	2.35%	0.39%	0.55%	0.34%	1.07%
Potassium (K)	1.36%	0.62%	1.9%	0.22%	1.36%
Calcium (Ca)	3.5%	0.26%	0.37%	0.04%	0.62%
Magnesium (Mg)	1.9%	0.25%	0.30%	0.27%	0.50%
Iron (Fe) (ppm)	27	55	36	33	373
Copper (Cu) (ppm)	6	7	41	5	6
Manganese (Mn) (ppm)	3	6	36	30	67.1
Zinc (Zn) (ppm)	47	18	67	20	66.2

[1]Data on proximate composition and mineral contents of fishmeal are from Glencross (2020); [2]Mineral contents of safflower oilseed meal are obtained from this study; [3]Data on proximate composition and mineral contents of soybean meal, wheat meal, and canola meal are from Hertrampf & Wiedad-Pascual (2000); *Cold press extraction process; **Solvent extraction process

4. Discussion

The average safflower seeds yield ha^{-1} is lower (0.72 tons) when compared with other oilseeds (Alizadeh et al. 2010), such as soybeans (2.34 tons), and rapeseeds (1.51 tons), peanuts (1.37 tons), and sunflowers (1.14 tons). Dubois et al. (2007) determined safflower oilseed meal contains a rich linoleic fatty acid.

The weight of a 1000-seed varies between 15-104 g with a coat percentage that varies between 18-59% of the total seed weight (Smith 1996; Bowles et al. 2010). It has been emphasized in several studies that these characteristics are influenced by genetic variation (Smith 1996; Amini et al. 2008; Ben Moumen et al. 2015). The oil extraction process, two of safflower product types are used (Knowles and Ashri 1995). One is the hull and labeled as a hulled safflower meal; the other is de-hulled (totally or partially) and labelled as a de-hulled safflower meal. Safflower meal in which the coat is not separated is commonly used in the safflower variety (Balcı) we discussed in our study.

Safflower seeds used for oil production may be either cold-pressed, expeller-pressed, or solvent-extracted (GRDC 2010). In this study, it was determined that the cold-press extraction method was used to extract safflower oil.

Safflower oilseeds are cold-pressed in a cold-press machine with fixed parameters of 10 m output mold, 40 rpm screw rotation speed, and a maximum 40 °C output temperature. After each cold pressing, the oil (liquid phase) and meal (solid phase-meal) are collected and weighed, while the oil fraction is immediately filtered through a 40 mm sieve to separate the suspended materials. The oil is separated in a centrifuge that has been chilled to a constant temperature of 10 °C (Aydeniz et al. 2014; Aksoylu Özbek & Günç Ergönül 2020). Safflower producers in Türkiye extract oil from the safflower oilseed plant by using the cold-press method (RIPSA 2021). In animal nutrition, safflower seed meal can be used as a protein source, although nutritional value of safflower oilseed meal may vary depending on the amount of coat and oil extracted (Heuzé et al. 2015).

According to the findings and results of this research, it was determined that the use of safflower meal in animal feeds, either alone or in combination with another raw material, had no negative effects on the growth performance, feed conversion, and physiological characteristics of aquaculture species.

4.1. Proximate analysis

According to crude protein content, safflower meal is similar to wheat meal, which has lower protein ratios than fish meal, soybean meal, and canola meal. When the results of our study's analysis are compared with other in the literature it can be seen that the oil

content is higher than fish meal, soybean meal, wheat meal, and canola meal. In the studies in which safflower meal was used instead of fish meal and soybean meal in aquafeeds for protein sources up to 20%, no negative effects occurred in terms of growth performance and feed evaluation (Galicia-González et al. 2010; Ustaoglu Tiril & Kerim 2015; Çantaş & Yıldırım 2020). In addition, it can be used as a source of oil as well as a source of protein in fish feeds. In this case, it can benefit both in terms of sustainability and cost-effectiveness.

4.2. Comparing amino acids

The amino acid content of safflower oilseed meal is relatively low when compared to fish meal and soybean meal. It is remarkable that the content of arginine, histidine and phenylalanine percentages as vegetable protein sources in fish feeds are close to that of canola meal. Upon understanding the importance of the amount of amino acids in feeds, this should be taken into account in the feedstuff to be substituted for fishmeal. This is one of the factors that directly affect the growth and health in fish (Tibaldi & Kaushik 2005). Cysteine and methionine constitute the whole sulfur amino acid of fish. Methionine is an essential sulfur amino acid that plays an important functional role in initiating protein synthesis (Elesho et al. 2021). The nutritional value of the safflower oilseed meal is limited by sulfur amino acids. Rodehutscord (1997) determined that 2.77% lysine, 0.2% tryptophan. 0.13% leucine, and 1% isoleucine are required to achieve 95% maximum protein utilization. It has been determined that if the ratios are lower, the growth performance will be adversely affected. Tibaldi and Kaushik (2005) have determined that the optimum protein ratio for European sea bass and sea bream is Arg, 4.6-5.4; Lys 4.8-5.0; Phe 1.6-1.7; Ile 2.6; Leu 4.3-4.5; Val 2.9-3.0; Met+Cys. 2.3-2.4; Phe+Tyr. 2.6-2.9; His 1.3-1.7 respectively.

The levels of arginine, histidine, leucine, valine, and phenylalanine in rainbow trout diets are similar to those found in safflower oilseed meal according to the expected essential amino acid ratios. However, isoleucine, lysine, methionine, and threonine are not sufficient to meet the needs of fish in safflower oilseed meal. The arginine and histidine requirements of common carp and Nile tilapia are affordable with safflower oilseed meal alone. It seems unlikely that other essential amino acids can be supplemented with safflower oilseed meal alone and used together with other raw materials. Table 5 below shows the amino acid values of safflower oilseed meal and the essential amino acid levels required by rainbow trout, carp, and Nile tilapia.

Table 5- Essential amino acid requirements of some freshwater fish

Essential amino acids	***Safflower oilseed meal[1] (% of diet)***	***Rainbow trout[2] (% of diet)***	***Common carp[2] (% of diet)***	***Nile tilapia[2] (% of diet)***
Arginine	1.68	1.50	1.70	1.20
Histidine	0.81	0.80	0.90	1.00
Isoleucine	0.51	1.10	1.00	1.00
Leucine	1.21	1.50	1.40	1.90
Lysine	0.81	2.40	2.20	1.60
Methionine	0.13	0.70	0.70	0.70
Phenylalanine	0.93	0.90	1.30	1.10
Threonine	0.64	1.10	1.50	1.10
Valine	0.94	1.20	1.40	1.50

[1]Values based on this study; [2]Values based on dry matter (NRC 2011)

In Table 6, assuming that fish meal, which is the basic feed raw material, meets all the essential amino acid needs, a full score of 100 is obtained and it is shown at what rate other raw materials can be used in feeds. Safflower oilseed meal can be used directly in place of wheat meal in terms of all essential amino acids except methionine. In general, safflower oilseed meal can be used at a level of 20% instead of fish meal and soybean meal in terms of essential amino acids.

Table 6- Comparison of the essential amino acid content of raw materials

Essential amino acids	*Fish meal*[*]	*Safflower oilseed meal*[*]	*Soybean meal*[*]	*Wheat meal*[*]	*Canola meal*[*]
Arginine	100	43.07	87.94	18.46	58.46
Histidine	100	32.40	48.80	14.00	55.20
Isoleucine	100	17.00	73.00	18.33	40.33
Leucine	100	24.20	79.40	19.80	48.60
Lysine	100	15.28	50.37	7.73	38.11
Methionine	100	6.50	24.50	29.00	34.00
Phenylalanine	100	34.44	92.96	25.18	51.85
Threonine	100	25.60	96.40	17.20	64.80
Valine	100	25.40	67.56	19.45	44.86

[*]Assuming that fish meal, which is the basic feed raw material, meets all the essential amino acid needs, a full score of 100 is obtained and it is shown at what rate other raw materials can be used in feeds. Also, references to raw materials in Table 1 were used

In the studies of Ustaoglu Tiril & Kerim (2015), Çantaş & Yıldırım (2020) the essential amino acid and nutritional composition data of safflower meal are found to be similar to this study. Galicia-González et al. (2010), the quantity of amino acid in the safflower meal used in a study is relatively high compared to our study.The reasons for this can be listed as the type of safflower meal, the soil, environmental conditions, and maintenance differences. In safflower meal, the first limiting amino acid is methionine followed by isoleucine. These ratio changes are expected to be normal as a result of these differences. Diets relatively low in amino acids may have negatively affected the growth performance of the cultured species.

4.3. Comparing fatty acid

Compared to fish meal, soybean meal, wheat, and canola meal the analysis results show us that safflower oilseed is a good source of linoleic (61%) and oleic acid (26%). It is noteworthy that they are at similar levels compared to these raw materials (wheat meal and soybean meal). Lipids in the diet play an important role in fish nutrition as a source of body energy and essential fatty acids (Yıldız 2008). Essential fatty acids are important for osmoregulation in fish. In the absence of essential fatty acids, it is thought that digestion and absorption will be adversely affected and will have also negative effects on neural tissues over time (Glencross 2009). As a result, essential fatty acids are crucial in juvenile fish and crustacean feeds (Glencross 2009). In general, the requirements for n-3 HUFA in Mediterranean aquaculture species range from 0.64% to 2.2% in broodstocks diets and from 0.8% to 3.5% in juvenile feeds (Izquierdo 2005).

In rainbow trout and common carp, the requirement for 18:3n-3 varies between 0.7-1% and 0.5-1%, respectively. Furthermore, the requirement for n-3 PUFA in rainbow trout ranges between 0.4% and 0.5% (NRC 2011).

Galicia-González et al. (2010) studied three different safflower meals that had C18:1n-9 content as a fatty acid content. These contents were higher than the values in our study. In terms of C16:0, C18:3n-3 and C20:1n-11, it was determined that they had similar results to our study results. In our study, the C18:2n-6 (LA) value, which is one of the total PUFAs, was quite high. At the same time, the HUFA value was found to be 0.22

4.4. Comparison of mineral composition

It was discovered that safflower meal only meets the mineral substance levels required by the cultured species when combined with other raw materials. The essentiality macro minerals were calcium, phosphorus, magnesium, and potassium. Calcium and phosphorus play important roles in the development and maintenance skeletal system. The Ca to P ratio of fish bone may show some changes during the development stage of fish species, however, Calcium: Phosphorus reported in several fish species ranges from 1.6:1 to 2:1 (Lall & Kaushik 2021). In this study, the Ca:P ratio is close to 1.0. Magnesium is a modulator of ion channels, an important intracellular signaling molecule involved in nerve conduction, muscle contraction, and potassium transport, and a modulator of oxidative phosphorylation (Lall & Kaushik 2021). Safflower oilseed meal contains significant potassium, iron, and phosphorus from mineral elements. Safflower

meal has a higher potassium (0.62%) and calcium level (0.26%) than wheat meal. Comparing the magnesium content, it is found to be less than fishmeal, similar to soybean meal and wheat meal.

4.5. If we summarize the studies of safflower meal over aqua feeds

In rainbow trout feeds, Ustaoglu Tiril & Kerim (2015) used safflower meal at three different rates (10, 20, and 30). Their results indicate that safflower meal is a promising feed ingredient and can be used up to a concentration of 20% in the rainbow trout diet with no adverse effects on growth performance, nutrient digestibility, or body composition. Çantaş & Yıldırım (2020) used safflower meal instead of soybean meal in two different amounts (10% and 20%) in rainbow trout feeds and supplemented the feeds with phytase enzyme. The group, which can be considered suitable for rainbow trout due to its sustainability and reduced environmental impact due to decrease phosphorus excretion, was the group containing 20% safflower meal and 2000 IU/kg phytase instead of soybean meal. Ali Assaf (2018) used 0%, 25%, 50%, 75%, and 100% safflower meal instead of fish meal in herbivore ratfish (*Siganus rivulatus*) diets. His study's findings noted that as the rate of safflower meal increased, the feed efficiency and protein utilization decreased. It is thought that a 25% or more usage of safflower oilseed meal instead of fish meal is not appropriate in herbivorous marine fish species such as ratfish because it indirectly reduces protein intake (Ali Assaf 2018). In addition, according to Ali Assaf's (2018) study, this type of safflower meal is not appropriate for use as a substitute for fish meal. This is because the palatability of the feed is not accepted by the fish. It was also understood that there was uneaten feed at the bottom of the tank. Galicia-González et al. (2010) investigated the digestibility of three different safflower meals used in white-legged shrimp feeds in their study. It was revealed that high-protein safflower oilseed plant meal feeds performed the best. Turchini et al. (2019) drew attention to the need to determine the positive or negative interaction of this raw material with other feed ingredients during commercial feed production. As Glencross (2020) points out, seven important steps must be taken into account in safflower oilseed meal/oil as well as in other raw materials.

5. Conclusions

The safflower oilseed plant meal is found in the feed of many animals and cultured fish. Researchers have revealed that up to 20% protein source can be used experimentally, especially in rainbow trout diets. Safflower oilseed meal is more advantageous in terms of potassium percentage (0.62%) when compared to wheat meal. While the amount of arginine, histidine, leucine, valine, and phenylalanine needed in the nutrition of rainbow trout can be met with safflower oilseed meal, it seems unlikely that isoleucine, lysine, methionine, and threonine can be satisfied with safflower oilseed meal alone. The arginine and histidine levels required by carp and Nile tilapia can be fulfilled with a safflower meal. The use of aquaculture as a protein source in their feed is important for cost and sustainability. It is also predicted that it can be evaluated as a protein and lipid source as well as a linoleic and oleic acid supplement. Studies are needed testing different proportions of oilseed safflower meal in rations for more species. Knowing which raw materials can be used in what amounts can make a difference in terms of cost and sustainability and focus on future research. Considering the balance of amino acids, fatty acids, and energy, research on using safflower oil seed meal and safflower oil as dietary supplements for fish fills the existing gaps.

Data availability: Data are available on request due to privacy or other restrictions.

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References

Aksoylu Özbek Z & Günç Ergönül P (2020). Determination of physicochemical properties, fatty acid, tocopherol, sterol, and phenolic profiles of expeller–pressed poppy seed oils from Turkey. *Journal of the American Oil Chemists' Society* 97(6): 591-602**.** doi.org/10.1002/aocs.12337

Ali Assaf N (2018). Replacing fishmeal by safflower meal in the diet of the marine algaevorous teleost, *Siganus rivulatus*. *American University of Beirut* 2-6.

Alizadeh A, Ghorbani G, Alikhani M, Rahmani H & Nikkhah A (2010). Safflower seeds in corn silage and alfalfa hay based early lactation diets: A practice within an optimum forage choice. *Anim Feed Sci Tech* 155(1): 18-24. doi.org/10.1016/j.anifeedsci.2009.09.014

Amini F, Saeidi G & Arzani A (2008) Study of genetic diversity in safflower genotypes using agro-morphological traits and RAPD markers. *Euphytica* 163: 21-30. doi.org/10.1007/s10681-007-9556-6

AOAC (1990). Official Methods of Analysis (13th Ed.) *Association of Official Analytical Chemists*.

AOAC (1995). Official Methods of Analysis. (14th. Ed.) *Association of Official Analytical Chemists*.

AOAC (2002). Protein content in meat. 928.08. Official Method of Analysis (17th ed.). *Association of Official Analytical Chemists*.

AOAC (2006). Crude Fat Determination-Soxhlet Method. Meat technology information sheet. 1-3.

Apper-Bossard E, Feneuil A, Wagner A & Respondek F (2013). Use of vital wheat gluten in aquaculture feeds. *Aquatic Biosystems* 9: 21. doi.org/10.1186/2046-9063-9-21

Aydeniz B, Güneşer O & Yılmaz E (2014). Physico-chemical. sensory and aromatic properties of cold press produced safflower oil. *Journal of the American Oil Chemists Society* 91(1): 99-110. doi.org/10.1007/s11746-013-2355-4

Babaoğlu M (2017). Dünya'da ve Türkiye'de aspir bitkisinin tarihi. Kullanım alanları ve önemi. *Tarım Gündem Dergisi*. 6(36): 98-102.

Baümler E, Cuniberti A, Nolasco S M & Riccobene I C (2006). Moisture-dependent physical and compression properties of safflower seed. *J Food Eng* 72: 134-140. doi.org/10.1016/j.jfoodeng.2004.11.029

Ben Moumen A, Mansouri F, Richard G, Abid M, Fauconnier M L, Sindic M, Amrani A & Serghini Caid H (2015). Biochemical characterisation of the seed oils of four safflower (*Carthamus tinctorius*) varieties grown in north-eastern of Morocco. *International Journal of Food Science & Technology* 50(3): 804-810. doi.org/10.1111/ijfs.12714

Bilgin Ö, Çarlı U, Erdoğan S, Maviş M E, Göksu Gürsu G & Yılmaz M (2018). Karadeniz'de (Sinop Yarımadası Civarı avlanan İzmarit Balığı *Spicara smaris* (Linnaeus. 1758) Etinin LC-MS/MS kullanarak amino asit içeriğinin tespiti ve ağırlık-boy ilişkisi. *Türk Tarım ve Doğa Bilimleri Dergisi* 6(2): 130-136. doi.org/10.30910/turkjans.556589

Bowles V G, Mayerhofer R, Davis C, Good A G & Hall J C (2010). A phylogenetic investigation of Carthamus combining sequence and microsatellite data. *Plant systematics and evolution* 287(1): 85-97. doi.org/10.1007/s00606-010-0292-3

Czesny S & Dabrowski K (1998). The effect of egg fatty acid concentrations on embryo viability in wild and domesticated walleye (*Stizostedion vitreum*). *Aquatic Living Resources* 11(6): 371-378. doi.org/10.1016/S0990-7440(99)80002-3

Çantaş İ B & Yıldırım Ö (2020). Supplementation of microbial phytase with safflower meal in rainbow trout (*Oncorhynchus mykiss*): the effects on growth, digestibility, environmental and serum biological parameters. *Journal of Chemistry* 2020. doi.org/10.1155/2020/4634796

Çulha Erdal Ş, Eyidoğan F & Ekmekçi Y (2021). Comparative physiological and proteomic analysis of cultivated and wild safflower response to drought stress and re-watering. *Physiology and Molecular Biology of Plants 27*(2): 281-295. doi.org/10.1007/s12298-021-00934-2

Dajue L & Mündel H H (1996). Safflower *Carthamus tinctorius* l. promoting the conservation and use of underutilized and neglected crops. Institute of Plant Genetics and Crop Plant Research. Gatersleben/International Plant Genetic.

Dubois V, Breton S, Linder M, Fanni J & Parmentier M (2007). Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential. *Eur J Lipid Sci Technol* 109(7): 710-732. doi.org/10.1002/ejlt.200700040

Ecoport (2010). Safflower (*Carthamus tinctorius*) seeds and oil meal. http://www.ecoport.org

Ekin Z (2005). Resurgence of Safflower (*Carthamus tinctorius* L.) Utilization: a global view. *Journal of Agronomy* 4(2): 83-87. doi.org/10.3923/ja.2005.83.87

Elesho F E, Sutter D A H, Swinkels M A C, Verreth J A J, Kröckel S & Schrama J W (2021). Quantifying methionine requirement of juvenile African catfish (*Clarias gariepinus*). *Aquaculture* 532: 736020. doi.org/10.1016/j.aquaculture.2020.736020

Emongor V (2010). Safflower (*Carthamus tinctorius* L.) the Underutilized and Neclected Crop: A Review. *Asian Journal of Plant Sciences* 9(6): 299-306. doi.org/10.3923/ajps.2010.299.306

FAO (1996). FAO Training Series (V.21/1). Coche, A.G. & Muir, J.F. Simple methods for aquaculture, Management for freshwater fish culture: Fish Stocks and Farm Management. www.fao.org/fishery/docs/CDrom/FAO_Training/FAO_Training/General/x6709e/x6709e10.htm

FAO (2011). Fishstat Plus: Universal software for fishery statistical time series. Aquaculture production: quantities 1950-2009; aquaculture production: values 1984-2009; capture production: 1950-2009; commodities production and trade: 1950-2008; total production: 1970-2009, Vers. 2.30. Available at. FAO Fisheries Department, Fishery Information, Data and Statistics Unit.

FAO (2017). FAO Statistical Databases. Available from http://faostat3.fao.org (last consult: 2017/10/03).

FAO (2020). FAO Statistical Databases. Available from http://faostat3.fao.org

FAOSTAT (2017). *Food and Agriculture Organization of the United Nations*. 2017.

FAOSTAT (2020). World production of safflower seeds in 2018; World Regions/Crops/Production from pick lists". United Nations *Food and Agriculture Organization*.

Folch J, Lees M & Sloane Stanley G H (1957). A simple method for the isolation and purification of total lipids from animal tissues. *The Journal of Biological Chemistry* 226(1): 497-509. doi.org/10.1016/s0021-9258(18)64849-5

Galicia-González A, Goytortúa-Bores E, Palacios E, Civera-Cerecedo R, Moyano-López F J, Cruz-Suárez L E & Ricque-Marie D (2010). Chemical composition and digestibility of three mexican safflower meals used as ingredients in diets for Whiteleg shrimp. *Litopenaeus vannamei*. *Journal of world aquaculture society* 41(2): 191-202. doi.org/10.1111/j.1749-7345.2010.00359.x

Gilbert J (2008). International safflower production an overview. 7th *International Safflower Conference. Australian Oilseeds Federation*.

Glencross B D (2009). Exploring the nutritional demand for essential fatty acids by aquaculture species. *Reviews in aquaculture* 1: 71-124. doi.org/10.1111/j.1753-5131.2009.01006.x

Glencross B D (2020). A feed is still only as good as its ingredients: An update on the nutritional research strategies for the optimal evaluation of ingredients for aquaculture feeds. *Aquacult Nutr* 2020 26(6): 1871-1883. doi.org/10.1111/anu.13138

GRDC (2010). Raising the bar with better safflower agronomy. *GRDC - Grain research and development Corporation.*

Hekmatpour F & Mozanzadeh M T (2021). Legumes Sustainable Alternative Protein Sources for Aquafeeds. *Legumes Research* doi.org/10.5772/intechopen.99778

Hertrampf J & Wiedad-Pascual F (2000). Handbook on Ingredients for Aquaculture Feeds. *Klumer Academic Publishers*.

Heuzé V, Tran G, Chapoutot P, Renaudeau D, Bastianelli D & Lebas F (2015). Safflower (*Carthamus tinctorius*) seeds and oil meal. *Feedipedia*.

Heuzé V, Tran G, Sauvant D, Lessire M & Lebas F (2020). Rapeseed meal. *Feedipedia*. IFFO (2021).

Izquierdo M (2005). Essential fatty acid requirements in Mediterranean fish species. *CIHEAM* 91-102.

Kinupp V F & Lorenzi H (2014). Plantas alimentícias não convencionais (PANC) no Brasil: guia de identificação. aspectos nutricionais e receitas ilustradas. Nova Odessa: *Instituto Plantarum de estudos da flora Ltda*.

Kiron V, Kulkarni A, Dahle D, Vasanth G, Lokesh J & Elvebo O (2016). Recognition of purified beta 1,3/1,6 glucan and molecular signalling in the intestine of Atlantic salmon. *Dev Comp Immunol* 56: 57-66. doi:10.1016/j.dci.2015.11.007.

Knowles P F & Ashri A. (1995) Safflower *Carthamus tinctorius* (Compositae). *Evolution of crop plants* 47-50.

Köprücü K & Sertel E (2012). The effects of less-expensive plant protein sources replaced with soybean meal in the juvenile diet of grass carp (*Ctenopharyngodon idella*): growth. nutrient utilization and body composition. *Aquaculture international* 20(3): 399-412. doi.org/10.1007/s10499-011-9471-7

Lall S P & Kaushik S J (2021). Nutrition and metabolism of minerals in fish. *Animals* 11(9): 2711. doi.org/10.20944/preprints202108.0088.v1

MacIntosh S C, Shaw M, Connelly M & Yao Z J (2021). Food and Feed Safety of NS-B5ØØ27-4 Omega-3 Canola (Brassica napus): A New Source of Long-Chain Omega-3 Fatty Acids. *Frontiers in nutrition* 8: 716659. doi.org/10.3389/fnut.2021.716659

Mansouri F, Moumen A B, Richard G, Fauconnier M L, Sindic M, Elamrani A & Caid H S (2018). Proximate composition, amino acid profile, carbohydrate and mineral content of seed meals from four safflower (*Carthamus tinctorius* L.) varieties grown in north-eastern Morocco. OCL 25(2): A202.

Mejicanos G, Sanjayan N, Kim I H & Nyachoti C M (2016). Recent advances in canola meal utilization in swine nutrition. *Journal of Animal Science and Technology* 58:7. doi.org/10.1186/s40781-016-0085-5

Meshram P D, Puri R G & Patil H V (2011). Epoxidation of Wild Safflower (*Carthamus oxyacantha*) Oil with Peroxy Acid in Presence of Strongly Acidic Cation Exchange Resin IR-122 Ascatalyst. *International Journal Of Chem Tech Research* 3(2011): 1152-1163.

Metcalfe L D & Schmitz A A (1961). The rapid preparation of fatty acid esters for gas chromatographic analysis. *Analytical Chemistry* 33(3): 363-364. doi.org/10.1021/ac60171a016

Murashita K, Fukada H, Takahashi N, Hosomi N, Matsunari H, Furuita H, Oku H & Yamamoto T (2015). Effect of feed ingredients on digestive enzyme secretion in fish. *Bulletin of Fisheries Research Agency* 40: 69-74.

Nikolić N, Radulović N, Momcilović B, Nikolić G, Lazić M & Todorovic Z (2008). Fatty acids composition and rheology properties of wheat and wheat and white or brown rice flour mixture. *European Food Research and Technology* 227(5): 1543-1548. doi.org/10.1007/s00217-008-0877-z

NRC (2011). Nutrient Requierments fish and shrimp. National Research Council. Division on Earth and Life Studies. Board on Agriculture and Natural Resources. Committee on the Nutrient Requirements of Fish and Shrimp. *National Academies Press* 392.

OECD/FAO (2022). OECD-FAO Agricultural Outlook 2022-2031, OECD Publishing, Paris, doi.org/10.1787/f1b0b29c-en

Oliveira X S M, Palma A S, Reis B R, Franco C S, Marconi A P, Shiozaki F A G, Reis L, Salles M S & Netto A S (2021). Inclusion of soybean and linseed oils in the diet of lactating dairy cows makes the milk fatty acid profile nutritionally healthier for the human diet. *Plos one* 16(2): e0246357. doi.org/10.1371/journal.pone.0246357

Oyen L P A & Umali B E (2007). *Carthamus tinctorius* L. Record from Protabase. PROTA *Plant Resources of Tropical Africa / Ressources végétales de l'Afrique tropicale*.

Pahlavani M H (2005). Some technological and morphological characteristics of safflower (Carthamus tinctorius L.) from Iran. *Asian Journal of Plant Sciences* 4(3): 234-237. doi.org/10.3923/ajps.2005.234.237

RİPSA (2021). Personal communication with Onur ÖZŞAHİN who is the manager of RİPSA safflower company in Kayseri.

Rodehutscord M, Becker A, Pack M & Pfeffer E (1997). Response of rainbow trout (Oncorhynchus mykiss) to supplements of individual essential amino acids in a semipurified diet, including an estimate of the maintenance requirement for essential amino acids 1, 2, 3. *J Nutr*. 127(6): 1166-75. doi.org/10.1093/jn/127.6.1166.

Sarker P K, Kapuscinski A R, Lanois A J, Livesey E D, Bernhard K P & Coley M L (2016). Towards sustainable aquafeeds: complete substitution of fish oil with marine microalga *Schizochytrium* sp. improves growth and fatty acid deposition in juvenile Nile tilapia (*Oreochromis niloticus*). *PloS One* 11(6): e0156684. doi.org/10.1371/journal.pone.0156684

Smith J R (1996). Safflower. *The American Oil Chemists Society*.

Storebakken T, Refstie S & Ruyter B. (2000). Soy-product as fat and protein sources in fish feeds for intensive aquaculture. *Soy In Animal Nutrition Federation Of Animal Science Societies Savoy* 2: 127-170.

Suárez J A, Gaxiola G, Mendoza R, Cadavid S, Garcia G, Alanis G, Suárez A, Faillace J & Cuzon G (2009). Substitution of fish meal with plant protein sources and energy budget for white shrimp *Litopenaeus vannamei* (Boone. 1931). *Aquaculture* 289(1-2): 118-123. doi.org/10.1016/j.aquaculture.2009.01.001

Suprayudi M A, Inara C, Ekasari J, Priyoutomo N, Haga Y, Takeuchi T & Satoh S (2015). Preliminary nutritional evaluation of rubber seed and defatted rubber seed meals as plant protein sources for common carp *Cyprinus carpio* L. juvenile diet. *Aquaculture research* 46(12): 2972-2981. doi.org/10.1111/are.12452

Technovit (2014). Importance of aquaculture nutrition. tecnovit.net/en/news-tecnovit/importance-aquaculture-nutrition

Thacker P & Widyaratne G (2012). Effects of expeller pressed camelina meal and/or canola meal on digestibility. performance and fatty acid composition of broiler chickens fed wheat–soybean meal-based diets. *Archives of Animal Nutrition* 66(5): 402-415. doi.org/10.1080/1745039x.2012.710082

Tibaldi E & Kaushik S J (2005). Amino acid requirements of Mediterranean fish species. *Mediterranean fish nutrition* 59-65.

Turchini G M, Trushenski J T & Glencross B D (2019). Thoughts for the future of aquaculture nutrition: realigning perspectives to reflect contemporary issues related to judicious use of marine resources in aquafeeds. *North Am J Aquaculture* 81(1): 13-39. doi.org/10.1002/naaq.10067

USEPA (2007). "Method 3051A (SW-846): Microwave Assisted Acid Digestion of Sediments, Sludges, and Oils," Revision 1. Washington, DC.

Ustaoglu Tiril S & Kerim M (2015). Evaluation of safflower meal as a protein source in diets of rainbow trout (*Oncorhynchus mykiss*.Walbaum. 1792). *Journal of Applied Ichthyology* 31(5): 895-899. doi.org/10.1111/jai.12807

Ustaoğlu Tiril S, Dernekbasi S, Karayucel I, Kerim M & Akyuz A P (2016). Effects of canola and safflower oil supplementation in diets on growth performance and fatty acid composition of Russian sturgeon (*Acipencer gueldenstaedtii* Brandt, 1833). *The Israeli Journal of Aquaculture-Bamidgeh* 68: 1222-1234. doi.org/10.46989/001c.20840

Yau S K & Ryan J (2010). Response of rainfed safflower to nitrogen fertilization under Mediterranean conditions. *Industrial Crops and Products* 32(3): 318-323. doi.org/10.1016/j.indcrop.2010.05.008

Yıldız M (2008). Fatty acid composition of some commercial marine fish feeds available in Turkey. *Turk. J. Vet. Anim. Sci* 32(3): 151-158.

Zheng J, Dan Z, Jiang D, Tang Z, Zhu S, Li Q, Li X, Mai K & Ai Q (2022). Evaluation of Six Selected Commercial Fermented Soybean Meal by Feeding Juvenile Turbot (*Scophthalmus maximus* L.). *Aquaculture Nutrition* 2022: 1-13. doi.org/10.1155/2022/3822758

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Effect of Nitrogen and Boron Treatments on Harvest Index and Nitrogen Use Efficiency in Sugar Beet

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ABSTRACT

The use of optimum nitrogen (N) and boron (B) fertilizers is important for yield and quality in sugar beet production. Therefore, it is necessary to determine the rate of use and recycling parameters of the applied nutrients by the plants in order to optimize the yield and quality in production. This study aims is to examine the effect of different doses of N and B treatment on sugar harvest index (SHI), nitrogen harvest index (NHI), and N use efficiency parameters and to determine the economic optimum nitrogen rates (EONR) in sugar beet. The experiment was set up in a randomized block factorial design with three replications. Five doses of N (0, 90, 180, 270, and 360 kg N ha^{-1}) and four doses of B (0, 2, 4, and 6 kg B ha^{-1}) were applied in the study. According to the results of the research, the SHI decreased statistically significantly with the increase of dose of the N treatment, but the NHI was not affected by the N treatment. The physiological efficiency of N in taproot dry matter yield and physiological efficiency of N in sugar yield decreased statistically significantly ($p<0.01$) with the increase in the dose of N treatment. A similar case was observed in the parameters of nitrogen agronomic efficiency (NAgE) and nitrogen uptake efficiency (NUpE). The increase in B treatment doses statistically significantly ($p<0.01$) increased the NAgE in the first year. The EONR, calculated using the quadratic model, was found to be 240 kg N ha^{-1} on average of two years. As a result, the N use potential decreased with the increase of N doses applied to sugar beet. The use of EONR can be recommended for optimum yield and quality in the region.

Keywords: Sugar harvest index, Physiological efficiency of nitrogen, Nitrogen agronomic efficiency, Nitrogen uptake efficiency, Economic optimum nitrogen rates

1. Introduction

Nitrogen (N) is one of the most commonly applied nutritional elements in sugar beet production. In agricultural production, plants' N fertilizer use capacity varies between 30.2-53.2% and N losses can increase up to 70% as a result of excessive and incorrect treatments (Anas et al. 2020). The use of excessive N fertilizers increases the input cost of farmers and causes an increase in nitrate concentration in ground and surface waters and eutrophication in the coastal ecosystem, thus negatively affecting biodiversity (Smil 2011). On the other hand, N applied to sugar beet more than needed increases the taproot yield (Sulfab et al. 2017) and decreases the sugar yield (Cimrin 2001). Therefore, N fertilizer should be applied at the optimum dose without reducing the yield and quality of sugar beet. For this reason, it is recommended to calculate nitrogen use efficiency (NUE) and harvest index (HI) and to apply the optimum dose of fertilizer. The purpose of NUE parameters is to evaluate the performance of crop growing systems determine the losses of N applied to the soils and to provide optimum nutrients to the crops (Fixen et al. 2014). NUE parameters can also be expressed as the relationship between the ability of the cultivated plant to take up the available N from the soil and the dry matter production (Hirose 2012).

While there are many calculations of NUE in wheat, maize or other grains, these calculations are quite limit in sugar beet. N in beta beet crops is required for (i) canopy (Malnou et al. 2006) and (ii) sugar storage in root cells (Milford & Watson 1971). However, N is not a compound of sugar and is the main storage product of sugar beet (Hoffman et al. 2005). Therefore, the calculation of HI and NUE parameters in sugar beet is different from cereals (Laufer et al. 2016).

The first goal of sugar beet producers should be to obtain a high sugar yield. The sugar harvest index (SHI), which is also expressed as the ability of the plant to produce (Porker et al. 2020), is defined as the amount of sugar produced per one unit of dry matter (Laufer et al. 2016).

The nitrogen harvest index (NHI) in plants is an important feature that shows how efficiently the applied N is used. NHI in sugar beet is calculated similarly to grains. While NHI is calculated in grains by the ratio of the amount of N taken up by the grain to the total amount of N taken up (Fageria 2014), in sugar beet it is calculated by the ratio of the N taken up by the taproot of the sugar beet to the total amount of N taken up (Laufer et al. 2016).

In order to facilitate the NUE studies, Moll et al. (1982) divided the NUE into two components in the 1980s: (i) physiological NUE and (ii) N uptake efficiency. On the other hand, Fageria et al. (2008) asserted that the agronomic efficiency of N is also an important component in NUE calculations.

While physiological efficiency of nitrogen (NPE) is calculated in cereals as the grain yield per one unit of N taken up by the plant (Yilmaz 2015), it is calculated separately for the taproot dry matter yield and sugar yield in sugar beet. While the NPE in the taproot dry matter of sugar beet is the taproot dry matter yield obtained per one unit of N taken up by the plant (tap - root + leaf), the NPE in sugar yield is expressed as the sugar yield obtained per unit of N taken up by the plant (taproot + leaf) (Laufer et al. 2016). N uptake efficiency is calculated in corn plant by the ratio of the N taken up by the above-ground parts to the applied N dose (Buyuk 2016). Unlike the corn plant, significant amounts of N are taken up by both the taproot and the leaf in sugar beet; therefore, it has been suggested that it is more appropriate to formulate nitrogen uptake efficiency (NUpE) in sugar beet as the ratio of the total amount of N uptake (taproot + leaf) to the amount of N dose applied (Good et al. 2004).

N agronomic efficiency a feature that more closely reflects the direct impact of a unit of fertilizer applied on production, is associated with economic returns (Fixen et al. 2014). N agronomic efficiency is calculated in sugar beet as the taproot dry matter yield per unit of N fertilizer (Fageria et al. 2008).

In their study, Laufer et al. (2016) administered six different doses of N (0, 40, 80, 120, 160, and 200 kg N ha^{-1}) in six different locations (DE10, DE11, NL10, NL11, DK10, DK11) in Germany (DE), Netherlands (NL), and Denmark (DK) between 2010 and 2011 and determined the NHI and NUE. They reported that, with increasing doses of N treatment, SHI decreased up to 0.619, 0.617, 0.605, 0.601, 0.590, and 0.579 in these six locations, respectively. They also reported that while the N treatments did not have a statistically significant effect on NHI, the physiological efficiency of N in taproot dry matter yield (NPE_{TDMY}) decreased from 128.5 to 84.9 (18120 kg ha^{-1}, 22260 kg ha^{-1}) with increasing doses of N. Moreover, they asserted that the physiological efficiency of nitrogen in sugar yield (NPE_{SY}) decreased from 95.7 to 63.1 with increasing doses of N.

Increasing NUE must be achieved in agricultural production systems by maintaining yield and quality. While NUE can be increased by rational management of N fertilizer, it is limited by boron (B) deficiency. Using this synergistic relationship between N and B is an effective strategy to increase efficiency and improve NUE (Zhang et al. 2015; Wang et al. 2021). The use of B fertilizer in sugar beet directly affects the taproot yield because it increases the sugar ratio (Mekdad 2015), has a positive effect on the formation of healthy cell walls, and increases the indolacetic acid (IAA) (Marschner 2012). However, B has an important role in N_2 fixation and nitrate assimilation. Camacho-Cristobal et al. (2008) reported that nitrate uptake is low in both leaves and roots in areas where B is deficient.

Shivay et al. (2017) applied urea fertilizer to the wheat plant by coating it with borax containing 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% B and reported that the nitrogen agronomic efficiency (NAgE) increased with the increase of B concentration, and the highest NAgE was obtained in the treatment containing 0.5% B. Also, Pooniya et al. (2018) reported that the yield and NUE of maize increased with the treatment of urea fertilizer by coating it with B.

Hellal et al. (2009) used soil applied N and foliar applied B fertilizers and examined their effect on the yield of sugar beet and the distribution and ratio of nutrients in taproots and shoots. They administered 3 different doses of N (60, 80, and 100 mg N kg^{-1}) and 4 different doses of B (0, 20, 50, and 100 mg B L^{-1}) and reported that the combined treatment of 100 mg N kg^{-1} + 50 mg B L^{-1} yielded the maximum taproot yield, maximum shoot yield, and maximum nutrient balance. In conclusion, they asserted that N, K, and Fe concentrations increased in taproots and shoots due to the positive interaction between N and B.

N fertilizers are the most commonly used fertilizers in sugar beet. Irrational and excessive use of N fertilizers increases the taproot yield of the sugar beet but decreases the sugar ratio, which is the most important quality character, and thus decreases the SHI and NUE. The main goal in sugar beet production is to increase sugar yield without compromising quality. For this reason, it is very important

to determine the economic optimum N dose in order to prevent the use of the excessive amount of N fertilizer and to improve NUE parameters.

Although several different models are widely used to describe the response of the yield to the applied N fertilizer, it has been reported that the most suitable model for sugar beet is the Quadratic Model (Cerrato & Blackmer 1990; Sayili & Akca 2004). Rezvani et al. (2013) took into account the yield of sugar beet while determining economic optimum nitrogen rates (EONR), but Ilbas et al. (1996) took into account sugar yield and leaf yield in addition to sugar beet yield while determining EONR.

In the interviews made with the agricultural stakeholders and farmers in the region where the study was conducted, it was determined that they applied nitrogenous fertilizer (300-400 kg N ha^{-1}) to the sugar beet well above the needed, and the B content was generally found to be low in the region's soils. Therefore, it is necessary to investigate the use of N and B fertilizers for optimum yield and quality in the region. Researches on NUE parameters in sugar beet in Türkiye are also limited. Therefore, it is important to determine the NUE parameters and HI of N applied to sugar beet, which has a large share in agricultural production. The aim of this study is to determine the effect of the treatment of different doses of N and B fertilizers on NUE parameters and HI in sugar beet production and to develop a proposal about EONR.

2. Material and Methods

2.1. Study area

This research was carried out for two consecutive years (2017 and 2018) in the district of Elbistan, Kahramanmaras. Before the experiments, soil samples were taken from the study area, and their texture, pH, EC, lime, organic matter, available Ca, K, Mg, P, B, Cu, Fe, Zn, Mn, and N-min were determined. According to the results of physical and chemical analyzes, deficient P, Fe and Zn were applied homogeneously at the beginning of the experiment (2017). In 2018, only N and P fertilizers were applied. The experiment was set up in a randomized block factorial design with three replications. The fertilizers were applied to an area of 20 m^2 (2.50 m x 8 m), and 18 m^2 (2.25 m x 8 m) of this area was used to collect data due to the edge effect. The doses of N fertilizer (0, 90, 180, 270, 360 kg N ha^{-1}) were applied in two splits in 2017 and 2018, the first half was applied in the form of ammonium sulfate at planting and the other half in the form of urea before the first irrigation. The doses of B fertilizer (0, 2, 4, and 6 kg B ha^{-1}) were applied only in 2017. In order to distribute the B homogeneously in the parcels, it was dissolved in water and mixed with a rake after it was sprayed on the soil.

In the experiment, the seeds of the sugar beet variety "Aranka" were used, and sowing was done in the first half of April in both years. After the planting, singling and rarefying were performed, and 144 plants (8000 plants per da^{-1}) were left in each parcel. Irrigation was carried out with the treatment of the same amount of water to each parcel at the same time, taking into account the need for plants.

2.2. Sugar beet harvest and yield calculations

After completing the vegetation period and reaching technological maturity, sugar beets were harvested in October in both years. After removing the heads and leaves of the harvested beets, the beet taproots were counted and weighed, and the yield per hectare was calculated using the average taproot weight. The leaves of 10 randomly selected sugar beets were weighed, and the leaf yield per decare was calculated. The samples taken from the taproot and leaves were dried in an oven at 65 °C until they reached a constant weight in order to calculate the dry matter yield. Then, N concentrations of dry leaf and taproot samples were determined (Equation 2.1). For the amount of N removal up by sugar beet taproot and leaves and the sugar yield were calculated using the following Equation 2.2:

Nutrient removal (kg ha^{-1}) = Dry matter yield (kg ha^{-1}) x N concentration (%)/100 (2.1)

Sugar yield (kg ha^{-1}) = Sucrose concentration (%) x Taproot yield (kg ha^{-1})/100 (2.2)

2.3. Method

The texture class of the soils was identified using the bouyoucus hydrometer method reported by Gee & Bauder (1986), and pH and EC values were determined by pH and EC meter using the method reported by Demiralay (1993), Rhoades (1996). Organic matter content was determined using the modified Walkley-Black method (Nelson et al. 1996), total lime content using Scheibler calcimeter (Allison & Moodie 1965), and plant-available Ca, Mg, and K using the 1 N ammonium acetate (NH_4OAC, pH=7) method (Helmke & Sparks 1996). Plant-available phosphorus was determined using the 0.5 M $NaHCO_3$ method (Olsen & Sommers 1982), and extractable Fe, Cu, Zn, and Mn using the DTPA method (Lindsay & Norvell 1978). The available B in soils was determined using mannitol-$CaCl_2$ method

(Cartwright et al. 1983), the N concentration in the leaves using the Kjeldahl method reported by Bremner (1996), and the N-min concentration in the leaves using the method reported by Bremner (1965). The sugar content was determined by mixing the chopped sugar beet samples with 0.3% aluminum sulphate solution, then filtering and using a polarimeter (Kavas & Leblebici 2004).

2.4. Formulas for calculating the nitrogen use efficiency parameters

The formulas developed for the calculation of HI, NHI, and NUE parameters (NPE_{TDMY}, NPE_{SY}, NAgE, NUpE) in sugar beet are given in Table 1 (Good et al. 2004; Hoffmann 2006; Fageria 2008; Ciampiti & Vyn 2012).

Table 1- The terminology used to calculate harvest indices and NUE parameters in sugar beet

Used terminology	*Formula*
Harvest index (HI)	
Sugar harvest index	$HI_S = SY/PDMY$
Nitrogen harvest index	$NHI = TNU_P/PNU_P$
Nitrogen use efficiency (NUE)	
Physiological efficiency of nitrogen in taproot dry matter yield	$NPE_{TDMY} = TDMY/PNU_P$
Physiological efficiency of nitrogen in sugar yield	$NPE_{SY} = SY/PNU_P$
Nitrogen agronomic efficiency	$NAgE = (N_{fertilizedTDMY}-N_{unfertilizedTDMY})/N_{applied}$
Nitrogen uptake efficiency	$NUpE = PNU_P-N_{applied}$

S: Sugar, SY: Sugar yield, PDMY: Total (taproot + leaf) Dry matter yield, TNUp: N taken up by taproot (kg da^{-1}), PNUp: Total (taproot + leaf) N taken up (kg da^{-1}), TDMY: Taproot dry matter yield

2.5. Determination of critical dose of nitrogen and economical optimum nitrogen rates

In this study, the relationship between N fertilization and sugar yield was calculated with the quadratic, quadratic-plateau, linear-plateau models (Ceratto & Blackmar 1990) obtained from the Sigmaplot program. It was determined that the relationship between yield and fertilizer dose in determining the optimum economical N rates was best explained by the quadratic model (Equation 2.3).

The Quadratic Model Equation is given below;

$$Y = a+bX+cX^2 \qquad (2.3)$$

In this formula, Y: Sugar yield, X: Nitrogen dose, a: Inception coefficient, b: Linear coefficient, and c: Quadratic coefficient.

The critical dose of nitrogen (CD) is determined by setting the first derivative of the quadratic model equation to zero. This also refers to the N dose corresponding to the maximum yield (Dikici 2007). The EONR are calculated by equating the first derivative of the quadratic model formula with the fertilizer product price ratio (Equation 2.4).

$$EONR = (PR-b)/2c \qquad (2.4)$$

In this equation, PR refers to the fertilizer-product price ratio. In the study, only sugar yield values obtained from N fertilized plots were used to calculate the critical dose and the economic optimum dose of N (N_0B_0, $N_{90}B_0$, $N_{180}B_0$, $N_{270}B_0$, $N_{360}B_0$).

2.6. Statistical analysis

In the study, variance analysis was performed according to the randomized blocks factorial experiment design using the "JMP 13.2.0" package program. Tukey's multiple comparison test was used to determine the difference between treatments in statistically significant results (SASS 1999).

3. Results and Discussion

3.1. General characteristics of the soils of the experiment area

Some physical and chemical properties of soils were given in Table 2.

Table 2- Some physical and chemical properties of soils

Texture	*Sand*	*Loam*	*Clay*	*Lime*	*OM*	*pH*	*EC*
	%	*%*	*%*	*%*	*%*		*ds m^{-1}*
CL	31.4	31.8	36.7	33	2.15	7.97	2.15

OM: Organic matter EC: Electrical Conductivity CL: Clay Loam

The texture of the experimental area soil was clay loam. The soil pH value was 7.97, and it was slightly alkaline (Saglam 2012). The soil is very calcareous with a lime content of 32.7%, and its organic matter content is 2.15% (middle class) (Gucdemir 2006). According to the limit values reported by Alparslan et al. (1998), the soil's available phosphorus (P) was low, its potassium (K) and magnesium (Mg) contents were high, calcium content (Ca) was very high, manganese (Mn) and copper (Cu) contents were sufficient, and B, iron (Fe), and zinc (Zn) contents were deficient (Table 3).

Table 3- Some macro and micro nutrient contents of soils

Ca mg kg^{-1}	*K mg kg^{-1}*	*Mg mg kg^{-1}*	*P mg kg^{-1}*	*B mg kg^{-1}*	*Cu mg kg^{-1}*	*Fe mg kg^{-1}*	*Zn mg kg^{-1}*	*Mn mg kg^{-1}*	*N-min mg kg^{-1}*
7659	473	877	11	0.56	1.50	3.20	0.47	2.80	4.20

3.2. Harvest index

3.2.1. Sugar harvest index

SHI refers to the sugar produced per unit of dry matter amount (Table 4). The effect of N and B fertilizers applied at different doses on SHI is given in Table 5. With the increase of N fertilizer treatment doses, SHI decreased, and the differences between the treatment doses were found to be statistically significant ($p<0.01$). In the first year, the highest SHI (0.74) was found to be in the N_0 treatment, while the lowest SHI (0.65) was in the N_{27} treatment. In the second year, the highest SHI (0.72) was found to be in the N_0 treatment, while the lowest SHI (0.63) in the N_{18}, N_{27}, N_{36} treatments. While the B treatment did not cause a significant effect on SHI, the NxB interaction had a significant effect in the second year, and the highest SHI was found to be in the N_0xB_2 treatment. Laufer et al. (2016) reported that SHI decreased significantly with increasing N doses. In this study, SHI values did not change significantly with the increase of N treatment dose in both years, except for the control and 90 kg N ha^{-1} dose. Malnou et al. (2008) reported that the sugar forming capacity of the dry matter decreased with the increase in the amount of N applied to sugar beet. While the increase of N applied to sugar beet increases leaf and taproot yield (Mampa et al. 2017), it causes a decrease in sugar content (Cimrin 2001). McDonnell et al. (1966) stated that every 23 kg ha^{-1} N added to sugar beet causes a 0.1% decrease in sugar ratio.

Table 4- Effect of N and B fertilization on the dry matter yields and amounts of N removed in sugar beet sections in 2017 and 2018

	Dry matter yields						*Amounts of N removed in sugar beet sections*					
	Leaf (kg ha^{-1})		*Taproot (kg ha^{-1})*		*Total (kg ha^{-1})*		*Leaf (kg ha^{-1})*		*Taproot (kg ha^{-1})*		*Total (kg ha^{-1})*	
Treatments	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
N_0B_0	1803	1703	11150	11220	12953	12923	69.2	71.8	93.6	111	162	183
N_0B_2	1979	1462	11368	11657	13347	13119	80.9	58.6	114	93.0	195	151
N_0B_4	2171	1617	10696	12166	12868	13783	75.3	66.3	95.8	88.6	171	154
N_0B_6	2324	1785	11248	12690	13543	14476	74.2	67.2	109	99.4	185	160
$N_{90}B_0$	2089	1846	11985	13038	14075	14885	85.8	75.0	128	139	214	214
$N_{90}B_2$	2896	2152	13087	13538	15984	15690	78.0	84.9	124	119	219	204
$N_{90}B_4$	2526	1970	12873	13169	15399	15139	90.6	81.3	127	126	218	208
$N_{90}B_6$	2688	2079	13262	13939	15950	16018	96.9	84.0	154	148	258	232
$N_{180}B_0$	2663	2819	13250	14885	15913	17705	122	110	143	164	265	275
$N_{180}B_2$	2495	2216	12435	14352	14930	16569	115	91.5	145	168	260	260
$N_{180}B_4$	2365	2252	13620	14922	15985	17174	93.2	91.7	141	164	234	256
$N_{180}B_6$	2691	2236	16314	15802	19005	18039	100	89.7	158	178	258	268
$N_{270}B_0$	3265	2568	14567	14805	17833	17374	130	111	164	182	295	293
$N_{270}B_2$	2779	1971	15303	14946	18082	16917	112	86.1	175	181	289	267
$N_{270}B_4$	2703	2715	14513	14635	17217	17350	132	116	186	172	319	288
$N_{270}B_6$	2729	2364	14131	14423	16861	16788	82.0	103	170	177	278	280

Table 4. Continued

$N_{360}B_0$	3148	2506	14695	16840	17843	19347	123	105	215	188	339	294
$N_{360}B_2$	3250	3485	14895	15084	18145	18569	170	151	178	181	345	332
$N_{360}B_4$	3204	3147	15427	16394	18631	19542	134	126	180	206	314	332
$N_{360}B_6$	3339	3078	14898	15885	18238	18963	134	133	204	209	339	342
Minimum	1803	1462	10696	11220	12953	12923	69.2	58.6	93.6	88.6	162	151
Maximum	3339	3485	16314	16394	19005	19542	170	133	215	209	339	342
Mean	2655	2298	13486	14219	16140	16518	104	95.1	150	154	257	249

3.2.2. Nitrogen harvest index

According to the results of variance analysis applied N, B doses and NxB interaction did not have a statistically significant effect on the NHI of sugar beet (Table 5). In this study, NHI values were found to be between 0.51-0.63 ($N_{360}B_2$-$N_{360}B_0$) in 2017 and between 0.54-0.67 ($N_{360}B_2$-$N_{270}B_2$) in 2018. In a study on the N uptake of sugar beet, it was determined that 0.44 of the N absorbed by the plant was in the leaves and 0.56 in the taproot (Noshad et al. 2012). Ebmeyer & Hoffmann (2021), in their study on N uptake and use in sugar beet genotypes, determined that sugar beet leaf and root N contents were close to each other. Laufer et al. (2016) argued that N treatments did not have a significant effect on the NHI index in sugar beet, and this was du e to the ability of sugar beet to assimilate the existing N in the taproot and leaf parts.

Table 5- Effect of N and B fertilization on the DF (Degrees of freedom), p value and mean values of SHI (Sugar Harvest Index) and NHI (Nitrogen Harvest Index) for the years 2017 and 2018

	Treatments	***SHI***		***NHI***	
		2017	***2018***	***2017***	***2018***
	N_0B_0	0.68±0.079	0.67±0.098	0.57±0.020ab	0.60±0.028
	N_0B_2	0.77±0.013	0.78±0.042	0.58±0.036ab	0.61±0.050
	N_0B_4	0.75±0.043	0.70±0.038	0.56±0.011ab	0.57±0.034
	N_0B_6	0.75±0.052	0.74±0.070	0.59±0.026ab	0.58±0.072
	$N_{90}B_0$	0.78±0.088	0.72±0.018	0.60±0.050ab	0.67±0.070
	$N_{90}B_2$	0.66±0.090	0.66±0.037	0.56±0.031ab	0.58±0.088
	$N_{90}B_4$	0.73±0.059	0.72±0.008	0.58±0.013ab	0.60±0.011
	$N_{90}B_6$	0.68±0.066	0.67±0.033	0.59±0.040ab	0.63±0.021
	$N_{180}B_0$	0.67±0.024	0.61±0.023	0.53±0.076ab	0.59±0.015
	$N_{180}B_2$	0.70±0.026	0.64±0.057	0.56±0.075ab	0.64±0.063
	$N_{180}B_4$	0.64±0.010	0.63±0.014	0.60±0.010ab	0.64±0.029
	$N_{180}B_6$	0.67±0.050	0.64±0.022	0.61±0.028ab	0.66±0.049
	$N_{270}B_0$	0.63±0.043	0.63±0.037	0.55±0.020ab	0.62±0.016
	$N_{270}B_2$	0.64±0.030	0.63±0.011	0.60±0.032ab	0.67±0.017
	$N_{270}B_4$	0.66±0.025	0.62±0.066	0.58±0.031ab	0.59±0.024
	$N_{270}B_6$	0.67±0.006	0.65±0.049	0.61±0.011ab	0.63±0.058
	$N_{360}B_0$	0.65±0.023	0.56±0.046	0.63±0.023a	0.64±0.027
	$N_{360}B_2$	0.68±0.050	0.66±0.027	0.51±0.027b	0.54±0.033
	$N_{360}B_4$	0.68±0.037	0.63±0.017	0.57±0.055ab	0.62±0.047
	$N_{360}B_6$	0.71±0.050	0.66±0.058	0.60±0.038ab	0.61±0.046
Effect	***DF***	***p value***	***p value***	***p value***	***p value***
N	4	<0.01	<0.01	ns	ns
B	3	ns	ns	ns	ns
NxB	12	ns	ns	<0.05	ns

Means sharing the same letter, within a column, don't differ significantly at p<0.01; p<0.05
ns: non-significant

3.3. Nitrogen use efficiency parameters

3.3.1. Physiological efficiency of nitrogen in taproot dry matter yield

The effect of N fertilizer applied to sugar beet on NPE_{TDMY} was found to be statistically significant ($p<0.01$) for both years (Table 6). The highest values were obtained in treatments where N was not applied in both years. This can be attributed to the more efficient use of N in the soil in the production of taproot dry matter. It has been reported that in plant production the treatment of N above the optimum dose decreases the benefits of N fertilizer, increases the nitrogen losses, and decreases N use rate (Karam 2002).

Although NPE_{TDMY} was high in the control group without N fertilizer treatment, taproot dry matter yield and sugar yield were low in sugar beet (Table 7). It can be asserted that with the increase in the amount of N , the taproot dry matter yield and sugar yield increase, but the physiological efficiency of N decreases (Laufer et al. 2016). The effect of B treatment on NPE_{TDMY} was not significant. The highest NPE_{TDMY} value of NxB interaction was found to be N_0xB_0 in the first year.

3.3.2. Physiological efficiency of nitrogen in sugar yield (NPE_{SY})

The effect of N and B fertilizers applied to sugar beet at different doses on NPE_{SY} is given in Table 6. Physiological efficiency of N in sugar yield decreased with the increase in N fertilizer treatment doses, and the differences between the treatment doses were found to be statistically significant ($p<0.01$). While B treatment and NxB interaction did not cause significant changes in NPE_{SY} in the first year, significant differences were observed in 2 kg B ha^{-1} treatment in the second year due to high sugar yield (Table 7). In the NxB interaction, the highest NPE_{SY} value was found to be 68.1 in the N_0xB_2 treatment, while the lowest was 36.7 in the $N_{360}xB_6$ treatment.

Table 6- Effect of N and B fertilization on the degrees of freedom (DF), *p*-value and mean values of physiological efficiency of nitrogen in taproot dry matter yield (NPE_{TDMY}) and physiological efficiency of nitrogen in sugar yield (NPE_{SY}) for the years 2017 and 2018

	Treatments	***NPE_{TDMY}***		***NPE_{SY}***	
		2017	***2018***	***2017***	***2018***
	N_0B_0	68.3±2.39a	61.0±1.97	54.3±5.41	47.5±6.30
	N_0B_2	58.0±2.05a-e	76.9±5.29	53.0±0.53	68.1±6.78
	N_0B_4	62.5±2.47a-c	78.4±3.05	56.4±1.20	62.3±4.74
	N_0B_6	60.8±6.47a-d	80.5±16.0	55.4±5.22	68.0±11.7
	$N_{90}B_0$	56.0±2.58b-f	60.8±3.00	52.0±9.53	46.6±1.72
	$N_{90}B_2$	59.7±4.83a-e	68.1±17.9	48.2±4.63	54.6±18.4
	$N_{90}B_4$	59.1±2.76a-e	63.6±7.19	51.9±6.03	50.8±9.54
	$N_{90}B_6$	51.4±4.12c-g	59.9±0.48	42.4±6.34	45.0±1.18
	$N_{180}B_0$	49.9±3.03d-g	54.0±1.87	40.5±2.59	39.7±1.23
	$N_{180}B_2$	48.3±7.15e-g	55.1±4.22	40.7±3.49	40.7±3.55
	$N_{180}B_4$	58.4±5.30a-e	58.8±9.40	44.4±4.14	43.2±5.04
	$N_{180}B_6$	63.3±4.16ab	59.3±6.14	49.7±4.23	43.5±6.10
	$N_{270}B_0$	49.3±1.49d-g	50.3±1.31	38.1±3.30	37.4±1.64
	$N_{270}B_2$	52.9±2.76b-g	55.7±2.11	40.1±2.85	40.3±1.67
	$N_{270}B_4$	45.6±2.40fg	50.7±2.15	35.8±1.05	37.5±4.97
	$N_{270}B_6$	50.8±2.67d-g	51.3±2.43	40.8±2.12	39.2±3.47
	$N_{360}B_0$	43.3±2.96g	57.1±2.44	34.3±1.76	37.0±2.02
	$N_{360}B_2$	43.1±2.60g	45.3±1.97	35.8±2.73	37.0±1.08
	$N_{360}B_4$	48.9±3.35e-g	49.5±4.83	40.2±0.76	37.4±3.48
	$N_{360}B_6$	43.9±1.09g	46.6±3.55	38.3±3.27	36.7±4.87
Effect	***DF***	***p value***	***p value***	***p value***	***p value***
N	4	<0.01	<0.01	<0.01	<0.01
B	3	ns	ns	ns	ns
NxB	12	<0.01	ns	ns	ns

Means sharing the same letter, within a column, don't differ significantly at $p<0.01$; $p<0.05$, ns: non-significant

NPE_{SY} at the N_0 dose was found to be approximately 12% lower in the first year than in the second year. The reason for this is due to the difference in the total amount of N taken up although there was no significant difference between the sugar yields in the N_0 doses in the two years (Table 7). Laufer et al. (2016) reported that NPE_{SY} decreased from 95.7 to 63.1 with the increase of N doses applied to sugar beet. The difference in this regard between the present study and the study of Laufer et al. (2016) is due to the difference in sugar and taproot dry matter yields. It has been reported that sugar beet, which takes up the N in the soil in the area where N treatment is not applied, uses the N more effectively in sugar production, while increasing the N treatment dose causes an increase in the vegetative part of the plant and a decrease in the physiological efficiency of N in sugar yield (Allison et al. 1996). Many researchers stated that B application increases the yield of sunflowers, chickpeas and beans (Ceyhan et al. 2007; 2008; Harmankaya et al. 2008).

Table 7- Effect of N and B fertilization on the sugar yield (kg ha^{-1}) for the years 2017 and 2018

Treatments	*Sugar yield*	
(kg ha^{-1})	*2017*	*2018*
N_0B_0	8820	8710
N_0B_2	10390	10340
N_0B_4	9660	9570
N_0B_6	10260	10790
$N_{90}B_0$	11000	10770
$N_{90}B_2$	10610	10480
$N_{90}B_4$	11280	11040
$N_{90}B_6$	10910	10770
$N_{180}B_0$	10750	10950
$N_{180}B_2$	10540	10590
$N_{180}B_4$	10370	10990
$N_{180}B_6$	12820	11560
$N_{270}B_0$	11250	11000
$N_{270}B_2$	11590	10810
$N_{270}B_4$	11430	10790
$N_{270}B_6$	11340	11030
$N_{360}B_0$	11670	10880
$N_{360}B_2$	12330	12340
$N_{360}B_4$	12670	12400
$N_{360}B_6$	12990	12470
Minimum	8820	8710
Maximum	12990	12470
Mean	11130	10910

3.3.3. Nitrogen agronomic efficiency (NAgE)

When the NAgE data of the sugar beet were analyzed, it was observed that the NAgE decreased statistically significantly ($p<0.01$) with the increase in N treatment in the first year (Table 8). However, with the increase of B treatment doses in the first year, the NAgE increased statistically significantly ($p<0.01$) up to 10.8, 12.3, 16.9, and 18.0, respectively. In the first year, the highest NAgE (28.3) was found to be in the $N_{180}xB_6$ treatment, while the lowest NAgE (5.92) in the $N_{180}xB_2$ treatment. It has been reported that the increase in NAgE value with the increase in B treatment doses is due to the positive effect of B fertilization applied to sugar beet on taproot yield (Durak & Ulubas 2017). NAgE is highly affected by environmental factors. Especially the residues of the previous year's agricultural products and the remaining mineral N in the soil can affect the NAgE value (Jacops et al. 2018). This can explain our result that NAgE was higher in the first year than in the second year and caused statistically significant differences. Atar et al. (2017) reported that NAgE decreased from 9.1 to 7.6 with the increase of N doses (75, 125 kg N ha^{-1}) in the 2011/2012 crop production year in their study which took into account the grain yield in wheat. It has been determined that N application to sugar beet increases yield, but application of more N than the plant needs reduces agronomic efficiency and decreases root quality parameters (Varga et al. 2022). In the study, in

which the effect of N application doses (120, 240, 360 kg N ha^{-1}) on different wheat varieties was investigated, it was reported that NAgE value decreased significantly with the increase of N doses (Belete et al. 2018). Fixen et al. (2014) determined that NAgE value in corn, rice, and wheat varied between 15 and 30. Shivay et al. (2017) reported that when the urea fertilizer applied to the wheat plant was covered with B, it increased from 10.5 in the control group to 14.1 with the increase in the amount of B used in the NAgE coating.

3.3.4. Nitrogen uptake efficiency

It was observed that NUpE decreased significantly ($p<0.01$) with increasing N treatment doses in both years of the study. The effect of B treatment on NUpE was not significant. The highest NUpE value of NxB interaction was found to be $N_{90}xB_6$ in both years. The lowest NUpE value was obtained in the $N_{360}xB_4$ treatment in the first year and in the $N_{360}xB_0$ treatment in the second year (Table 8). The decrease in NUpE may be due to the increased N losses caused by increased N doses. In a similar study conducted in wheat, it was reported that the NUpE value decreased significantly with increasing doses of N (30, 60, 90, 120 kg N ha^{-1}) (Haile et al. 2012). Keeney and Olson (2009) suggested that NUpE value decreases with increasing N doses and that N fertilization should be done in splits, not all at once, in order to increase NUpE value. Buyuk (2006), in his study on maize, asserted that NUpE decreased from 5.8 to 2.1 with increasing N treatment doses.

3.4. Critical dose of nitrogen and economic optimum nitrogen rates

Table 8- Effect of N and B fertilization on the degrees of freedom (DF), P value and mean values of nitrogen agronomic efficiency (NAgE) and nitrogen uptake efficiency (NUpE) for the years 2017 and 2018

	Treatments	***NAgE***		***NUpE***	
		2017	***2018***	***2017***	***2018***
	N_0B_0	-	-	-	-
	N_0B_2	-	-	-	-
	N_0B_4	-	-	-	-
	N_0B_6	-	-	-	-
	$N_{90}B_0$	9.28±2.17de	20.1±13.7	2.38±0.26b	2.38±0.16
	$N_{90}B_2$	19.1±4.12a-d	20.8±20.3	2.43±0.06b	2.27±0.41
	$N_{90}B_4$	24.1±3.30ab	11.1±6.40	2.42±0.13b	2.31±0.19
	$N_{90}B_6$	22.7±9.06a-c	13.8±12.6	2.87±0.22a	2.58±0.08
	$N_{180}B_0$	11.6±6.05c-e	20.3±8.96	1.47±0.07c	1.52±0.04
	$N_{180}B_2$	5.92±1.39e	14.9±2.33	1.44±0.15cd	1.44±0.06
	$N_{180}B_4$	16.2±0.19b-e	15.3±9.89	1.30±0.11c-e	1.42±0.14
	$N_{180}B_6$	28.3±1.44a	17.2±4.14	1.43±0.10cd	1.49±0.18
	$N_{270}B_0$	12.6±0.61b-e	13.2±2.91	1.09±0.08d-f	1.08±0.05
	$N_{270}B_2$	14.5±1.23b-e	12.1±3.02	1.07±0.06ef	0.99±0.02
	$N_{270}B_4$	14.1±2.66b-e	9.14±4.15	1.18±0.04c-f	1.06±0.08
	$N_{270}B_6$	10.7±1.50de	6.42±1.74	1.03±0.07ef	1.04±0.03
	$N_{360}B_0$	9.84±4.97de	15.6±0.30	0.94±0.02ef	0.81±0.02
	$N_{360}B_2$	9.79±4.62de	9.51±1.40	0.96±0.08ef	0.92±0.02
	$N_{360}B_4$	13.1±3.66b-e	11.7±3.15	0.87±0.01f	0.92±0.07
	$N_{360}B_6$	10.2±2.10de	8.87±2.18	0.94±0.04ef	0.95±0.11
Effect	***DF***	***p value***	***p value***	***p value***	***p value***
N	4	<0.01	ns	<0.01	<0.01
B	3	<0.01	ns	ns	ns
NxB	12	<0.01	ns	<0.01	<0.01

Means sharing the same letter, within a column, don't differ significantly at $p<0.01$; $p<0.05$ ns: non-significant

For an accurate fertilizer dose recommendation, only the N doses used in the experiment are not sufficient, it is very important to determine the intermediate doses. The critical N dose, the intermediate dose at which maximum efficiency is obtained, should not be interpreted as an economic rate.

According to the quadratic model ($Y=8972.9+167.61x-2.724x^2$) for the N treatment doses and sugar yield data in first year, the highest sugar yield was obtained at the critical N dose of 11551 kg ha^{-1} and 307 kg ha^{-1}. According to the quadratic model ($Y=8911+192.82x-3.946x^2$) for the second year's data, the highest sugar yield was obtained at the critical N dose of 11260 kg ha^{-1} and 244 kg ha^{-1} (Table 9). The differences in critical N doses between the two years are due to sugar yield (Table 7).

Table 9- The coefficients of the quadratic equation (a, b, c) and the critical dose of nitrogen (CD), maximum yield, economic optimum nitrogen rates (EONR) calculated in 2017 and 2018

Quadratic	*a*	*b*	*c*	*CD*	*Max. Yield*	*EONR*	*R^2*
Model				*(kg ha^{-1})*	*(kg ha^{-1})*	*(kg ha^{-1})*	
2017	8972.9	167.61	-2.724	307	11551	265	0.83
2018	8911	192.82	-3.946	244	11260	207	0.91

In both years, N doses applied to the experiment area were divided into two: half of them in the form of ammonium sulfate (21% N) and the remaining half in the form of urea (46% N). In determining the EONR, the price ratio was calculated by taking into account the price of 1 kg of pure N, based on ammonium sulfate (20.8%) + urea (46%) fertilizers, and the price of 1 kg of sugar determined by the purchase price of sugar beet (Table 10).

Table 10- Determination of price ratio with ammonium sulfate, urea fertilizers (Anonymous 2021) and sugar price (Anonymous 2020) in 2017 and 2018

Years	*A. sulfate*	*Urea*	*Nitrogen*	*Sugar*	*Nitrogen/sugar*
	(kg/$)	*(kg/$)*	*(kg/$)*	*(kg/$)*	
2017	0.95	0.69	0.82	0.36	2.26
2018	1.23	0.90	1.07	0.30	2.91

The EONR were determined as 265 kg ha^{-1} in 2017 and 207 kg ha^{-1} in 2018. The EONR differences in both years are due to the fact that the quadratic model used is very much affected by price changes. Dikici (2007) reported that EONR reached the lowest value because fertilizer prices increased too much compared to wheat prices. Ilbas et al. (1996) reported the EONR as 150 kg N ha^{-1} for the N fertilizer applied by dividing into two in order to provide the highest sugar yield. The reason why their low value was lower than ours was because their sugar yield was.

Marlander et al. (2003) stated that the amount of N fertilizer applied to sugar beet is closely related to soil mineralization. Although the N requirement of sugar beet is 200-250 kg ha^{-1} on average (Varga et al. 2022), some researchers have stated that the amount of N needed by sugar beet can be reduced by adding 100-150 kg N ha^{-1} to the soil through mineralization. In Greece, where sugar beet is widely grown, sugar beet yield (taproot and sugar yield) is maximized when N treatment dose is >200 kg N ha^{-1} (Tsialtas & Maslaris 2005). Neeteson and Wadman (1987) and Stevens et al. (2008), reported that the optimum dose of N is more than 200 kg N ha^{-1} in the Netherlands and USA. When interpreted considering the coefficient of determination in order to achieve maximum sugar yield in the soils of the region, the EONR to be applied in 2018 (R^2= 0.91) will be 207 kg N ha^{-1}, while sugar yield decreases with the N treatment dose exceeding 244 kg N ha^{-1}. Therefore, in the calculation made by combining the data of the two years, the economic optimum N dose was obtained in the treatment of approximately 240 kg N ha^{-1}. In studies carried out by different researchers, 200-250 kg ha^{-1} N application has been suggested for maximum efficiency in sugar beet (Armstrong & Milford 1985; Draycott 1993; Lopez-Bellido et al. 1994).

Considering the economic optimum N level, with the reduction of the amount N applied in fertilization, HI and NUE parameters improved and fertilizer cost decreased, contributing to both the farmer and the country's economy. Moreover, using economic optimum level of N rather than large amounts of N fertilizers decreases the negative effects of N fertilizers on the environment.

4. Conclusions

In this study, N and B fertilizers were applied to sugar beet grown as the main product in Kahramanmaras Elbistan in Türkiye, the effects of this treatment on SHI and NUE parameters (NPE_{TDMY}, NPE_{SY}, NAgE, NUpE) were examined, and critical dose of N and EONR were

calculated. As a result of the research, SHI values decreased with increasing N treatment doses. N and B fertilizers had no significant effect on NHI. The NPE_{TDMY} and NPE_{SY} values of N utilization efficiency parameters decreased with increasing N fertilizer treatment doses. While the effect of N and B treatment on NAgE was found to be statistically significant in the first year, no significant effect was observed in the second year. While the NAgE value decreased with the increase of the N treatment doses, but it increased with the increase of the B treatment doses. The NUpE value decreased from 2.52 to 0.93 in the first year and from 2.38 to 0.90 in the second year with the increase in N treatment doses.

The EONR was 265 kg N ha^{-1} in 2017 and 207 kg N ha^{-1} in 2018. The EONR was obtained as 240 kg N ha^{-1} in the calculation made with the quadratic model by combining the two years' data. In the study, HI and NUE parameters, which are a reflection of the N taken up by the plant, decreased for per unit N applied in plant production. The HI and NUE parameters can also be evaluated as an indicator of the yield and quality relations of plant products. Therefore, it can be recommended to apply economical optimum N doses in terms of fertilizer economy, yield and quality in the research area.

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References

Allison L E & Moodie C D (1965). Carbonate," In: Black, C. A., Ed., Methods of soil analysis. *Chemical and microbiological properties Agronomy* 1379-1398.

Allison M F, Armstrong M J K, Jaggard W A, Todd D & Milford G F J (1996). An analysis of the agronomic, economic and environmental effects of applying N fertilizer to sugar beet (Beta vulgaris). *The Journal of Agricultural Science* 127: 475-486. doi.org/10.1017/S0021859600078709

Alpaslan M, Gunes A & Inal A (1998). Experiment Technique Book. Ankara University, Faculty of Agriculture Publication, Ankara, No:1501:57

Anas M, Liao F, Verma K K, Sarwar M A, Mahmood A, Chen Z L, Li Q, Zeng X P, Liu Y & Li Y R (2020). Fate of nitrogen in agriculture and environment agronomic, ecophysiological and molecular approaches to improve nitrogen use efficiency. *Biological Research* 53(47): 1-20. doi.org/10.1186/s40659-020-00312-4

Anonymous (2020). Turkish Sugar Sector Report 2020.

Anonymous (2021). Plant Nutrition Statistics, Fertilizer and diesel prices according to years.

Atar B, Kara B & Kucukyumuk B (2017). Determination of Nitrogen Efficiency in Winter Bread Wheat Cultivars. *Journal of Agricultural Sciences* 23(1): 119-127.

Armstrong M J & Milford G F J (1985). The nitrogen nutrition of sugar beet the background to the requirement or sugar yield and amino-N accumulation. *British Sugar Beet Review* 53(4): 42-44.

Belete F, Dechassa N, Molla A & Tona T (2018). Effect of nitrogen fertilizer rates on grain yield and nitrogen uptake and use efficiency of bread wheat (Triticum aestivum L.) varieties on the Vertisols of central highlands of Ethiopia. *Agriculture & Food Security* 7: 78 doi.org/10.1186/s40066-018-0231-z

Bremner J M (1965). Inorganic Forms of Nitrogen. *Methods of Soil Analysis: Part 2 Chemical and Microbiological Properties, 9.2* doi.org/10.2134/agronmonogr9.2.c33

Bremner J M (1996). Nitrogen Total. *Chemical Methods* 1085-1112. doi.org/10.2136/sssabookser5.3.c37

Buyuk G (2006). The effect of varying nitrogen doses at different periods to the nitrogen use efficiency, grain yield and quality of the corn genotypes at Cukurova conditions. *Cukurova University*, *Institute of Science and Technology, Department of Soil Science and Plant Nutrition*.

Camacho-Cristobal J J, Rexach J & Gonzalez-Fontes A (2008). Boron in plants: Deficiency and toxicity. *Journal of Integrative Plant Biology* 50: 1247-1255. doi.org/10.1111/j.1744-7909.2008.00742.x

Cartwright B, Tiller K G, Zarcinas B A & Spouncer L A (1983). The chemical assessment of the boron status of soils. *Australian Journal of Soil*

Research 21(3): 321-332. doi.org/10.1071/SR983032

Cerrato M E & Blackmer A M (1990). Comparison of models for describing; corn yield response to nitrogen fertilizer. *Agronomy Journal* 82(1): 138-143. doi.org/10.2134/agronj1990.00021962008200010030x

Ceyhan E, Onder M, Harmankaya M, Hamurcu M & Gezgin S (2007). Response of chickpea cultivars to application of boron in boron-deficient calcareous soils. *Communications in Soil Science and Plant Analysis* 38(17-18): 2381-2399.

Ceyhan E, Onder M, Oztürk O, Harmankaya M, Hamurcu M & Gezgin S (2008). Effects of application boron on yields, yield component and oil content of sunflower in boron-deficient calcareous soils. *African Journal of Biotechnology* 7(16): 2854-2861.

Ciampiti I A & Vyn T J (2012). Physiological perspectives of changes over time in maize yield dependency on nitrogen uptake and associated nitrogen efficiencies: a review. *Field Crops Research* 133: 48-67. doi.org/10.1016/j.fcr.2012.03.008

Cimrin K M (2001). The Effect of Fertilization on N, P, K Content and Uptake of Sugar beet. *Yuzuncu Yil University*, *Journal Agriculture of Science* 11(1): 5-10.

Demiralay İ (1993). Soil Physical Analysis. *Ataturk University Faculty of Agriculture*.

Dikici H (2007). Effect of Different Response Models and Fertilizer-Crop Prices on Economic Optimum Nitrogen Levels. *KSU Journal of Science and Engineering* 10(2): 89-93.

Draycott A P (1993). In The Sugar Beet Crop: Science into Practice. *Nutrition* 239-278.

Durak A & Ulubas G (2017). The Effects of Boron Fertilization to Yield Properties in Sugar beet. *Turkish Journal Of Agriculture-Food Science and Technology* 5(7): 745-749. doi.org/10.24925/TURJAF.V5I7.745-749.1152

Ebmeyer H & Hoffmann C M (2021). Efficiency of nitrogen uptake and utilization in sugar beet genotypes. Field Crops Research 274: 108334. doi.org/10.1016/j.fcr.2021.108334

Fageria N K, Baligar V C & Li Y C (2008). The Role of Nutrient Efficient Plants in Improving Crop Yields in the Twenty First Century. *Journal of Plant Nutrition* 31(6): 1121-1157. doi.org/10.1080/01904160802160680

Fageria N K (2014). Nitrogen Harvest Index and its Association With Crop Yields. *Journal of Plant Nutrition* 37(6): 795-810. doi.org/10.1080/01904167.2014.881855

Fixen P, Brentrup F, Bruulsema T, Garca F, Nortons R & Zingore S (2014). Nutrient/Fertilizer Use Efficiency: Measurement, Current Situation and Trends. *Managing Water and Fertilizer for Sustainable Agricultural Intensification*.

Gee G W & Bouder J W (1986). Particle Size Analysis. Methods of Soil Analysis. Part I Agronomy, (9). *American Society of Agronomy.*

Good A G, Shrawat A K & Muench D G (2004). Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? *Trends in Plant Science* 9(12): 597-605. doi.org/10.1016/j.tplants.2004.10.008

Gucdemir IH (2006). Turkey Fertilizer and Fertilization Guide. *In Ministry of Agriculture and Rural Affairs* 5:231.

Haile D, Nigussie D & Ayana A (2012). Nitrogen use efficiency of bread wheat: Effects of nitrogen rate and time of application. *Journal of Soil Science and Plant Nutrition* 12(3): 389-410.

Harmankaya M, Onder M, Hamurcu M, Ceyhan E & Gezgin S (2008). Response of common bean (*Phaseolus vulgaris* L.) cultivars to foliar and soil applied boron in boron-deficient calcareous soils. *African Journal of Biotechnology* 7(18): 3275-3282.

Hellal F A, Taalab A S & Safaa, A M (2009). Influence of nitrogen and boron nutrition on nutrient balance and sugar beet yield grown in calcareous soil. *Ozean Journal of Applied Science* 2(1): 1-10.

Helmke P A & Sparks D L (1996). Lithium, Sodium, Potassium, Rubidium, and Calcium. Sparks D.L. Methods of Soil Analysis: Part 3. *Chemical Methods* 551-574.

Hirose T (2012). Leaf-level nitrogen use efficiency: definition and importance. *Oecologia* 169(3): 591-597. doi.org/10.1007/s00442-011-2223-6.

Hoffmann C M, Kenter C & Bloch D (2005). Marc concentration of sugar beet (Betavulgaris L.) in relation to sucrose storage. *Journal Science Food Agriculture* 85(3): 459-465. doi.org/10.1002/jsfa.2002

Hoffmann C M (2006). Physiological basis of the yield potential of sugar beet. *SugarInd* 131(12): 863-870.

Ilbas A I, Yildirim B, Arslan B & Gunel E (1996). A research on the examination of relations between nitrogen dosages and yield of sugar beet and determination of natural and economic optimum nitrogen dosage. *Yuzuncu Yil University Journal of Agricultural Sciences* 6(1): 97-113.

Jacobs A, Koch H J & Marlander B (2018). Preceding crops influence agronomic efficiency in sugar beet cultivation. *Agronomy for Sustainable Development* 38: 5. doi.org/10.1007/s13593-017-0469-z

Karam F, Mounzer O, Sarkis F & Lahoud R (2002). Yield and nitrogen recovery of lettuce under different irrigation regimes. *Journal of Applied Horticulture* 4(2): 70-76. doi.org/10.37855/jah.2002.v04i02.21

Kavas M F & Leblebici M J (2004). Quality and Operation Control Laboratories Handbook, Turkey Sugar Factories Inc. General Directorate, Ankara, pp.85-196.

Keeney D & Olson R (2009). Sources of nitrate to ground water. *Critical Reviews Environmental* 16(3): 257-304. doi.org/10.1080/10643388609381748

Laufer D, Nielsen P O, Wilting H, Koch J & Marlander B (2016). Yield and nitrogen use efficiency of fodder and sugar beet (Beta vulgaris L.) in contrasting environments of northwestern Europe. *European Journal of Agronomy* 73: 124-132. doi.org/10.1016/j.eja.2015.11.008

Lindsay W L & Norvell W A (1978). Development of a DTPA Soil Test for Zinc, Iron, Manganese and Copper. *Soil Science Society of America Journal* 42(3): 421-428. doi.org/10.2136/sssaj1978.03615995004200030009x

Lopez-Bellido L, Castillo J E & Fuentes M (1994). Nitrogen uptake by autumn sown sugar beet. *Fertilizer Research* 38: 101-109. doi.org/10.1007/BF00748770

Malnou C S, Jaggard K W & Sparkes D L (2006). A canopy approach to nitrogen fertilizer recommendations for the sugar beet crop. *European Journal of Agronomy* 25(3): 254-263. doi.org/10.1016/j.eja.2006.06.002

Malnou C S, Jaggard K W & Sparkes D L (2008). Nitrogen fertilizer and the efficiency of the sugar beet crop in late summer. *European Journal of Agronomy* 28(1): 47-56. doi.org/10.1016/j.eja.2007.05.001

Mampa S S, Maboko M M, Soundy P & Sivakumar D (2017). Nitrogen application and leaf harvesting improves yield and nutritional quality of beetroot. *HortTechnology* 27(3): 337-343. doi.org/10.21273/horttech03599-16

Marlander B, Hoffmann C, Koch H J, Ladewig E, Merkes R, Petersen J & Stockfisch N (2003). Environmental Situation and Yield Performance of the Sugar Beet Crop in Germany:Heading for Sustainable Development. *Journal of Agronomy and Crop Science* 189(4): 201-226. doi.org/10.1046/j.1439-037X.2003.00035.x

Marschner P (2012). Marschner's Mineral Nutrition of Higher Plants. *Academic Press 672.*

McDonnell P M, Gallagher P A, Kearney P & Carroll P (1966). Fertilizer use and sugar beet quality in Ireland. In: Potassium Symposium 1966, *International Potash Institute* 10: 107-126.

Mekdad A A A (2015). Sugar Beet Productivity as Affected by Nitrogen Fertilizer and Foliar Spraying with Boron. *International Journal of Current Microbiology and Applied Sciences* 4(4): 181-196.

Milford G F & Watson D J (1971). The Effect of Nitrogen on the Growth and Sugar Content of Sugar Beet. *Annals of Botany* 35(2): 287-300. doi.org/10.1093/oxfordjournals.aob.a084478

Moll R H, Kamprath E J & Jackson W A (1982). Analysis and Interpretation of Factors Which Contribute to Efficiency of Nitrogen Utilization. *Agronomy Journal* 74(3): 562-564. doi.org/10.2134/agronj1982.00021962007400030037x

Neeteson J J & Wadman W P (1987). Assessment of economically optimum application rates of fertilizer N on the basis of response curves. *Fertilizer Research* 12: 37-52.

Nelson D W, Sommers L E, Madison W I & Series B (1996). Total carbon, organic carbon and organic matter. *Methods of Soil Analysis* 3: 961-1010. doi.org/10.2134/agronmonogr9.2.2ed.c29

Noshad H, Abdollahian Noghabi M & Babaee B (2012). Effect of Nitrogen and Phosphorous Application on the Efficiency of Nitrogen Uptake and Consumption in Sugar Beet (Beta vulgaris L.). *Iranian Journal of Field Crop Science* 43(3): 529-539. doi.org/10.22059/ijfcs.2012.29049

Olsen S R & Sommers E L (1982). Phosphorus Soluble in Sodium Bicarbonate, Methods of Soil Analysis, Part 2, *Chemical and Microbiological Properties* 404-430.

Pooniya V, Shivay Y S, Madan P & Bansal R (2018). Relative Performance of Boron, Sulphur and Zinc Coatings onto Prilled Urea For Increasing Productivity and Nitrogen Use Efficiency In Maize. *Experimental Agriculture* 54(4): 577-591. doi.org/10.1017/S0014479717000254

Porker K, Straight M & Hunt J R (2020). Evaluation of G × E × M Interactions to Increase Harvest Index and Yield of Early Sown Wheat. *Sec. Crop and Product Physiology* 11: 14. doi.org/10.3389/fpls.2020.00994

Rezvani S M, Noruzi, A, Azari K & Jafari A M (2013). Determination of an appropriate model for optimum use of N fertilizer in furrow irrigation. *Journal of Sugar beet* 29(1): 53-69.

Rhoades J D (1996). Salinity: Electrical Conductivity and Total Dissolved Solids. *Methods of Soil Analysis* 417-435.

Saglam T M (2012). Soil Chemistry. Namık Kemal University Faculty of Agriculture. Tekirdag, pp.238

SASS (1999). Sas user's guide: Statistic. statistical anal. systems institute inc.

Sayili M & Akca H (2004). Comparison of different models for describing sugar beet yield response to nitrogen fertilizer. *Asian Journal of Plant Science* 3(4): 529-531. doi.org/10.3923/ajps.2004.529.531

Shivay Y S, Prasad R, Pooniya V, Pal M & Bansal R (2017). Response of spring wheat to boron coated urea and its effect on nitrogen use efficiency. *Journal of Plant Nutrition* 40(13): 1920-1927. doi.org/10.1080/01904167.2016.1270313

Smil V (2011). Nitrogen cycle and world food production. *Word agriculture scientific* 2(1): 9-13.

Stevens W B, Violett R D, Skalsky S A & Mesbah A O (2008). Response of eight sugar beet varieties to in- creasing nitrogen application: I. Root, sucrose, and top yield. *Journal of American Society of Sugar Beet Technologist* 45(3-4): 65-83.

Sulfab H A, Bakhit B E M I, Hagalla E, Karima A, Asha I & Marajan W A (2017). Effect of Nitrogen Fertilizer on Growth, Yield and Sucrose Concentration of Sugar beet (Beta vulgaris L.) Under Saline Soil in El Kadaro, Sudan. *Journal of Agriculture and Research* 3(12): 5-19.

Tsialtas J T & Maslaris N (2005). Effect of N fertilization rate on sugar yield and non-sugar impurities of sugar beets (Beta vulgaris) grown under Mediterranean conditions. *Journal of Agronomy and Crop Science* 191: 330-339. doi.org/10.1111/j.1439-037X.2005.00161.x

Varga I, Jovic J, Rastija M, Kulundzinc A M, Zebec V, Loncaric Z, Iljkic D & Antunovic M (2022). Efficiency and Management of Nitrogen Fertilization in Sugar Beet as Spring Crop: A. Review. *Nitrogen* 3(2): 170-185. doi.org/10.3390/nitrogen3020013

Wang Y, Zhao Z, Wang S, Shi L & Xu F (2021). Genotypic differences in the synergistic effect of nitrogen and boron on the seed yield and nitrogen use efficiency of Brassica napus. *Journal of the Science of Food and Agriculture* 102(9): 3563-3571. doi.org/10.1002/jsfa.11700

Yilmaz G F (2015). Determination of Relations Among Nitrogen use Efficiencies and Yield and Quality Properties of Some Bread Wheat Grown ın the Central Anatolia Region. Konya

Zhang X, Davidson E A, Mauzerall D L, Searchinger T D, Dumas P & Shen Y (2015). Managing nitrogen for sustainable development. *Nature* 528: 51-59. doi.org/10.1038/nature15743

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Effects of Different Types of Irrigation Water Quality and Silicon Doses on Fruit Yield, Chlorophyll and Carotenoid Contents of Tomato (*Lycopersicon esculentum L.*) under Soilless Culture Technique

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ABSTRACT

This study was conducted to determine the effects of different irrigation water quality and silicon doses on leaf soil plant analysis development meter readings, chlorophyll content and carotenoid contents of tomato plants. Tybiff Aq tomato seedlings were grown in 3-liter pots filled with 1100 g of 1:1 peat-perlite mixture for 70 days. Four different types of irrigation water quality were prepared with the use of sea and tap water. Irrigation waters included i) Full seawater, ii) ½ seawater + ½ tap water, iii) ¼ seawater + ¾ tap water, iv) full tap water (control). Each irrigation water was supplemented with silica gel ($SiO_2.x\ H_2O$) at 0, 0.5, 1 and 2 mM Si doses. Nutrient solutions were supplied to meet macro and micronutrient requirements of tomato plants. Leaf chlorophyll-a, chlorophyll-b and total chlorophyll contents significantly increased with increasing tap water ratios of the irrigation water. Significant increases were observed in chlorophyll-a, chlorophyll-b and total chlorophyll contents with increasing silicon doses. Such increases achieved with silicon treatments were more remarkable for chlorophyll-a and total chlorophyll contents. Leaf chlorophyll-a, chlorophyll-b and total chlorophyll contents significantly decreased with increasing leaf sodium, chlorine and magnesium contents, but significantly increased with increasing leaf active iron and potassium contents. Leaf chlorophyll-a, chlorophyll-b and total chlorophyll contents increased with increasing leaf calcium contents, but such increases were not significant. Leaf carotenoid contents significantly increased with increasing tap water ratios of the irrigation water. Effects of silicon doses on leaf carotenoid contents varied with the type of irrigation water. The 0.5 mM silicon supplementation into tap water significantly increased carotenoid contents.

Keywords: Tomato, Seawater, Tap water, Silicon, Chlorophyll, Carotenoid

1. Introduction

Since 1966 the use of seawater for agriculture was often studied. Despite intensive research and projects, only few organisms have been found, which can be grown with seawater: some mangrove trees and shrimps. Even today there is still no considerable use of seawater irrigation. Some halophytic vascular plants, however, can fulfil their whole lifecycle with seawater. But they also grow better on half seawater concentration. In many thousands of other projects (with many cash crops) the use of only 10-20% seawater concentration has been tried. But even this concentration is often too high and spoils the soils in their structure, especially if not an efficient leaching is applied. A sustainable agriculture based on irrigation with seawater on a large scale seems to be still a utopic illusion (Breckle 2009).

Alternative water sources for irrigation can represent a valid help for the preservation of the already overexploited freshwater. In particular, seawater is considered a realistic option in agriculture, either desalinized or blended with freshwater (Yermiyahu et al. 2007). Even if in recent years numerous large-scale seawater desalination plants have been built, such a technique is anyhow very energivourous and thus presents environmental concerns (Elimelech & Phillip 2011). Besides, desalination cost has decreased because of technical improvements in a world of increasing fossil fuel prices (Karagiannis & Soldatos 2008). Nevertheless, in developing countries, often characterized by water shortage, desalination has been generally excluded because of the economic conditions (Wade

2001). A different option is to use seawater as a complementary irrigation source at concentrations not harmful for the cultivated crops. Seawater is the most abundant source of water of the planet and its specific composition represents a well balanced ionic environment for plants (Boyko 1966). In fact, despite its very high chloride content (about 75% NaCl and 10% of $MgCl_2$), seawater is rich in all nutritive elements needed by plants,

Soilless systems offer an important alternative to soil cultivation in case of soil and/or water issues, as for example, among the most important, water shortage and salinization (Olympios 1999). The three main soilless systems are liquid hydroponics, solid media culture and aeroponics. Hydroponics is further categorized in open or closed systems, depending on the collection and reuse (i.e. in closed systems) of the nutrient solution until its depletion. Solid media culture systems can in the same way be open or closed and several substrates are used for plants anchorage (i.e. perlite, vermiculite, coconut coir), as long as characterized by water and air holding capacity and by an easy drainage. Aeroponics, in the end, enables the maximum utilization of space by growing plants with roots suspended in air sprayed every 2-3 minutes, plants getting nutrients and water from the solution film that adheres on roots (Hussain et al. 2014). This diversity of techniques makes soilless culture adaptable to very dissimilar situations, with the common potential application in providing food in areas characterized by soil and water availability issues (Sheikh 2006).

Net photosynthesis is an indicator of biomass production and resultant growth. Thus, environmental stress factors influencing plant growth and development also influence photosynthesis. Therefore, the changes in plant photosynthetic activity under stress conditions somehow reveal information about general health of the plants. Such changes in photosynthetic activity are considered to be a sensor of stress in plants, algae and cyanobacteria (Biswal et al. 2011). Stress factors, including soil salinity, play a great role in photosynthetic pigment quantity, light absorbance of these pigments and resultant primary photochemical reactions, structural organization of thylakoid membranes and units, electron transport and rate of CO_2 fixation reactions (Mittal et al. 2012). For more than a century, researchers have been working on effects of salts around the roots. Salinity has osmotic effects on plants and toxic effects on plant nutrition (Levitt 1980). Tomato plants are moderately sensitive to salinity (Maas & Hoffman 1977). Besides, Alian et al. (2000) indicated significant differences in salt tolerance of tomato cultivars. In soilless culture, salinity of the root zone could be modified through changing nutrient solution composition or alteration of irrigation frequency and it was reported that tomato could tolerate salt concentrations of root zone of between 2.5-2.9 dS m^{-1} without any losses in yields (Sonneveld & Van der Burg 1991). The salt levels of growing environment vary based on sensitivity of the cultivars and environmental conditions (Li et al. 2001). Irrigation water to be used in soilless culture should have an EC value of less than 0.5 dS m^{-1}, sodium concentration of less than 35 ppm, chlorine concentration of less than 50 ppm, bicarbonate concentration of less than 250 ppm and boron concentration of less than 0.5 ppm. Irrigation water pH values should be between 5.0 and 7.0 (Gül 2012). Negative impacts of excessive salt levels on plant growth and development could be summarized as 1) Reduction in plant water uptake (water stress), 2) Inhibition of uptake of some nutrients, 3) Specific ion effect (salt stress) (Marschner 1995).

Zhu & Gong (2014) reported that silicon treatments increased water uptake of roots, reduced water loss of leaves, provided nutrient balance and improved photosynthesis rates. It was also reported that silicon treatments increased antioxidant enzyme activity, non-antioxidant enzyme contents, thus prevented plants from oxidizing effect of salt, provided contributions to osmatic regulation, thus increased activity of photosynthetic enzymes. Researchers also indicated that silicon treatments reduced sodium accumulation in roots and shoots.

Coşkun et al. (2016) indicated that although silicon is not an essential element for plants, it provides various contributions to plant growth and development under stress conditions like salinity and drought. Silicon provides suberization, lignification and silicification in cell wall, and then reduce transpiration of water and salt-induced oxidative damage. Recent studies have revealed that Si addition under salinity stress can increase water content in plants through increasing root water absorption (Zhu et al. 2015; Wang et al. 2015). In tomato, Li et al. (2015) found that Si promoted root growth and root hydraulic conductance, thereby increasing root water uptake and further improving leaf water content.

Cell wall is the outer most layer of the plant cells and composed of polysaccarite and polymer secretions of the cells. Cell wall is a supporting cover with a primary functions to regulate cell volume and designate cell shape. Under salt stress conditions, Na^+ is accumulated at high concentrations in apoplast. The accumulated Na^+ distords ionic bondings of sturcutural members like pectine in cell wall or negatively incluences apoplastic enzymes, thus prenvents cell wall from performing basic functions (Rengel 1992).

Another harmfull effect of salt stress in on cell membrane. Cell membrane is a semi-permiable memberane composed of double phopholite layers and proteins embedded into this layer. Salt stress triggers the change in lypid composition of cell membrane and results in cell membrane damage. The changes in lypid composition are resulted from the changes in activity of enzymes participating into lypid synthesis, degredations (disintegration, destraction) or hydrolysis of phospholypids (Huang 2006).

Besides reductions in water potential, NaCl also impairs ion balance of the cell, thus negatively incluence plant growth and development. High NaCl uptakes increase cell Na^+ and Cl^- levels and reduce Ca^{+2}, K^+ and Mg^{+2} concentrations (Parida & Das 2005). The Na^+ intrusion into cell destructs membrane potential and facilitate passive intrusion of Cl^- into the cell through ion cannels (Niu et al. 1995; Tuteja 2007).

This study was conducted to determine the effects of different irrigation water quality and silicon doses on leaf soil plant analysis development (SPAD) meter readings, chlorophyll content and carotenoid contents of tomato plants.

2. Material and Methods

The experiment was carried *out* in a greenhouse environment. Plastic pots of 3 liters were filled with 1100 g substrate (1:1 peat:perlite mixture) materials. The particle diameter of the perlite used in the experiment varies between 0-6 mm. Its pH is 7.0 and its volume weight is 80-90 kg/m^3, whereas peat is uniformly brown, strongly to almost completely decomposed (H7-H9), and moderately acidic (pH 5.5).

Tybiff Aq tomato seedlings were planted into the pots as to have one seedling per pot. Four different types of irrigation water qualities were applied. Irrigation water quality included: i) Full seawater, ii) ½ seawater + ½ tap water, iii) ¼ seawater + ¾ tap water, iv) Full tap water (control).

Irrigation waters were supplemented with silica gel (SiO_2 xH_2O) (0, 0.5, 1 and 2 mM Si). Experiments were conducted in randomized plots 4×4 factorial design with 3 replications. From planting to harvest (70 days), following nutrient solutions were applied to tomato plants as recommended by Alpaslan et al. (1998):

1.25 mM KH_2PO_4; 15 µM Fe (FeEDDHA); 4.25 mM Ca $(NO_3)_2 \cdot 4H_2O$; 10 µM Mn ($MnCl_2$); 1.25 mM NH_4NO_3; 5 µM Zn ($ZnSO_4 \cdot 7H_2O$); 4.0 mM KNO_3; 30 µM B (H_3BO_3); 2.0 mM $MgSO_4.7H_2O$; 0.75 µM Cu ($CuSO_4 \cdot 5H_2O$); 1.75 mM K_2SO_4;

0.5 µM Mo $[(NH_4)_6Mo_7O_{24}4H_2O]$

Soilless growing mediums included a mixture of peat and perlite (1:1) in pots and were brought to the field capacity with 4 different irrigation applications according to the experimental subjects. Tomato seedlings were planted in the solid growing media brought to the field capacity. Growing medium have a high water-holding capacity (135%) to supply water to seedlings.

From planting to the first fruit set, 150 mL nutrient solution and 300 mL irrigation water was applied in each day; from the first fruit set to the harvest, 300 mL nutrient solution and 600 mL irrigation water was applied in each day. Following the application of irrigation water and nutrient solution, experimental pots were freely drained through the holes provided at the bottom of each pot.

2.1. Leaf analysis

Leaf potassium, calcium, magnesium, sodium and chlorine contents of tomato plants were determined in accordance with Kacar & İnal (2008). Fresh leaf samples were used to determine chlorophyll-a, chlorophyll-b and carotenoid contents in accordance with Arnon (1946). and Withan et al. (1971).

SPAD meter readings were taken from mid-point of the leaves with the use of portable SPAD meter device (Konica Minolta SPAD-502 Plus). Active iron contents were determined in dry leaf samples with the use of an AAS device (Oserkowsky 1933).

2.2. Irrigation water analysis

The pH, EC, SAR values, carbonate, bicarbonate, chlorine, calcium, magnesium, sodium contents (Sağlam 2008); sulphate (Kacar 1994); boron (Bayraklı 1987) contents of different irrigation waters were determined. SAR values were calculated with the use of the following equation:

$$SAR = \frac{Na^+}{\sqrt{\frac{Ca^{+2} + Mg^{+2}}{2}}}$$

where; Na^+, me L^{-1}, Ca^{+2}+Mg^{+2}, me L^{-1}

Irrigation water analysis results are provided in Table 1.

Table 1- Chemical properties of irrigation waters prepared from sea and tap water

Irrigation water parameter	*Full seawater*	*½ seawater + ½ tap water*	*¼ seawater + ¾ tap water*	*Full tap water*
pH	8.05	8.20	8.0	7.70
$EC_{25\,^{o}C}$, dS m^{-1}	62.40	37.20	18.70	0.70
$CO_3^{=}$, me L^{-1}	0.46	0.25	0.03	0.00
HCO_3^{-}, me L^{-1}	6.13	5.12	4.69	3.56
Cl^{-}, me L^{-1}	316.40	168.10	89.20	8.70
$SO_4^{=}$, me L^{-1}	6.40	7.30	4.80	0.80
Ca^{++}, me L^{-1}	10.90	7.25	5.40	3.40
Mg^{++}, me L^{-1}	62.14	36.59	20.10	2.32
Na^{+}, me L^{-1}	220.70	89.80	43.05	0.75
B, mg L^{-1}	1.42	1.07	0.91	0.71
SAR	36.54	19.20	12.10	0.44

2.3. Statistical analysis

Experimental data were subjected to variance analysis (ANOVA) in accordance with randomized plots 4×4 factorial experimental design with the use of SPSS 17.1 software. Significant means were compared with the use of Duncan's test at $p<0.05$ level. Correlation analysis was conducted to identify the relationships of some leaf nutrients with chlorophyll and carotenoid contents.

3. Results and Discussion

3.1. Evaluation of the agricultural suitability of irrigation waters

Entire parameters of full seawater, ½ seawater + ½ tap water and ¼ seawater + ¾ tap water were not suitable for irrigations to be conducted in soilless culture and field farming. On the other hand, entire parameters of tap water were ideal for irrigations (Sağlam 2008).

3.2. Effects of different irrigation waters and silicon doses on tomato fruit yield

Effects of different irrigation waters and silicon doses on tomato fruit yield are given in Table 2.

Table 2- Effects of different irrigation waters and silicon doses on tomato fruit yield

Parameters	*Irrigation waters*	*Si doses, mM*				*Average*
		0.0	*0.5*	*1.0*	*2.0*	
Fresh fruit yield, g $plant^{-1}$	Seawater	289.67	362.68	254.10	225.87	283.08D
	½ seawater + ½ tap water	618.15	660.35	674.30	692.43	661.31C
	¼ seawater + ¾ tap water	1181.43	1203.33	1078.87	1042.47	1126.53B
	Tap water	2051.40	2226.05	1971.77	2115.17	2091.10A
	Average	1035.16	1113.11	994.76	1018.98	

As can be inferred from Table 2, effects of irrigation waters on fruit yield were found to be significant at $p<0.01$ level, but the effects on silicon doses were not found to be significant.

Fruit yields significantly decreased with increasing seawater ratio of the irrigation water. While tap water treatments had an average fruit yield of 2091.05 g $plant^{-1}$, fruit yield per plant decreased to 1126.11 g $plant^{-1}$ with ¼ seawater-containing irrigation water, to 661.30 g $plant^{-1}$ with ½ seawater-containing irrigation water and to 283.08 g $plant^{-1}$ with full seawater. The correlations between seawater ratio of the irrigation water and yield loss revealed that 20% yield loss was seen at 6.5% seawater ratio, 40% yield loss was seen at 25.2% seawater ratio, 50% yield loss was seen at 43.45% seawater ratio, 80% yield loss was seen at 80.49% seawater ratio and 86.51% yield loss was seen at 100% seawater ratio (full seawater). Kahlaoui et al. (2011) indicated that tomato fruit yields were badly influenced when 70% of plant water need was met with saline irrigation waters.

3.3. Effects of irrigation water quality and silicon doses on fruit yield, chlorophyll and carotenoid contents of tomato plant

Effects of irrigation water treatments and silicon doses on leaf SPAD meter readings, chlorophyll-a, chlorophyll-b and total chlorophyll contents of tomato plants and variance analysis results for these effects are respectively provided in Table 3 and Table 4.

Table 3- Effects of irrigation water treatments and silicon doses on leaf SPAD meter readings, chlorophyll-a, chlorophyll-b and total chlorophyll contents of tomato plants

Parameters	*Irrigation water*	*Si doses, mM*				*Average*
		0.0	*0.5*	*1.0*	*2.0*	
SPAD meter reading	Seawater	48.81d-h	38.24h	44.13f-h	45.35e	**44.13C**
	½ seawater + ½ tap water	73.57ab	77.83a	63.17b-d	70.74a	**71.20A**
	¼ seawater + ¾ tap water	82.73a	53.18d-g	71.70ab	70.20a	**69.45A**
	Tap water	55.90c-f	59.53b-e	40.13gh	61.97b	**54.38B**
	Average	**65.25A**	**57.19BC**	**54.78C**	**61.94AB**	
Chlorophyll-a, mg g^{-1} FW	Seawater	0.98	0.46	0.84	1.09	**0.84B**
	½ seawater + ½ tap water	0.89	0.79	0.88	0.85	**0.85B**
	¼ seawater + ¾ tap water	1.16	1.17	1.29	1.48	**1.28A**
	Tap water	1.14	1.30	1.56	1.54	**1.39A**
	Average	**1.04BC**	**0.93C**	**1.14AB**	**1.24A**	
Chlorophyll-b, mg g^{-1} FW	Seawater	0.41	0.20	0.34	0.43	**0.35C**
	½ seawater + ½ tap water	0.35	0.32	0.39	0.36	**0.36C**
	¼ seawater + ¾ tap water	0.49	0.46	0.51	0.58	**0.51B**
	Tap water	0.51	0.55	0.70	0.62	**0.59A**
	Average	**0.44AB**	**0.38B**	**0.49A**	**0.50A**	
Total chlorophyll, mg g^{-1} FW	Seawater	1.39	0.66	1.19	1.52	**1.19B**
	½ seawater + ½ tap water	1.23	1.12	1.27	1.22	**1.21B**
	¼ seawater + ¾ tap water	1.66	1.63	1.81	2.06	**1.79A**
	Tap water	1.65	1.85	2.26	2.17	**1.98A**
	Average	**1.48BC**	**1.31C**	**1.63AB**	**1.74A**	

FW: fresh weight

Table 4- Variance analysis results for the effects of irrigation water treatments and silicon doses on leaf SPAD meter readings, chlorophyll-a, chlorophyll-b and total chlorophyll contents of tomato plants

Parameters	*Variation*							
	Irrigation water		*Silicon dose*		*Irrigation water x silicon dose*		*Error*	
	DF	*MS*	*DF*	*MS*	*DF*	*MS*	*DF*	*MS*
SPAD meter readings	3	1991.889**	3	265.063**	9	214.802**	32	61.105
Chlorophyll-a, mg g^{-1} FW	3	0.97**	3	0.22**	9	0.07	32	0.04
Chlorophyll-b, mg g^{-1} FW	3	0.177**	3	0.033*	9	0.010	32	0.008
Total chlorophyll, mg g^{-1} FW	3	1.97**	3	0.41**	9	0.13	32	0.08

*significant at p<0.05 level, **significant at p<0.01 level, FW: fresh weight

As can be inferred from Table 4, effects of irrigation waters, silicon doses and irrigation water × silicon dose interactions on leaf SPAD meter readings were found to be significant at p<0.01 level. Effects of irrigation waters on chlorophyll-a, chlorophyll-b and total chlorophyll contents were found to be significant at p<0.01 level and effects of silicon doses on the same parameters were respectively found to be significant at p<0.01, p<0.05 and p<0.01 levels. Effects of irrigation water × silicon dose interactions on leaf chlorophyll-a, chlorophyll-b and total chlorophyll contents were not found to be significant.

As compared to tap water, seawater supplementations into irrigation water significantly increased SPAD meter readings. Leaf SPAD meter reading was 44.13 in full seawater irrigations, 71.33 in ½ seawater + ½ tap water irrigations, 69.47 in ¼ seawater + ¾ tap water

irrigations and 53.64 in full tap water irrigations. Effects of silicon treatments on SPAD meter readings varied with the type of irrigation water (Figure 1).

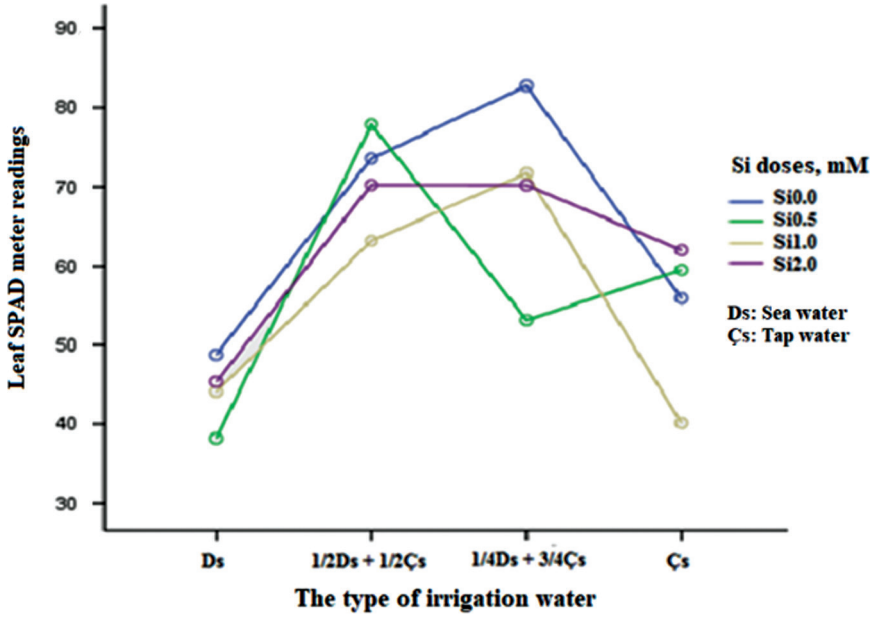

Figure 1- Effects of irrigation water × silicon dose interactions of leaf SPAD meter readings

Leaf chlorophyll-a, chlorophyll-b and total chlorophyll contents increased significantly with increasing tap water ratios of the irrigation water. While chlorophyll-a, chlorophyll-b and total chlorophyll contents were respectively measured as 0.84, 0.35 and 1.19 mg g^{-1} fresh weight (FW) in full seawater irrigations, the values were respectively measured as 0.85, 0.36 and 1.21 mg g^{-1} FW in ½ seawater + ½ tap water irrigations, as 1.27, 0.51 and 1.79 mg g^{-1} FW in ¼ seawater + ¾ tap water irrigations and as 1.39, 0.59 and 1.98 mg g^{-1} FW in full tap water irrigations (Table 3).

Leaf chlorophyll-a, chlorophyll-b and total chlorophyll contents increased significantly with increasing silicon doses. Such increases achieved with silicon treatments were more remarkable for chlorophyll-a and total chlorophyll contents (Table 3). On the other hand, effects of silicon doses on chlorophyll-a, chlorophyll-b and total chlorophyll contents were similar in different irrigation waters.

Photosynthetic pigment quantity of the plants generally decreases under salt stress. Agastian et al. (2000) indicated that salt treatments result in chlorosis in old leaves at early stage and in case of prolonged stress durations, these leaves were subjected to abscission. However, Wang & Nil (2000) reported increased chlorophyll content of Amaranthus plants subjected to salt stress. In previous studies, as compared to the control plants, decreased total chlorophyll and protochlorophyllide contents were reported in *Greviela arenaria*, total chlorophyll and chlorophyll-a contents in tomato, chlorophyll-a and chlorophyll-b contents in *Bruguiera parviflora* and total chlorophyll content in paddy (Kennedy & Fillippis 1999; Khavarinejad & Mostofi 1998; Alamgir & Ali 1999). Chutipaijit et al. (2011) asserted that the changes encountered in chlorophyll contents of plants under salt stress could be used as a sensitive indicator for cellular metabolisms. Maxwell & Johnson (2000) indicated changes in photosynthetic pigment biosynthesis as an apparent effect of salt stress on plants.

Tavakkoli et al. (2016) investigated the effects of alkalinity stress generated through the use of alkaline irrigation waters and growing media on development and physiological traits of gerbera. Researchers indicated that under irrigation water-induced alkalinity stress conditions, with increasing sodium carbonate levels from 0 to 40 mM, significant decreases were seen in plant growth and development, glutamine synthetase activity, leaf relative water content, chlorophyll-a, chlorophyll-b and total chlorophyll contents and carotenoid contents. Alkalinity stress-induced decreases in vegetative development, leaf relative water content, glutamine synthetase enzyme activity and photosynthetic pigment quantity were lower in coconut fiber media than the other growth media. Researchers pointed out that alkalinity stress resulted from high sodium carbonate of irrigation water could be eliminated with the use of proper substrate materials.

Dannon & Wydra (2004) reported that silicon supplementation into nutrient solution of tomato plants grown in hydroponic culture reduced the incidence of *Ralstonia solanacearum*-induced bacterial wilt disease. It was reported that silicon treatments reduced the

harmful effects of NaCl salinity on growth and development of tomato plants (Stamatakis et al. 2003). Stimulating effects of silicon treatments on growth and development of tomato plants subjected to NaCl salt reduced sodium and chlorine uptakes (Stamatakis et al. 2003), improved water status of the plant (Romero-Aranda et al. 2006) and increased superoxide dismutase and catalase enzyme activities. Thusly, it was indicated that superoxide dismutase and catalase enzyme activities prevented plant tissues from oxidative damage of the salt (Al-Aghabary et al. 2004). Silicon was also reported to increase net photosynthesis rate of tomato plants exposed to NaCl. Such an effect of silicon was attributed to increased leaf chlorophyll contents and phytochemical efficiency of Photosystem II (Romero-Aranda et al. 2006).

3.4. Effects of irrigation treatments and silicon doses on leaf carotenoid contents of tomato plants

Effects of different irrigation waters and silicon doses on leaf carotenoid contents of tomato plants and variance analysis results for these effects are respectively provided in Table 5 and Table 6.

Table 5- Effects of different irrigation waters and silicon doses on leaf carotenoid contents of tomato plants

Parameter	*Irrigation water*	*Si doses, mM*				*Average*
		0.0	*0.5*	*1.0*	*2.0*	
Carotenoid, mg g^{-1} Fresh weight	Seawater	0.159cd	0.095d	0.146cd	0.185b-d	0.146C
	½ seawater + ½ tap water	0.147cd	0.157cd	0.146cd	0.146cd	0.149C
	¼ seawater + ¾ tap water	0.206bc	0.194b-d	0.208bc	0.243bc	0.213B
	Tap water	0.201bc	0.394a	0.241bc	0.262b	0.274A
	Average	0.178	0.209	0.185	0.209	

Table 6- Variance analysis results for the effects of different irrigation waters and silicon doses on leaf carotenoid contents of tomato plants

Parameter	*Variation*							
	Irrigation water		*Silicon doses*		*Irrigation water x silicon doses*		*Error*	
	DF	*MS*	*DF*	*MS*	*DF*	*MS*	*DF*	*MS*
Carotenoid, mg g^{-1} Fresh weight	3	0.044**	3	0.003	9	0.008**	32	0.003

*Significant at $p<0.05$ **Significant at $p<0.01$

Effects of irrigation water and irrigation water × silicon doses interactions on leaf carotenoid contents were found to be significant at $p<0.01$ level, but the effects of silicon doses were not found to be significant (Table 6).

Leaf carotenoid contents significantly increased with increasing tap water ratios of the irrigation water. Leaf carotenoid content was identified as 0.146 mg g^{-1} FW in full seawater irrigations, as 0.149 mg g^{-1} FW in ½ seawater + ½ tap water irrigations, as 0.213 mg g^{-1} FW in ¼ seawater + ¾ tap water irrigations and as 0.274 mg g^{-1} FW in full tap water irrigations (Table 5).

Effects of silicon doses on leaf carotenoid contents varied with the type of irrigation water. The 0.5 mM silicon supplementation into tap water significantly increased carotenoid contents. The greatest carotenoid content (0.394 mg g^{-1} FW) was obtained from 0.5 mM dose of full tap water irrigations. Effects of silicon supplementations on carotenoid contents were not found to be significant in the other irrigation waters (Figure 2).

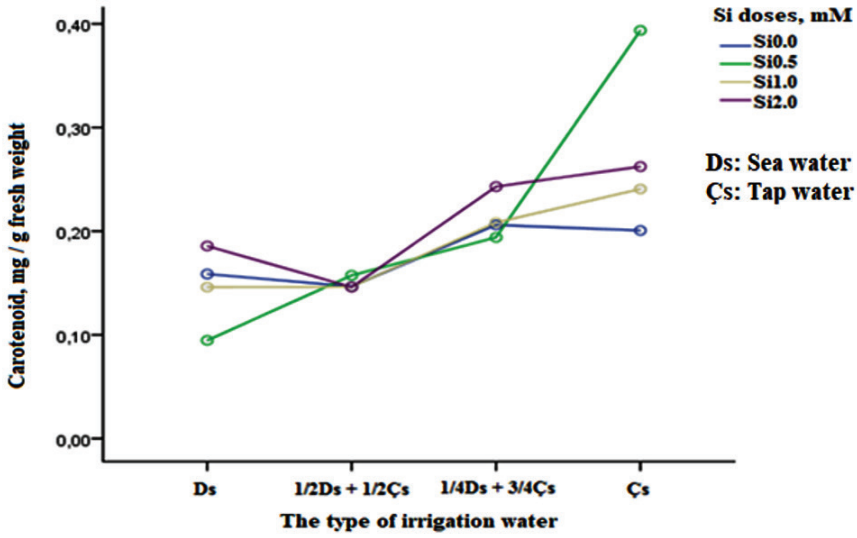

Figure 2- Effects of irrigation water and silicon doses on leaf carotenoid contents of tomato plants

It was reported that salt stress reduced quantity of photosynthetic pigments (chlorophyll and carotenoid) in light harvesting complexes (LHC) of photosynthesis systems (Parida & Das, 2005).

3.5. Correlation coefficients of the relation between leaf SPAD values, chlorophyll-a, chlorophyll-b, total chlorophyll, carotenoid contents and leaf nutrients

The correlation coefficients of the relations between leaf SPAD values, chlorophyll and carotenoid contents in the leaf and some element contents in the leaf are given in the Table 7.

Table 7- The correlation coefficients of the relations between chlorophyll and carotenoid contents in the leaf and some element contents in the leaf

Chlorophyll and carotenoid content in the leaf	*Correlation coefficients (r)*					
	Some element contents in the leaf					
	Na	*Cl*	*Ca*	*Mg*	*K*	*Active Fe*
Chlorophyll-a	-0.681**	-0.734**	0.477	-0.574*	0.602*	0.761**
Chlorophyll-b	-0.701**	-0.761**	0.469	-0.597*	0.641**	0.814
Total chlorophyll	-0.690**	-0.744**	0.476	-0.584*	0.620*	0.778
SPAD values	-0.489	-0,285				
Carotenoid	-0.595*			-0.498		0.541*

*significant at p<0.05, **significant at p<0.01

Correlations of SPAD meter readings with leaf sodium and chlorine contents were also not found to be significant (r=-0.489 and r=-0.285).

Leaf active iron contents had significant positive correlations with chlorophyll-a, chlorophyll-b and total chlorophyll contents (r=0.761**, r=0.814** and 0.778**, respectively). In other words, chlorophyll-a, chlorophyll-b and total chlorophyll contents significantly increased with increasing active iron contents of the leaves. On the other hand, leaf sodium contents had significant negative correlations with chlorophyll-a, chlorophyll-b and total chlorophyll contents (r=-0.681**, r=-0.701** and r=-0.690**). In other words, chlorophyll-a, chlorophyll-b and total chlorophyll contents significantly decreased with increasing leaf sodium contents.

Leaf magnesium contents had significant negative correlations with chlorophyll-a, chlorophyll-b and total chlorophyll contents (r=-0.574*, r=-0.597* and r=-0.584*, respectively) indicating significantly decreasing chlorophyll-a, chlorophyll-b and total chlorophyll contents with increasing leaf magnesium contents.

Leaf chlorine contents had significant negative correlations with chlorophyll-a, chlorophyll-b and total chlorophyll contents ($r=-0.734^{**}$, $r=-0.761^{**}$ and $r=-0.744^{**}$) also indicating significantly decreasing chlorophyll-a, chlorophyll-b and total chlorophyll contents with increasing leaf chlorine contents.

Leaf potassium contents had significant positive correlations with chlorophyll-a, chlorophyll-b and total chlorophyll contents ($r=0.602^{*}$, $r=0.641^{**}$ and $r=0.620^{*}$, respectively). In other words, chlorophyll-a, chlorophyll-b and total chlorophyll contents significantly increased with increasing leaf potassium contents. On the other hand, leaf calcium contents had insignificant positive correlations with chlorophyll-a, chlorophyll-b and total chlorophyll contents ($r=0.477$, $r=0.469$ and $r=0.476$, respectively). In other words, chlorophyll-a, chlorophyll-b and total chlorophyll contents increased with increasing leaf calcium contents. Salt stress significantly affects morphology, physiology and fruit weight of tomato. Salinity also adversely affects the shoot dry weight, leaf area, leaf chlorophyll content and also fruit weight/plant mostly at 8 dS m^{-1}. Exogenous application of Ca^{2+} significantly mitigates the adverse effects of salinity on plant biomass production or morphology, physiology and fruit production. The plant height, leaf number/plant, branch number/plant, dry weight of shoot/plant, leaf chlorophyll content, fruit weight/plant were increased with the application of calcium in saline condition compared to without calcium (Parvin & Haque 2015).

There was a significant positive correlations between leaf active iron content and carotenoid content ($r=0.541^{*}$), in other words, carotenoid contents significantly increased with increasing leaf active iron content. Correlations coefficients for correlations of leaf carotenoid contents with leaf sodium and magnesium contents were respectively identified as $r=-0.595^{*}$ and $r=-0.498$.

4. Conclusion

Leaf chlorophyll-a, chlorophyll-b and total chlorophyll contents significantly increased with increasing tap water ratios of the irrigation water. Significant increases were observed in chlorophyll-a, chlorophyll-b and total chlorophyll contents with increasing silicon doses. Such increases achieved with silicon treatments were more remarkable for chlorophyll-a and total chlorophyll contents.

Leaf chlorophyll-a, chlorophyll-b and total chlorophyll contents significantly decreased with increasing leaf sodium, chlorine and magnesium contents, but significantly increased with increasing leaf active iron and potassium contents. Leaf chlorophyll-a, chlorophyll-b and total chlorophyll contents increased with increasing leaf calcium contents, but such increases were not significant.

Leaf carotenoid contents significantly increased with increasing tap water ratios of the irrigation water. Effects of silicon doses on leaf carotenoid contents varied with the type of irrigation water. The 0.5 mM silicon supplementation into tap water significantly increased carotenoid contents.

There were significant positive correlations between leaf active iron content and carotenoid content ($r=0.541^{*}$), thus, carotenoid contents significantly increased with increasing leaf active iron contents. Correlations coefficients for the correlations of leaf carotenoid contents with leaf sodium and magnesium contents were respectively identified as $r=-0.595^{*}$ and $r=-0.498$.

If seawater with high salt content is to be used for agricultural purposes, it must be diluted with tap water or it is necessary to reduce the stress effect of salinity on plant production by adding silicon to seawater.

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References

Agastian P, Kingsley S J & Vivekanandan M (2000). Effect of salinity on photosynthesis and biochemical characteristics in mulberry genotypes. *Photosynthetica* 38: 287-290.

Al-Aghabary K, Zhu Z & Shi Q (2004). Influence of silicon supply on chlorophyll content, chlorophyll fluorescence, and antioxidative enzyme activities in tomato plants under salt stress. *Journal of Plant Physiology* 27(12): 2101-2115. doi.org/10.1081/PLN-200034641 Alamgir A N M & Ali M Y (1999). Effect of salinity on leaf pigments, sugar and protein concentration and chloroplast ATP_{az} activity of rice (Oryza sativa L.). *Bangladesh Journal of Botany* 28(2): 145-149.

Alian A, Altman A & Heuer B (2000). Genotypic difference in salinity and water stress tolerance of fresh market tomato cultivars. *Plant Science* 152(1): 59-65. doi.org/10.1016/S0168-9452(99)00220-4

Alpaslan M, Güneş A, Taban S, Erdal İ & Tarakcıoğlu C (1998). Tuz stresinde çeltik ve buğday çeşitlerinin kalsiyum, fosfor, demir, bakır, çinko ve mangan içeriklerindeki değişmeler, *Turkish Journal of Agriculture and Forestry* 22: 227-233.

Arnon D (1949). Copper Enzymes in Isolated Chloroplasts. Polyphenoloxidase in Beta Vulgaris. *Plant Physiology* 24(1): 1-15. doi.org/10.1104/pp.24.1.1

Bayraklı F (1987). Toprak ve bitki analizleri. Ondokuz Mayıs Üniversitesi Ziraat Fakültesi, Samsun, Türkiye (in Turkish).

Biswal B, Joshi P N, Raval M K & Biswal U C (2011). Photosynthesis, a global sensor of environmental stress in green plants: stress signalling and adaptation. *Current Science* 101(1): 47-56.

Breckle SW (2009). Is sustainable agriculture with seawater irrigation realistic? In: Ashraf M, Öztürk M, Athar H R (eds) Salinity and water stress-improving crop efficiency, tasks for vegetation science-44. Springer, the Netherlands, pp 187-196.

Boyko H (1966). Salinity and Aridity New Approaches to Old Problems. *Salinity and Aridity* 3-22. doi.org/10.1007/978-94-017-6014-0_1

Chutipaijit S, Cha-um S & Sompornpailin K (2011) High contents of proline and anthocyanin increase protective response to salinity in Oryza sativa L. spp. Indica, *Australian Jounral of Crop Science* 5(10): 1191-1198.

Coşkun D, Britto D T, Huynh W & Kronzucker H (2016). The role of silicon in higher plants under salinity and drought stress. *Frontiers in Plant Science* 18(7): 1072. doi.org/10.3389/fpls.2016.01072

Dannon E A & Wydra K (2004). Inretaction between silicon amendment, bacterial wilt development and phenotype of Ralstonia solanacearum in tomato genotypes. *Physiological and Molecular Plant Pathology* 64(5): 233-243. doi.org/10.1016/j.pmpp.2004.09.006

Elimelech M & Phillip W A (2011). The future of seawater desalination: energy, technology, and the environment. *Science* 333: 712-718. doi.org 10.1126/science.1200488

Gül A (2012). Topraksız Tarım 2. *Baskı, Hasat Yayıncılık Limited Şirketi*, İstanbul, Türkiye.

Huang B (2006). Cellular membranes in stress sensing and regulation of plant adaptation to abiotic stresses. *Plant-environment İnteractions* 3: 1-26.

Hussain A, Iqbal K, Aziem S, Mahato P, & Negi A K (2014). A review on the science of growing crops without soil (soilless culture) – a novel alternative for growing crops. *International Journal of Agriculture and Crop Sciences* 2227.

Kacar B (1994). Bitki ve toprağın kimyasal analizleri. Ankara ÜnİversitesiZiraat Fakültesi Eğitim, Araştırma ve Geliştirme Vakfı.

Kacar B & İnal A (2008). Bitki Analizleri, 1. Basım. Nobel Yayınları, Ankara, Türkiye.

Kahlaoui B, Hachicha M, Rejeb S, Misle E, Rouaissi M, Rejeb M N & Hanchi B (2011). Effect of saline water on tomato under subsurface drip irrigation: yield and fruit quality. *Australian Journal of Basic and Applied Sciences* 5(9): 517-529.

Karagiannis I C & Soldatos P G (2008). Water desalination cost literature: review and assessment. *Desalination*, 223(1-3): 448-456. doi.org/10.1016/j.desal.2007.02.071

Kennedy B F & De Fillippis L F (1999). Physiological and oxidative response to NaCl of the salt tolerant Grevillea ilicifolia and the salt sensitive Grevillea arenaria. *J Plant Physiology* 155(6): 746-754. https://doi.org/10.1016/S0176-1617(99)80092-3

Khavarinejad R A & Mostofi Y (1998). Effects of NaCl on photosynthetic pigments, saccharides, and chloroplast ultrastructure in leaves of tomato cultivars. *Photosynthetica* 35: 151-154.

Levitt J (1980). Responses of plants to environmental stresses. *Academic Press* 365-434.

Li H, Zhu Y, Hu Y, Han W, Gong H (2015). Beneficial effects of silicon in alleviating salinity stress of tomato seedlings grown under sand culture. *Acta Physiologiae Plantarum* 37: 71. doi: 10.1007/s11738-015-1818-7

Li Y, Stanghellini C & Challa H (2001). Effect of electrical conductivity and transpiration on production of greenhouse tomato (Lycopersicon esculentum L.). *Scientia Horticulturae* 88: 11-29.

Maas E V & Hoffman G J (1977). Crop salt tolerance – current assessment. *Journal of Irrigation and Drainage Division American Society of Civil Engineers* 103(2): 115-134.

Marschner H (1995). Mineral nutrition of higher plants. *Academic Press*, New York, USA pp 657-680.

Maxwell K & Johnson G N (2000). Chlorophyll fluorescence-A practical guide. *Journal of Experimental Botany* 51(345): 659-668. doi.org/10.1093/jexbot/51.345.659

Mittal S, Kumari N & Sharma V (2012). Differential response of salt stress on *Brassica juncea*: Photosynthetic performance, pigment, proline, D1 and antioxidant enzymes. *Plant Physiology and Biochemistry* 54: 17-26.

Niu X, Bressan R A, Hasegawa P M & Pardo J M (1995). Ion homeostasis in NaCl stress environments. *Plant Physiology* 109: 735-742. doi.org/10.1104/pp.109.3.735

Olympios C M (1999). Overview of soilless culture: Advantages, constraints and perspectives for its use in Mediterranean countries. *Cahiers Options Méditerranéennes* 31: 307-324.

Oserkowsky J (1933). Quantitative relation between chlorophyll and iron in green and chlorotic pear leaves. *Plant Physiology* 8(3): 449-468.

Parida A K & Das A B (2005). Salt tolerance and salinity effect on plant: A review. *Ecotoxicology and Environmental Safety* 60(3): 324-349. doi.org/10.1016/j.ecoenv.2004.06.010

Parvin K & Haque N (2015). Response of tomato plant under salt stress: Role of Exogenous calcium. *Journal of Plant Sciences* 10(6): 222-233.

Rengel Z (1992). The role of calcium in salt toxicity. *Plant, Cell & Environment* 15(6): 625-632. doi.org/10.1111/j.1365-3040.1992.tb01004.x

Romero-Aranda M R, Jurado O & Cuartero J (2006). Silicon alleviates the deleterious salt effect on tomato plant growth by improving plant water status. *Journal of Plant Physiology* 163(8): 847-855.

Sağlam T (2008). Toprak ve suyun kimyasal analiz yöntemleri. *Namık Kemal Üniversitesi Ziraat Fakültesi* 2: 1-154.

Sheikh B A (2006). Hydroponics: key to sustain agriculture in water stressed and urban environment. *Pakistan Journal of Agriculture and Agricultural Engineering and Veterinary Sciences* 22: 53-57.

Sonneveld C & Van Der Burg A M M (1991). Sodium chloride salinity in fruit vegetable crops in soilless culture. *Netherlands Journal of Agricultural Science* 39: 115-122.

Stamatakis A, Savvas D, Papadantonakis N, Lydakis-Simantiris N & Kefalas P (2003). Effects of silicon and salinity on fruit yield and quality of tomato grown hydroponically. *Acta Horticulturae* 609: 141-149.

Tavakkoli M M, Roosta H R & Hamidpour M (2016). Effects of alkali stress and growing media on growth and physiological characteristics of Gerbera plants. *Journal of Agricultural Science and Technology* 18: 453-466.

Tuteja N (2007). Mechanisms of high salinity tolerance in plants, *Methods in Enzymology* 428: 419-438. https://doi.org/10.1016/S0076-6879(07)28024-3

Wade N M (2001). Distillation of plant development and cost update. *Desalination* 136(1-3): 3-12. doi.org/10.1016/S0011-9164(01)00159-X

Wang S, Liu P, Chen D, Yin L, Li H, Deng X (2015). Silicon enhanced salt tolerance by improving the root water uptake and decreasing the ion toxicity in cucumber. *Frontiers in Plant Science* 6: 759. doi: 10.3389/fpls.2015.00759

Wang Y & Nil N (2000). Changes in chlorophyll, ribulose biphosphate carboxylase–oxygenase, glycine betaine content, photosynthesis and transpiration in Amaranthus tricolor leaves during salt stress, The *Journal of Horticultural Science and Biotechnology* 75(6): 623-627.

Withan F H & Blayedes D F & Devlin R M (1971). Experiments in Plant Physiology. *Van Nostrand Reinhold Co* 55-58.

Yermiyahu, U, Tal A, Ben-Gal A, Bar-Tal A, Tarchitzky J & Lahav O (2007). Rethinking desalinated water quality and agriculture. *Science* 318(5852) 920-921. doi.org/10.1126/science.1146339

Zhu Y & Gong H (2014). Beneficial effects of silicon on salt and drought tolerance in plants agronomy for sustainable development, *Springer Verlag/ Edp Sciences/INRA* 34(2): 455-472.

Zhu Y X, Xu X B, Hu Y H, Han W H, Yin J L, Li H L & Gong H J (2015). Silicon improves salt tolerance by increasing root water uptake in *Cucumis sativus* L. *Plant Cell Reports* 34: 1629-1646.