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Research Article

Investigation of the Effect of Immersion and Ultrasound Pretreatments on Some Quality Characteristics of Pears Dried in Vacuum-Assisted Double-Sided Infrared Dryer

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Ultrasound

Abstract: In this study, the effects of different immersion solutions (maltodextrin and ascorbic acid), solution concentrations (7 and 14% for maltodextrin, 0.5 and 1% for ascorbic acid), immersion times (1, 5, and 10 minutes) and ultrasound amplitudes (0%, 50% and 100%) applied as pretreatment on pears dried in a vacuum-assisted two-way infrared dryer were investigated. Moisture, pH, and acidity values of fresh pear were determined as 81.15%, 4.50 and 0.26%, respectively. Glucose, fructose, sucrose, and total sugar values were calculated as 14.76, 26.51, 6.63, and 47.90 g $100g^{-1}$ DM, respectively. Total phenolic content amount, DPPH, and ABTS values were found to be 2461.35 mg GAE kg^{-1} DM, 407.76, and 206.79 mmol Trolox eq. kg^{-1} DM. Increasing ascorbic acid concentration prevented HMF formation with increasing ultrasound amplitude and duration, while increasing maltodextrin concentration increased HMF content, especially with increasing immersion duration. In terms of individual phenolics, ellagic acid was not detected in fresh pears, while ellagic acid was detected in dry samples together with chlorogenic acid and rutin. As the ultrasound duration applied in pretreatments increased, the amounts of all individual phenolic compounds in the samples, except for rutin, increased. In the sensory evaluation, the highest approval score was obtained from samples kept in 0.5% ascorbic acid solution and 50% ultrasound amplitude for 10 minutes. As a result, it was determined that the quality of pears dried in vacuum-supported two-way infrared dryers could be increased with the pretreatment combinations, and products with low HMF, high phenolic compounds, antioxidant activity, and approval levels could be produced.

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1. Introduction

Pear is a thin-skinned, soft-core, juicy, and delicious fruit belonging to the Pyrus genus of the Rosaceae family (Kuş, 2016; Kurt et al, 2023). Pears are mostly consumed dried, fresh, in pear juice, or canned. Drying has an important place in these consumption methods. In addition to their table consumption, dried pears are also used as an intermediate product in the production of different products

(fruit ice cream, yogurt, cereals, bakery products) for various purposes in the food industry (Özaydın and Özçelik, 2016). In recent years, many innovative dryers have been developed such as fluidized bed, lyophilization, microwave, infrared, spray, and roller dryers, considering many issues such as drying time, product quality, energy consumption, space occupied, investment, and operating costs. However, industrial scale dryers have disadvantages such as high initial investment costs, high energy consumption, and the inability to obtain the desired characteristics, especially color, in some foods (Seçkin and Taşeri, 2015; Ar and Ocak, 2022). Infrared drying is one of the drying methods that has gained speed in the food industry in recent years. It has many advantages such as shortening the drying time compared to other technologies, energy saving, low investment, and operating costs, homogeneous heat distribution, easy combination with many other applications (lyophilization, vacuum, ultrasound, etc.), and providing the opportunity to obtain a higher quality dry product compared to many drying technologies (Wang and Sheng, 2006). Successful drying results, especially with the combination of infrared, vacuum, and ultrasound, have been reported in the literature. Salehi and Kashaninejad (2018) reported that both the infrared lamp power and the vacuum level were effective on the drying time of lemon slices dried with a vacuum-assisted infrared dryer and that when the infrared power level was increased by 100 W, moisture diffusion increased approximately 10 times. Kouchakzadeh and Haghighi (2011) compared conventional and vacuum-assisted infrared technologies in the drying of pistachios and found that the infrared drying time was 8-10 times faster than conventional drying on average. Dujmić et al (2013) dried pear slices in an infrared dryer at 70 °C with (24 kHz) and without ultrasound treatment and noted that ultrasound treatment had a significant effect on the textural properties of pear samples such as chewiness, hardness, and elasticity.

In order to increase drying efficiency and product quality in the drying processes applied to fruits and vegetables, various pretreatments such as immersion in various solutions (ascorbic acid, citric acid, maltodextrin, tocopherols, etc.), application of alkaline solution, boiling, salting, and ultrasound are applied (Şahin et al., 2012; Fadıllah et al., 2024). The use of L-ascorbic acid in pre-treatments provides advantages against color darkening, and when taken into the body, it performs an important physiological function in terms of health by destroying free radicals owing to its strong antioxidant activity. For this reason, in recent years, studies on the use of some natural antioxidants such as L-ascorbic acid as pre-treatment before drying have intensified (Şen, 2013). In a study where different solutions were used as pretreatment, hot red peppers were dried in a tray dryer at 70 ºC. It was recorded that the total phenolic content (TPC) results of the pretreated dried red peppers varied between 271.34-312.93 g 100 g^{-1} DM and the highest TPC amount belonged to the peppers dipped in ascorbic acid solution (Wiriya et al., 2009). Francisco et al. (2008) investigated the effect of different concentrations of ascorbic acid (0-0.1 g/g) on the drying of 5 mm thick, 4-7 cm diameter chopped pear slices in a conventional dryer at 60-70 ºC. The results were evaluated using the response surface method and they stated that optimum drying was achieved when the 5 cm diameter sliced samples were dried at 63 °C after immersion in 0.075% ascorbic acid solution.

Maltodextrins, which have a wide range of use in different sectors, especially in the food and pharmaceutical industries, are chemicals obtained as a result of partial hydrolysis of corn starch by acidic or enzymatic methods and have a dextrose equivalence (DE) of 20 or less (Söbüçovalı and Özer, 2014). One of the areas where maltodextrins are used in the food industry is drying technology. In studies where high drying temperatures are applied, it has been stated that maltodextrin increases the quality of fruit powder. Moser et al. (2017) stated that phenolic compounds are better stabilized in grape powders using maltodextrin and that shelf life can be extended. In a study in which Sapollida fruit powder was obtained, Sapollida fruit juices were coated with maltodextrin at different concentrations (10, 20, 30, 40, and 50 g L^{-1}) and then dried with a spray dryer. It was found that 20 and 30 g L^{-1} maltodextrin preserved color and phenolic compounds better (Chong and Wong, 2017).

In this study, the effects of immersion pretreatments (maltodextrin and ascorbic acid) and ultrasound (0%, 50%, and 100%) on some physicochemical properties, sensory properties, and phenolic compounds of pears dried with a vacuum-assisted double-sided infrared dryer were investigated.

2. Material and Methods

In this study, pears of the Deveci variety collected from a local garden in Van province were used. The pears used for the study were collected in 2020 and all data were obtained in the same year.

HPLC (High Performance Liquid Chromatography) grade methanol, acetonitrile (HPLC grade), sodium carbonate, acetic acid, and ethanol used in the study were supplied by Merck (Darmstadt, Germany). Folin ciocalteu, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), HMF, chlorogenic acid, ellagic acid, and rutin standards were purchased from Sigma Aldrich (St. Louis, MO, USA). Food-grade maltodextrin (18-20 DE) and L-ascorbic acid were supplied by Tito (İstanbul, Türkiye).

2.1. Application of pretreatments

The collected pears were brought to the laboratory immediately after harvesting, washed, and cleaned. The pears were peeled with a manual fruit slicer (Hex, Istanbul) and sliced with an average thickness of 5 mm. The picture of the sliced pear slices is shown in Figure 1a. The pear slices were immersed in 7 and 15 °Bx maltodextrin (18-20 DE) solutions prepared with distilled water and 0.5% and 1.0% ascorbic acid solutions prepared with distilled water for 1, 5, and 10 min. During the immersion process, ultrasound (Bandelein Sonoplus HD, Germany) was applied using the Bandelin Sonoplus HD 3200 probe at 0%, 50% and 100% power levels. Each application was performed with five replications for each treatment group.

2.2. Vacuum-assisted double-sided infrared dryer

The drying process was conducted in a vacuum dryer (Uniterm, Ankara) in which 4 250 W infrared lamps were placed, 2 at the bottom and 2 at the top. The drying oven and its parts used are shown in Figure 1b. A thermocouple was placed inside the oven to monitor the instantaneous temperature. In infrared drying studies, the distance between the lamp and the dried material was adjusted to 12 cm from the bottom and top. In drying trials, temperature measurement was made with ±2 °C precision. The desired vacuum level in the dryer was provided by a vacuum pump (DOA-P730-BN, USA). In the study, 250 g of pear sample was placed on a perforated drying tray and dried for 120 minutes by applying 300 W infrared power and 250 mmHg vacuum pressure, which were determined to be suitable for pears in previous studies (Topuz, 2021; Topuz et al., 2023).

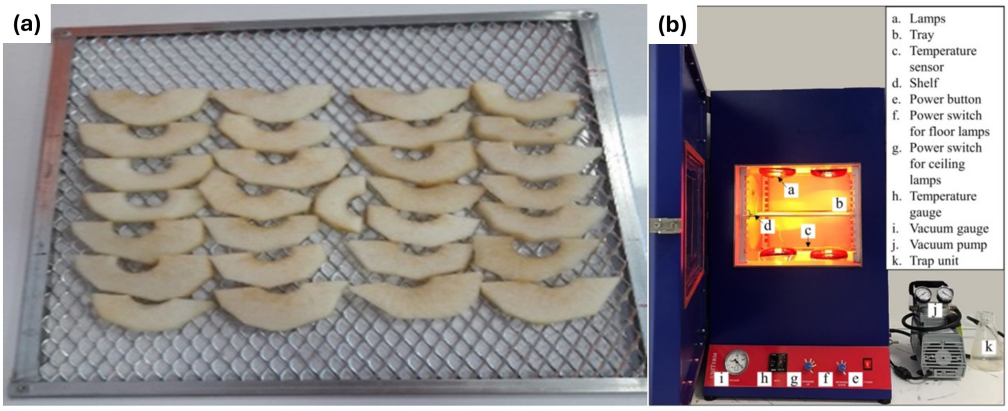

Figure 1. a) an image of sliced fresh pears, and b) parts of the vacuum-assisted two-way infrared dryer.

2.1.1. Color determination

The L* (light, dark), a* (redness, greenness), and b* (yellowness, blueness) values of the samples were measured and recorded with a Minolta CR 400 (Tokyo, Japan) color determination device (Anonymous, 1994). Color measurement was performed to determine the differences between the color values of the pear samples before and after drying and pre-treatment.

2.1.2. Moisture determination

The moisture content of the samples was determined by was determined by drying in an oven at 105 °C until a constant weight was achieved (AOAC, 2003).

2.1.3. pH and titration acidity

The pH and titration acidity analyses of the samples were performed according to AOAC (2003).

2.1.4. Rehydration rate (RR)

Dry samples (2 g) were placed in a glass beaker and 16 mL of pure water was added. The top of the beaker was covered with parafilm and left at room temperature for 24 hours. After 24 hours, the rehydrated pear slices were poured into the strainer, and the water was allowed to drain for 3-4 minutes. Excess water was removed with blotting paper and the rehydrated samples were weighed on a precision scale and the weights were recorded. RR was obtained by dividing the weight of the rehydrated sample by the initial dry weight (Xiaoyong et al., 2018).

2.1.5. Sugar analysis

For sugar determination, 0.5 g of sample was taken, 5 mL of distilled water was added, and left to stand at room temperature overnight. The next day, the rehydrated pears were carefully crushed in a mortar and the contents of the mortar were washed with 15 mL of pure water and transferred to a tube. The tube content was homogenized for 10 seconds at 12.000 rpm, then shaken at 180 rpm on a circular shaker (OS-3000, JEIO TECH, Korea) for 1 hour. Finally, the sample was centrifuged at 3600 *g* for 10 minutes. The supernatants obtained at the end of centrifugation were passed through a 0.45 PVDF syringe filter and injected into the HPLC device. An HPLC instrument (Shimadzu, Japan) equipped with an LC-20A gradient pump, RI detector (RID-20A, Shimadzu, Kyoto, Japan), CTO-10AS VP column oven, and DGA-14A degasser was used for sugar determination. Elution was carried out in isocratic mode with acetonitrile: water (4:1) at 1.3 mL min^{-1}. Separation of sugars was carried out with Intersil NH2 (4.6 x 250 mm ID, 5 µm) (GL Sciences Inc., Tokyo, Japan) column at 25 ºC column temperature. Peaks in the HPLC chromatogram were identified and quantified by comparing them with the peaks of fructose, glucose, and sucrose standard substances. The amounts of sugar components were given as g 100 g^{-1} DM (Hamzaoğlu et al. 2018).

2.1.6. Preparation of methanol extracts

0.5 g of dry samples were taken and 10 ml of methanol was added, homogenized at 12000 rpm for 10 seconds. Samples were shaken on a circular shaker for 2 hours and then centrifuged at 8000 *g* for 10 minutes. After the supernatant was removed, the same procedures were repeated twice more on the remaining part. The supernatants obtained were collected and the final volume was completed to 25 ml (Colaric et al. 2005). The extracts were stored in 25 ml amber bottles at -24 °C until used in TPC, DPPH, ABTS, and phenolic compound distribution analyses.

2.1.7. TPC

TPC analysis of pear samples was performed by modifying the method reported by Singleton and Rossi (1965). 2 mL of Folin Ciocalteu and 1.6 mL of 7% sodium carbonate solution were added to 0.4 mL of methanolic extract, respectively. After the mixture was left in the dark at room temperature for 1 hour, absorbance values were measured at 760 nm. The results were given as mg GAE kg^{-1} DM.

2.1.8. DPPH

3.6 mL of DPPH solution (0.025 g L^{-1} methanol) was mixed with 0.4 mL of methanolic extract in a test tube and then left in the dark for 60 minutes at room temperature. At the end of this period, sample absorbance was measured at 515 nm to determine the inhibition rate of the DPPH radical. The results were given as mmol TE g^{-1} DM (Pyo et al. 2004).

2.1.9. ABTS

ABTS analyses of samples were performed according to the method reported by Re et al. (1999). A 7 mM ABTS solution containing 2.45 mM potassium persulfate was prepared and kept in the dark at room temperature for 12-16 hours to form the stock ABTS+ radical solution. The stock radical solution was diluted with ethanol and the absorbance of the ABTS+ working solution was adjusted to be 0.70 ± 0.02 at 734 nm. Then, 40 µL of methanolic extract was mixed with 1960 µL of ABTS+ working solution and kept in the dark for 6 minutes before the absorbance was read at 734 nm. The results were expressed as mmol TE g^{-1} DM.

2.1.10. HMF content and phenolic compound distribution

The method proposed by Uğurlu et al. (2020) was modified for the determination of phenolic substance content and HMF content of pear samples. Methanolic extracts were passed through a 0.45 PVDF syringe tip filter and injected into the HPLC device. An HPLC device with an SPD-M20A DAD detector (Shimadzu, Japan) was used for analysis. Separation was performed on a Waters Symmetry C18 (250x4 mm ID, 5 µm) column (Waters, USA) at 25°C. The mobile phases were 2% acetic acid in water (A) and 0.5% acetic acid in water: acetonitrile (1:1, v/v) (B). The gradient program was: 10% B at 0 min, 55% B at 50 min, 100% B at 60 min and the flow rate was 1.0 mL min^{-1}. Peaks were identified by comparing their spectra and arrival times with those of standard substances. In the calculations, wavelengths of 280 nm for HMF, 320 nm for chlorogenic acid, and 360 nm for rutin and ellagic acid were used.

2.1.11. Sensory analysis

Sensory evaluations were performed by 8 semi-trained male and 7 female panelists using a 9-point hedonic scale. Samples were randomly coded with 3-digit numbers and presented to the panelists, who were asked to evaluate the samples according to color, smell, appearance, hardness degree, hardness appreciation, chewiness, taste, and general appreciation scales (Stokes et al., 2017).

2.1.12. Statistical evaluation

Pears were immersed in different solutions (maltodextrin and ascorbic acid) for different times (1, 5, and 10 min), at different ultrasound powers (50 and 100). After pretreatment, dried samples were analyzed and analyses were performed in 5 replicates. Analysis results were given as mean ±standard deviation. Differences between groups were determined by variance analysis (one-way ANOVA) using the IBM SPSS Statistic v20.0 package program. Duncan multiple comparison test was used to evaluate differences between means. P-values were calculated and significance was set at $P<0.05$.

3. Results and Discussion

3.1. Color measurement results of pears dried with different pretreatments

Color values of pretreated dried pears are given in Table 1. Due to browning reactions occurring on the surface during the drying process, the L* values of dry samples are lower and a* and b* values are higher than fresh samples. In terms of color values, small differences were detected between the concentrations used in both dipping solutions. However, only in ascorbic acid applications, the difference between the concentrations was found to be statistically significant ($P<0.05$). While the L* values of the samples treated with maltodextrin and the a* values of the samples treated with ascorbic acid decreased with increasing immersion time with ultrasound application, they increased with increasing immersion time for the samples without ultrasound application. The difference between samples with and without ultrasound for L* and a* values was found to be significant ($P<0.05$). When the pretreatment application times were examined, except for the samples where maltodextrin and ultrasound were used together, the L* and a* values increased as the time increased in all other samples, but the b* value decreased in all samples. However, only in the ascorbic acid immersion process, the changes in the L* and b* values were found to be statistically significant in terms of immersion time ($P<0.05$). Silva et al. (2016) stated that the L*, a*, and b* values of the melon samples immersed in sucrose solution and subjected to ultrasound increased after convective drying at 60°C compared to the initial sample. While the results of our study regarding the a* and b* values are consistent with the reported results, the L* value is inconsistent. This difference may be due to the immersion solution. In a different study, sliced fresh tomatoes were immersed in 1% citric acid and 1% ascorbic acid solution and dried at 65 and 75 ºC and under 10 kPa vacuum pressure. It was stated that the L*, a*, and b* values of the pretreated samples were higher than the untreated tomato samples at the end of drying (Şahin et al., 2012).

Table 1. L*, a*, and b* results of dried pears with different pretreatments

Concentration (°Bx)		Ultrasound (%)	Time (min.)	L*	a*	b*
Fresh pear		0	0	64.85±5.12	-0.04±0.01	13.23±2.41
Maltodextrin	7	0	1	49.95±0.65^{aA1}	13.27±0.86^{bA1}	25.08±2.18^{aA1}
			5	54.90±1.80^{aA1}	13.42±0.29^{bA1}	22.03±1.65^{aA1}
			10	57.94±3.75^{aA1}	14.51±1.05^{bA1}	18.43±1.10^{aA1}
		50	1	51,87±3.09^{aB1}	15.84±0.19^{bA1}	26.09±1.60^{aA1}
			5	50.29±2.04^{aB1}	15.66±0.17^{bA1}	25.58±1.53^{aA1}
			10	48.21±3.17^{aB1}	14.63±1.73^{bA1}	24.23±0.89^{aA1}
		100	1	52.71±2.59^{aB1}	15.46±1.18^{bA1}	26.00±1.29^{aA1}
			5	50.98±1.64^{aB1}	15.03±0.76^{bA1}	25.24±1.03^{aA1}
			10	50.26±2.14^{aB1}	14.69±0.93^{bA1}	23.56±1.15^{aA1}
	15	0	1	47.90±3.26^{aA1}	14.25±1.21^{aA1}	27.54±0.04^{aA1}
			5	50.20±1.94^{aA1}	14.74±1.88^{aA1}	25.79±2.57^{aA1}
			10	58.04±2.32^{aA1}	16.41±1.36^{aA1}	23.21±2.18^{aA1}
		50	1	53.29±3.23^{aB1}	14.98±0.49^{aA1}	26.82±1.62^{aA1}
			5	51.05±2.82^{aB1}	13.26±1.45^{aA1}	24.76±1.75^{aA1}
			10	48.97±0.77^{aB1}	13.17±1.70^{aA1}	24.27±1.28^{aA1}
		100	1	53.34±0.96^{aB1}	15.62±1.42^{aA1}	26.30±2.27^{aA1}
			5	52.15±1.42^{aB1}	14.48±0.81^{aA1}	24.05±1.19^{aA1}
			10	51.15±2.75^{aB1}	13.80±0.92^{aA1}	23.61±1.06^{aA1}
Ascorbic Acid	0.5	0	1	47.23±2.47^{aA1}	13.45±0.86^{aA1}	25.35±0.49^{aA1}
			5	51.55±3.33^{aA2}	14.26±2.00^{aA1}	23.38±1.93^{aA2}
			10	54.00±3.46^{aA3}	16.02±1.95^{aA1}	23.31±1.34^{aA3}
		50	1	50.78±2.11^{aA1}	13.57±0.58^{aB1}	25.22±1.70^{aA1}
			5	52.15±1.36^{aA2}	14.35±0.87^{aB1}	24.45±1.05^{aA2}
			10	54.93±2.61^{aA3}	16.40±0.22^{aB1}	24.07±1.61^{aA3}
		100	1	51.04±1.29^{aA1}	14.69±0.54^{aB1}	25.73±0.22^{aA1}
			5	53.32±1.59^{aA2}	15.70±0.77^{aB1}	25.60±2.13^{aA2}
			10	55.48±0.87^{aA3}	16.77±1.39^{aB1}	24.94±1.78^{aA3}
	1.0	0	1	50.54±3.36^{bA1}	13.88±0.50^{bA1}	27.53±2.79^{bA1}
			5	51.55±0.85^{bA2}	14.34±1.94^{bA1}	26.03±1.53^{bA2}
			10	54.28±2.59^{bA3}	14.70±0.09^{bA1}	25.64±1.34^{bA3}
		50	1	48.71±2.18^{bA1}	13.99±1.74^{bB1}	26.93±1.54^{bA1}
			5	51.26±1.45^{bA2}	15.74±0.78^{bB1}	26.47±2.02^{bA2}
			10	55.83±1.18^{bA3}	16.76±0.16^{bB1}	25.69±0.73^{bA3}
		100	1	52.91±2.60^{bA1}	14.35±0.72^{bB1}	27.45±1.53^{bA1}
			5	53.30±3.61^{bA2}	15.86±0.50^{bB1}	27.38±1.09^{bA2}
			10	56.09±1.42^{bA3}	16.81±1.61^{bB1}	26.16±0.08^{bA3}

Results are given as mean ± standard deviation. Lowercase letters indicate differences between concentrations for the same pretreatment agent applied according to Duncan's multiple comparison test, capital letters indicate differences between ultrasound powers for the same pretreatment agent applied, and numbers indicate differences between immersion times in the same pretreatment agent applied ($P<0.05$).

3.2. Results of moisture and chemical analyses of pears dried by applying different pretreatments

The results of moisture, pH, titratable acidity, and RR of pears dried by pretreatment are given in Table 2. Especially in long treatment periods, the moisture content of pears treated with maltodextrin was found to be lower than the moisture value of pears treated with ascorbic acid. This situation is thought to be due to the use of higher concentrations of maltodextrin causing an increase in dry matter in pears. In both pretreatment applications, as the pretreatment time increased, the dry matter amount of the samples increased and the moisture content in the obtained dry sample decreased ($P<0.05$). Similar results were also found by Nowacka et al. (2012) in apple drying. In terms of ultrasound application, it was noted that only in the application of maltodextrin at a concentration of 15 °Bx, the increase in ultrasound power significantly reduced the moisture content ($P<0.05$).

Compared to fresh Deveci pears, the pH values of the samples dried by pretreatment were lower and the titratable acidity values were higher. Especially in the samples treated with ascorbic acid, it was

determined that the pH values of the samples immersed in high concentrations were lower than the samples immersed in low concentrations ($P<0.05$). In both immersion solutions, it was found that the titratable acidity of the samples without ultrasound was lower and their pH was higher than the samples with ultrasound. While the difference between those with and without ultrasound application was found to be statistically significant in terms of both parameters in maltodextrin application, it was found to be significant only for titratable acidity in ascorbic acid application ($P<0.05$). It was noted that as the applied ultrasound power and immersion time increased, there was a decrease in the pH values of the samples and an increase in the titratable acidity values. As expected, this effect was more pronounced in the ascorbic acid application. Zlabur et al., (2019) reported that the titratable acidity (7.03% DM) of dried blueberry after ultrasound application as a pretreatment was higher than the titratable acidity (6.39% DM) of the sample without ultrasound application.

When the RR values of the samples were examined, it was determined that the RR increased as the immersion time increased in all pretreatment applications. It was found that especially the ultrasound application increased the RR values and this increase was the highest at high ultrasound power. The difference between concentrations and immersion times in both immersion solutions was found to be statistically significant in terms of RR ($P<0.05$). Liu et al. (2019b) reported that applying 24 W and 48 W ultrasound power to pear samples resulted in a 7.9% and 18.4% increase in RR values, respectively. It has been stated that 30 W and 60 W ultrasound power applied to strawberries to be dried similarly increased the RR values (Gamboa-Santos et al., 2014). In onions that were pretreated with 5% salt water, 5% maltodextrin/water mixture, 5% potato starch/water mixture, and soaked in tap water, it was determined that the RR value of the samples dried without dipping in maltodextrin was 5.45 g/g DM on average, while the RR value of those dried after dipping in maltodextrin increased to 5.74 g/g DM (Karaaslan et al., 2016).

Table 2. Moisture, pH, acidity, and RR of pretreated dry samples

Concentration (°Bx)		Ultrasound (%)	Time (min.)	Moisture (%)	pH	Acidity (%)	RR (g water g^{-1} DM)
Fresh Pear		0	0	81.15±4.55	4.50±0.67	0.26±0.03	-
Maltodextrin	7	0	1	21.09±2.99^{aA3}	4.19±0.33^{bB1}	0.28±0.04^{aA1}	3.39±0.05^{bA1}
			5	17.14±1.65^{aA2}	4.27±0.19^{bB1}	0.30±0.01^{aA1}	3.41±0.09^{bA1}
			10	16.21±1.38^{aA1}	4.31±0.01^{bB1}	0.31±0.04^{aA1}	3.59±0.11^{bA2}
		50	1	23.08±2.58^{aB3}	4.17±0.08^{bA1}	0.31±0.00^{aB1}	3.49±0.16^{bA1}
			5	20.16±0.11^{aB2}	4.23±0.02^{bA1}	0.32±002^{aB1}	3.72±0.12^{bA1}
			10	15.10±1.22^{aB1}	4.21±0.15^{bA1}	0.33±0.04^{aB1}	3.75±0.36^{bA2}
		100	1	23.42±0.97^{aC3}	4.12±0.29^{bA1}	0.32±0.00^{aB1}	3.48±0.09^{bA1}
			5	21.89±2.15^{aC2}	4.08±0.10^{bA1}	0.33±0.00^{aB1}	3.55±0.06^{bA1}
			10	15.03±1.95^{aC1}	4.01±0.03^{bA1}	0.35±0.01^{aB1}	3.79±0.04^{bA2}
	15	0	1	19.40±2.42^{aA3}	4.16±0.09^{aB1}	0.31±0.01^{bA1}	3.32±0.24^{aA1}
			5	16.79±1.64^{aA2}	4.23±0.14^{aB1}	0.32±0.05^{bA1}	3.40±0.20^{aA1}
			10	16.45±0.42^{aA1}	4.27±0.21^{aB1}	0.34±0.02^{bA1}	3.60±0.15^{aA2}
		50	1	19.77±0.98^{aB3}	4.14±0.05^{aA1}	0.32±0.00^{bB1}	3.16±0.19^{aA1}
			5	18.91±0.65^{aB2}	4.04±0.03^{aA1}	0.33±0.03^{bB1}	3.27±0.05^{aA1}
			10	15.60±1.55^{aB1}	4.02±0.03^{aA1}	0.35±0.03^{bB1}	3.67±0.02^{aA2}
		100	1	19.75±4.71^{aC3}	4.01±0.07^{aA1}	0.34±0.01^{bB1}	3.04±0.42^{aA1}
			5	14.36±1.25^{aC2}	3.90±0.08^{aA1}	0.35±0.01^{bB1}	3.81±0.18^{aA1}
			10	13.68±0.98^{aC1}	3.87±0.07^{aA1}	0.37±0.04^{bB1}	3.89±0.09^{aA2}

Table 2. Moisture, pH, acidity, and RR of pretreated dry samples (continued)

Concentration (°Bx)		Ultrasound (%)	Time (min.)	Moisture (%)	pH	Acidity (%)	RR (g water g^{-1} DM)
Ascorbic Acid	0.5	0	1	21.53±0.81^{aA3}	4.15±0.08^{bA1}	0.37±0.19^{aA1}	3.41±0.12^{aA1}
			5	19.85±0.68^{aA2}	4.14±0.41^{bA1}	0.40±0.20^{aA1}	3.55±0.40^{aA1}
			10	18.56±1.13^{aA1}	4.09±0.26^{bA1}	0.42±0.13^{aA1}	3.66±0.16^{aA2}
		50	1	20.05±3.77^{aA3}	4.07±0.26^{bA1}	0.40±0.10^{aB1}	3.31±0.17^{aA1}
			5	19.70±2.79^{aA2}	4.06±0.19^{bA1}	0.42±0.12^{aB1}	3.41±0.21^{aA1}
			10	18.36±2.71^{aA1}	3.98±0.11^{bA1}	0.44±0.40^{aB1}	3.73±0.34^{aA2}
		100	1	19.12±2.29^{aA3}	4.00±0.19^{bA1}	0.42±0.24^{aB1}	3.39±0.19^{aA1}
			5	18.72±2.24^{aA2}	3.96±0.02^{bA1}	0.43±0.29^{aB1}	3.53±0.27^{aA1}
			10	18.11±0.57^{aA1}	3.92±0.08^{bA1}	0.46±0.26^{aB1}	3.84±0.36^{aA2}
	1.0	0	1	19.41±1.55^{aA3}	4.13±0.04^{aA1}	0.39±0.11^{aA1}	3.40±0.30^{aA1}
			5	17.76±2.72^{aA2}	3.61±0.14^{aA1}	0.40±0.16^{aA1}	3.48±0.19^{aA1}
			10	17.63±0.69^{aA1}	3.56±0.06^{aA1}	0.45±0.21^{aA1}	3.75±0.20^{aA2}
		50	1	19.25±0.77^{aA3}	3.61±0.12^{aA1}	0.41±0.11^{aB1}	3.53±0.28^{aA1}
			5	17.65±1.63^{aA2}	3.56±0.19^{aA1}	0.42±0.13^{aB1}	3.72±0.13^{aA1}
			10	17.38±0.35^{aA1}	3.45±0.21^{aA1}	0.47±0.15^{aB1}	3.78±0.36^{aA2}
		100	1	18.01±0.66^{aA3}	3.45±0.27^{aA1}	0.42±0.24^{aB1}	3.11±0.31^{aA1}
			5	17.70±2.26^{aA2}	3.41±0.08^{aA1}	0.47±0.34^{aB1}	3.65±0.40^{aA1}
			10	17.27±0.08^{aA1}	3.39±0.64^{aA1}	0.48±0.40^{aB1}	3.90±0.28^{aA2}

Results are given as mean ± standard deviation. Lowercase letters indicate differences between concentrations for the same pretreatment agent applied according to Duncan's multiple comparison test, capital letters indicate differences between ultrasound powers for the same pretreatment agent applied, and numbers indicate differences between immersion times in the same pretreatment agent applied ($P<0.05$).

3.3. Sugar content of pears dried by different pretreatments

Sugar components and the total sugar amount of pear samples dried by pretreatment are given in Table 3. Glucose and fructose values of all samples dried by pre-treatment were higher than those of fresh pears, while sucrose values were lower. It is thought that this situation may be due to sucrose inversion during the drying process. When the applications are compared, the amounts of sugar components in the samples treated with maltodextrin are higher than in the samples treated with ascorbic acid. In addition, the results of the 15 °Bx concentration were higher than those of the 7 °Bx concentration in the maltodextrin treatment. It is thought that these differences are due to the transfer of sugar components from the maltodextrin solution to the pear slices during immersion. When the total value of sugar components is examined, it is seen that the total sugar content of the samples applied with maltodextrin is higher than fresh pears, while the samples applied with ascorbic acid are lower. This situation is thought to be due to the reverse movement of the sugar components during immersion in ascorbic acid and their transfer to the solvent. It was determined that increasing the ultrasound application time in slices applied with both maltodextrin and ascorbic acid decreased the amount of sugar. Şahin et al. (2012) immersed tomato slices in a solution consisting of 1% ascorbic acid and 1% citric acid before drying and stated that there was a decrease in sugar amounts after the drying process due to mass transfer from the tomato samples to the solution mixture during immersion. In our study, although the changes reported for sugar components were not in large ranges, the difference between both maltodextrin and ascorbic acid concentrations on glucose results was found to be statistically significant ($P<0.05$). The difference between ultrasound powers was found to be significant for fructose and sucrose values in maltodextrin application ($P<0.05$), while it was found to be significant for glucose and total sugar results in ascorbic acid application ($P<0.05$).

Table 3. The sugar content of pretreated dried pears

Concentration (°Bx)		Ultrasound (%)	Time (min.)	Glucose (g 100g^{-1} DM)	Fructose (g 100g^{-1} DM)	Sucrose (g 100g^{-1} DM)	Total (g 100g^{-1} DM)
Fresh Pear		0	0	14.76±2.15	26.51±3.42	6.63±1.13	47.90±5.02
Maltodextrin	7	0	1	19.20±3.06^{aA1}	30.15±6.65^{aB1}	2.02±0.03^{aAB1}	51.37±3.98^{aA1}
			5	20.03±2.95^{aA1}	30.24±6.13^{aB1}	4.00±0.52^{aAB1}	54.27±4.79^{aA1}
			10	20.00±3.11^{aA1}	30.18±5.88^{aB1}	3.83±0.77^{aAB1}	54.01±7.43^{aA1}
		50	1	19.35±2.32^{aA1}	29.23±2.34^{aA1}	1.94±0.88^{aB1}	50.52±6.11^{aA1}
			5	19.25±1.94^{aA1}	29.14±2.36^{aA1}	3.88±0.92^{aB1}	52.27±8.67^{aA1}
			10	19.22±3.08^{aA1}	29.38±4.55^{aA1}	3.21±0.96^{aB1}	51.81±4.89^{aA1}
		100	1	18.44±3.66^{aA1}	28.85±5.42^{aB1}	1.78±0.49^{aA1}	49.07±9.01^{aA1}
			5	18.40±3.07^{aA1}	28.31±5.67^{aB1}	3.85±0.41^{aA1}	50.56±8.26^{aA1}
			10	18.55±3.43^{aA1}	28.14±2.63^{aB1}	3.15±0.34^{aA1}	49.84±4.04^{aA1}
	15	0	1	19.78±2.88^{bA1}	31.82±7.42^{aB1}	2.88±0.67^{aAB1}	54.48±5.85^{bA1}
			5	21.77±2.56^{bA1}	31.05±5.44^{aB1}	3.13±0.21^{aAB1}	55.95±3.27^{bA1}
			10	21.08±1.93^{bA1}	31.01±5.21^{aB1}	3.36±0.47^{aAB1}	55.45±7.86^{bA1}
		50	1	19.49±3.42^{bA1}	30.36±5.07^{aA1}	2.80±0.40^{aB1}	52.65±6.28^{bA1}
			5	20.01±3.06^{bA1}	30.75±4.12^{aA1}	3.95±0.47^{aB1}	54.71±5.07^{bA1}
			10	20.44±3.13^{bA1}	30.91±5.47^{aA1}	3.98±0.59^{aB1}	55.33±6.90^{bA1}
		100	1	19.03±1.77^{bA1}	30.13±5.27^{aB1}	2.77±0.23^{aA1}	51.93±5.08^{bA1}
			5	19.85±1.96^{bA1}	29.73±7.11^{aB1}	3.92±0.29^{aA1}	53.50±6.99^{bA1}
			10	18.17±1.56^{bA1}	28.89±6.43^{aB1}	3.25±0.18^{aA1}	50.31±3.51^{bA1}
Ascorbic Acid	0.5	0	1	17.23±4.15^{bB1}	27.10±5.77^{aA1}	1.96±0.10^{aA1}	46.29±7.90^{aB1}
			5	17.18±2.01^{bB1}	27.13±6.19^{aA1}	1.39±0.18^{aA1}	45.70±5.20^{aB1}
			10	17.21±2.72^{bB1}	26.47±3.90^{aA1}	1.70±0.15^{aA1}	45.38±6.09^{aB1}
		50	1	17.16±3.77^{bA1}	26.89±2.78^{aA1}	1.88±0.13^{aA1}	45.93±2.99^{aA1}
			5	17.06±1.42^{bA1}	26.75±5.09^{aA1}	1.75±0.31^{aA1}	45.46±4.06^{aA1}
			10	17.02±3.56^{bA1}	25.23±3.75^{aA1}	1.56±0.11^{aA1}	43.70±5.33^{aA1}
		100	1	17.01±3.44^{bAB1}	26.66±3.77^{aA1}	1.41±0.07^{aA1}	45.08±4.79^{aAB1}
			5	17.02±2.90^{bAB1}	26.23±5.42^{aA1}	1.65±0.18^{aA1}	44.90±5.13^{aAB1}
			10	17.00±3.82^{bAB1}	25.18±2.08^{aA1}	1.50±0.27^{aA1}	43.68±5.04^{aAB1}
	1.0	0	1	17.44±1.14^{aB1}	27.16±5.67^{aA1}	1.93±0.18^{aA1}	46.53±6.47^{aB1}
			5	17.24±2.79^{aB1}	27.34±2.22^{aA1}	1.55±0.26^{aA1}	46.13±7.01^{aB1}
			10	17.51±2.55^{aB1}	27.86±3.90^{aA1}	1.72±0.05^{aA1}	47.09±2.69^{aB1}
		50	1	17.24±1.05^{aA1}	26.90±5.44^{aA1}	1.82±0.14^{aA1}	45.96±5.37^{aA1}
			5	17.87±2.16^{aA1}	26.83±3.99^{aA1}	1.33±0.18^{aA1}	46.03±7.18^{aA1}
			10	17.50±2.02^{aA1}	26.22±1.56^{aA1}	1.65±0.19^{aA1}	45.37±4.03^{aA1}
		100	1	17.13±3.66^{aAB1}	26.69±6.01^{aA1}	1.43±0.21^{aA1}	45.25±5.49^{aAB1}
			5	17.63±3.07^{aAB1}	25.93±2.78^{aA1}	1.84±0.27^{aA1}	45.40±1.58^{aAB1}
			10	17.12±2.33^{aAB1}	25.30±4.94^{aA1}	1.71±0.22^{aA1}	44.13±2.27^{aAB1}

Results are given as mean ± standard deviation. Lowercase letters indicate differences between concentrations according to Duncan's multiple comparison test, capital letters indicate differences between ultrasound powers, and numbers indicate differences between immersion times ($P<0.05$). Total: represents the sum of glucose, fructose, and sucrose amounts.

3.4. Results of HMF, phenolic compounds, and antioxidant activities of pears dried by different pretreatments

The HMF and phenolic substances of maltodextrin treated samples are presented in Table 4. While HMF and ellagic acid were not detected in fresh Deveci pear, chlorogenic acid and rutin were detected. In the samples dried after pretreatment, HMF and chlorogenic acid were detected and their amounts were determined along with chlorogenic acid and rutin. The samples with a longer immersion time (10 minutes) in a 15 °Bx maltodextrin solution showed higher HMF levels compared to all other treatments. The reason for this may be that more sugar is transferred to the pear slices at high concentration and long immersion time and Maillard reactions occur more during drying. At high immersion times (5 and 10 minutes), the HMF amounts of the samples applied with ascorbic acid were found to be lower than the samples applied with maltodextrin. It is noteworthy that the amount of HMF

decreased as the immersion time in ascorbic acid solutions increased in both ascorbic acid concentrations. The difference between immersion times on the amount of HMF in both maltodextrin and ascorbic acid applications was found to be statistically significant ($P<0.05$). Uğurlu et al. (2023) reported that the amount of HMF in apple chips dried with vacuum-assisted infrared ranged from 205.39 and 351.30 mg kg^{-1} DM. It was determined that the dominant phenolic compound in fresh and dried pears was chlorogenic acid. After immersion in both solutions, a decrease in the total rutin, chlorogenic acid, and phenolic compounds was found in dried samples compared to the fresh sample. This decrease is thought to be due to the transfer of phenolic compounds from pears to the immersion solutions during immersion and/or oxidation due to exposure to heat during drying. The detection of ellagic acid in dry samples, which cannot be detected in fresh samples, can be explained by the breakdown of ellagitannins during drying and the emergence of ellagic acid (Topuz et al., 2023). The values of the total chlorogenic acid and phenolic compounds of the samples dried by applying ascorbic acid pretreatment were found to be higher than the samples applied with maltodextrin. This situation is thought to be due to the antioxidant effect of ascorbic acid protecting the phenolic compounds. In addition, the values of the total chlorogenic acid and phenolic compounds increased as the infrared power and immersion times increased in both immersion solutions. This can be explained by the fact that bound phenolics become more free as the ultrasound power and time increase in the applications (Akdaş and Bakkalbaşı, 2017). While the difference between ultrasound powers in maltodextrin application was found to be significant for the total of chlorogenic acid and phenolic compounds ($P<0.05$), the difference between the applied concentrations, ultrasound powers, and immersion times in ascorbic acid application was found to be significant ($P<0.05$). The TPC, DPPH, and ABTS results of the samples are given in Table 5. It was noted that the TPC, DPP, and ABTS results of the pretreated dried samples were higher than the fresh Deveci pear. This is due to the transition of phenolic compounds from bound form to free form during drying and the increase in TPC amount and antioxidant activity. TPC, DPPH, and ABTS results were found to be higher in ascorbic acid applied samples compared to maltodextrin applied pears. This situation is thought to be due to the increase in the amount of antioxidant compounds in the samples due to the migration of ascorbic acid, a natural antioxidant, into the samples and the preservation of the existing phenolic compounds in the pear during drying. Wiriya et al. (2009) and Öztürk (2010) reported in their studies that among different immersion solutions, immersion in ascorbic acid provided the highest phenolic substance and antioxidant activity in the samples. In our study, as the immersion time increased in all applications, the TPC, DPPH, and ABTS values of the pear slices also increased. It was determined that the TPC, DPPH, and ABTS results of the samples from the immersion processes without ultrasound were higher than the samples with ultrasound. Similar results were also found by Nascimento et al. (2016) in the drying of dragon fruit peel at 70 ºC after ultrasound application. It was found that as the ultrasound power used in our study increased, the TPC amount and antioxidant activity values of the samples applied ascorbic acid decreased. In another study conducted on pear slices, it was found that the amount of TPC decreased as the applied ultrasound power level increased (Liu et al., 2019b). This may be due to the oxidation of phenolic compounds and ascorbic acid caused by ultrasound application. In samples immersed in maltodextrin solutions, as the ultrasound power increased, TPC and ABTS values decreased, while DPPH values showed a fluctuating change. In samples immersed in maltodextrin, the difference between immersion times was found significant for TPC, DPPH, and ABTS values ($P<0.05$), while the difference between ultrasound powers was found significant only for ABTS ($P<0.05$). In the samples immersed in ascorbic acid, the difference between the ultrasound powers applied on the TPC amount and the difference between the immersion times were found to be statistically significant ($P<0.05$), while the difference between the concentrations and the immersion times for the DPPH and ABTS results were found to be significant ($P<0.05$).

Table 4. HMF and phenolic substance distribution results of Deveci pears treated with maltodextrin and ascorbic acid (mg kg^{-1} DM)

Concentration (ºBx)		Ultrasound (%)	Time (min.)	HMF	Chlorogenic	Ellagic	Rutin	Total
Fresh Pear		0	0	-	43.68±6.15	-	10.64±1.88	54.32±5.12
Maltodextrin	7	0	1	1.72±0.03^{aA12}	14.23±3.01^{aA1}	0.21±0.03^{aA1}	0.10±0.01^{aA1}	16.26±2.10^{aA1}
			5	1.85±0.01^{aA1}	14.80±2.22^{aA1}	0.34±0.01^{aA1}	0.08±0.01^{aA1}	17.07±2.61^{aA1}
			10	2.05±0.01^{aA2}	18.00±2.78^{aA1}	0.37±0.06^{aA1}	0.05±0.01^{aA1}	20.47±3.27^{aA1}
		50	1	1.97±0.02^{aA12}	17.54±1.47^{aAB1}	0.24±0.01^{aA1}	0.13±0.01^{aB1}	19.88±2.09^{aAB1}
			5	1.24±0.01^{aA1}	18.57±2.21^{aAB1}	0.26±0.01^{aA1}	0.10±0.02^{aB1}	20.17±3.31^{aAB1}
			10	1.15±0.02^{aA2}	19.99±1.66^{aAB1}	0.41±0.02^{aA1}	0.07±0.01^{aB1}	21.62±2.18^{aAB1}
		100	1	1.44±0.03^{aA12}	18.78±4.11^{aB1}	0.33±0.04^{aA1}	0.14±0.02^{aA1}	20.69±3.71^{aB1}
			5	1.12±0.02^{aA1}	20.23±2.67^{aB1}	0.37±0.02^{aA1}	0.11±0.02^{aA1}	21.83±2.28^{aB1}
			10	0.74±0.01^{aA2}	21.45±3.05^{aB1}	0.44±0.03^{aA1}	0.09±0.01^{aA1}	22.72±3.79^{aB1}
	15	0	1	0.75±0.01^{aA12}	15.23±1.90^{aA1}	0.41±0.05^{bA1}	0.10±0.01^{aA1}	16.49±2.10^{bA1}
			5	1.31±0.02^{zA1}	16.43±1.04^{aA1}	0.55±0.06^{bA1}	0.11±0.01^{aA1}	18.40±3.34^{bA1}
			10	3.92±0.50^{aA2}	18.72±1.17^{aA1}	0.92±0.08^{bA1}	0.13±0.02^{aA1}	23.69±3.27^{bA1}
		50	1	0.70±0.01^{aA12}	15.68±3.41^{aAB1}	0.76±0.01^{bA1}	0.18±0.03^{aB1}	17.32±2.19^{bAB1}
			5	1.28±0.03^{aA1}	16.90±2.36^{aAB1}	0.95±0.02^{bA1}	0.25±.0.02^{aB1}	19.38±3.23^{bAB1}
			10	3.79±0.29^{aA2}	20.76±1.18^{aAB1}	1.21±0.03^{bA1}	0.36±0.02^{aB1}	26.12±2.07^{bAB1}
		100	1	0.56±0.01^{aA12}	16.06±1.10^{aB1}	0.87±0.02^{bA1}	0.27±0.01^{aA1}	17.76±1.08^{bB1}
			5	1.25±0.01^{aA1}	17.74±1.02^{aB1}	0.97±0.01^{bA1}	0.39±0.02^{aA1}	20.35±2.26^{bB1}
			10	3.50±0.48^{aA2}	24.21±3.25^{aB1}	1.45±0.01^{bA1}	0.44±0.02^{aA1}	29.60±4.23^{bB1}
Ascorbic Acid	0.5	0	1	1.87±0.02^{aA12}	22.82±2.46^{aA12}	0.08±0.01^{aA1}	0.06±0.01^{aA1}	24.83±3.44^{aA12}
			5	1.34±0.01^{aA1}	23.60±3.31^{aA1}	0.18±0.02^{aA1}	0.11±0.02^{aA1}	25.23±4.12^{aA1}
			10	0.92±0.01^{aA2}	26.56±3.45^{aA2}	0.20±0.01^{aA1}	0.13±0.00^{aA1}	27.81±4.03^{aA2}
		50	1	1.63±0.07^{aA12}	23.78±5.44^{aAB12}	0.11±0.02^{aA1}	0.11±0.02^{aA1}	25.63±5.20^{aAB12}
			5	0.97±0.02^{aA1}	25.60±4.19^{aAB1}	0.22±0.03^{aA1}	0.13±0.03^{aA1}	26.92±4.17^{aAB1}
			10	0.95±0.06^{aA2}	31.24±3.78^{aAB2}	0.29±0.01^{aA1}	0.17±0.01^{aA1}	32.65±5.23^{aAB2}
		100	1	1.38±0.31^{aA12}	23.82±4.43^{aB12}	0.14±0.03^{aA1}	0.14±0.02^{aA1}	25.58±3.45^{aB12}
			5	0.33±0.04^{aA1}	25.64±4.83^{aB1}	0.27±0.01^{aA1}	0.18±0.02^{aA1}	26.42±2.90^{aB1}
			10	0.27±0.02^{aA2}	34.44±2.09^{aB2}	0.32±0.05^{aA1}	0.24±0.01^{aA1}	35.27±2.57^{aB2}
	1.0	0	1	1.22±0.02^{aA12}	23.96±2.07^{bA12}	0.11±0.02^{bA1}	0.19±0.03^{bA1}	25.48±3.78^{bA12}
			5	1.90±0.03^{aA1}	29.98±2.68^{bA1}	0.13±0.01^{bA1}	0.20±0.01^{bA1}	32.21±4.27^{bA1}
			10	0.86±0.00^{aA2}	31.54±4.98^{bA2}	0.25±0.03^{bA1}	0.27±0.02^{bA1}	32.92±3.16^{bA2}
		50	1	0.85±0.03^{aA12}	32.15±5.17^{bAB12}	0.18±0.04^{bA1}	0.22±0.04^{bA1}	33.40±4.83^{bAB12}
			5	0.34±0.02^{aA1}	36.44±3.60^{bAB1}	0.27±0.01^{bA1}	0.29±0.03^{bA1}	37.34±2.19^{bAB1}
			10	0.25±0.08^{aA2}	41.04±4.18^{bAB2}	0.34±0.03^{bA1}	0.34±0.03^{bA1}	41.96±3.75^{bAB2}
		100	1	0.44±0.06^{aA12}	34.88±5.11^{bB12}	0.20±0.02^{bA1}	0.23±0.03^{bA1}	35.75±4.26^{bB12}
			5	0.30±0.17^{aA1}	37.69±4.18^{bB1}	0.31±0.01^{bA1}	0.34±0.01^{bA1}	38.64±4.39^{bB1}
			10	0.21±0.13^{aA2}	41.28±5.12^{bB2}	0.55±0.01^{bA1}	0.38±0.03^{bA1}	42.42±3.27^{bB2}

Results are given as mean ± standard deviation. Lowercase letters indicate differences between concentrations according to Duncan's multiple comparison test, capital letters indicate differences between ultrasound powers, and numbers indicate differences between immersion times ($P<0.05$). Total: represents the sum of HMF, phenolic acids, and rutin.

3.5. Results of TPC, DPPH and ABTS

The TPC, DPPH, and ABTS results of the samples are given in Table 5. It was noted that the TPC, DPPH, and ABTS results of the pretreated dried samples were higher than the fresh Deveci pear. This is due to the transition of phenolic compounds from bound form to free form during drying and the increase in TPC amount and antioxidant activity. TPC, DPPH, and ABTS results were found to be higher in ascorbic acid applied samples compared to maltodextrin applied pears. This situation is thought to be due to the increase in the amount of antioxidant compounds in the samples due to the migration of ascorbic acid, a natural antioxidant, into the samples and the preservation of the existing phenolic compounds in the pear during drying. In our study, as the immersion time increased in all applications, the TPC, DPPH, and ABTS values of the pear slices also increased. It was determined that the TPC, DPPH, and ABTS results of the samples from the immersion processes without ultrasound were higher than the samples with ultrasound. Similar results were also found by Nascimento et al. (2016) in the drying of dragon fruit peel at 70 ºC after ultrasound application. It was found that as the ultrasound power used in our study increased, the TPC amount and antioxidant activity values of the samples applied ascorbic acid decreased. In another study conducted on pear slices, it was found that the amount of TPC decreased as the applied ultrasound power level increased (Liu et al., 2019b). This may be due

to the oxidation of phenolic compounds and ascorbic acid caused by ultrasound application. In samples immersed in maltodextrin solutions, as the ultrasound power increased, TPC and ABTS values decreased, while DPPH values showed a fluctuating change. In samples immersed in maltodextrin, the difference between immersion times was found significant for TPC, DPPH, and ABTS values ($P<0.05$), while the difference between ultrasound powers was found significant only for ABTS ($P<0.05$). In the samples immersed in ascorbic acid, the difference between the ultrasound powers applied on the TPC amount and the difference between the immersion times were found to be statistically significant ($P<0.05$), while the difference between the concentrations and the immersion times for the DPPH and ABTS results were found to be significant ($P<0.05$).

Table 5. TPC, DPPH, and ABTS results of pears dried with different pretreatments

Concentration (°Bx)		Ultrasound (%)	Time (min.)	TPC (mg GAE kg^{-1} DM)	DPPH (mmol Trolox eq g^{-1} DM)	ABTS (mmol Trolox eq g^{-1} DM)
Fresh Pear		0	0	2461.35±300.79	407.76±25.15	206.79±22.68
Maltodextrin	7	0	1	2903.73±258.05^{aA1}	485.38±20.47^{aA1}	231.55±18.30^{aA1}
			5	3269.34±389.98^{aA2}	501.33±33.19^{aA1}	250.12±26.00^{aA1}
			10	3687.82±58.55^{aA3}	690.94±14.91^{aA2}	265.84±40.71^{aA2}
		50	1	2832.84±200.13^{aA1}	453.07±55.26^{aA1}	212.61±24.50^{aA1}
			5	3231.15±150.77^{aA2}	520.69±33.95^{aA1}	222.72±41.51^{aA1}
			10	3597.63±95.25^{aA3}	663.76±65.9^{aA2}	248.95±12.36^{aA2}
		100	1	2783.27±228.04^{aA1}	450.28±93.51^{aA1}	209.59±35.45^{aA1}
			5	3092.02±206.52^{aA2}	510.33±89.51^{aA1}	213.68±34.69^{aA1}
			10	3350.52±392.94^{aA3}	626.33±50.40^{aA2}	225.04±15.34^{aA2}
	15	0	1	2748.57±363.99^{aA1}	483.83±87.74^{aA1}	235.72±31.57^{aB1}
			5	3298.00±103.07^{aA2}	572.95±57.93^{aA1}	253.74±22.27^{aB1}
			10	4623.00±52.43^{aA3}	635.16±109.62^{aA2}	268.79±23.18^{aB2}
		50	1	2672.86±124.37^{aA1}	583.46±16.98^{aA1}	228.20±42.59^{aB1}
			5	2928.26±183.08^{aA2}	584.20±23.14^{aA1}	229.69±34.57^{aB1}
			10	4046.79±87.34^{aA3}	633.77±68.95^{aA2}	242.37±35.24^{aB2}
		100	1	2509.72±317.33^{aA1}	470.86±72.63^{aA1}	214.35±28.42^{aB1}
			5	2884.64±151.70^{aA2}	526.98±72.69^{aA1}	229.18±68.95^{aB1}
			10	3841.87±370.20^{aA3}	627.60±52.94^{aA2}	238.66±58.06^{aB2}
Ascorbic Acid	0.5	0	1	3640.66±383.91^{aB1}	649.49±117.26^{aA1}	255.34±31.74^{aA1}
			5	3742.57±332.14^{aB2}	659.75±81.54^{aA1}	266.35±42.37^{aA1}
			10	4222.89±196.29^{aB3}	712.05±71.56^{aA2}	305.15±44.43^{aA2}
		50	1	3469.41±351.28^{aB1}	644.26±39.87^{aA1}	237.21±12.41^{aA1}
			5	3917.68±253.80^{aB2}	671.29±16.54^{aA1}	254.10±22.22^{aA1}
			10	4198.88±445.30^{aB3}	693.36±15.14^{aA2}	282.54±49.30^{aA2}
		100	1	2591.49±300.14^{aA1}	618.07±31.09^{aA1}	216.98±22.25^{aA1}
			5	3903.12±120.31^{aA2}	627.73±91.03^{aA1}	253.43±30.06^{aA1}
			10	3934.57±400.49^{aA3}	646.24±45.91^{aA2}	278.17±23.10^{aA2}
	1.0	0	1	4182.44±245.02^{aB1}	714.74±27.16^{bA1}	315.61±27.62^{bA1}
			5	4543.37±52.04^{aB2}	728.75±24.25^{bA1}	396.43±12.90^{bA1}
			10	5490.56±318.57^{aB3}	818.85±153.38^{bA2}	451.27±10.47^{bA2}
		50	1	3247.15±457.21^{aB1}	706.22±42.97^{bA1}	284.02±14.04^{bA1}
			5	3375.44±493.41^{aB2}	738.17±142.01^{bA1}	393.43±27.00^{bA1}
			10	3805.28±45.04^{aB3}	769.46±8.60^{bA2}	415.86±107.68^{bA2}
		100	1	2865.96±380.02^{aA1}	698.55±35.28^{bA1}	270.73±22.30^{bA1}
			5	2988.59±300.68^{aA2}	715.32±24.98^{bA1}	344.48±22.46^{bA1}
			10	3770.01±432.19^{aA3}	742.75±36.33^{bA2}	410.75±39.63^{bA2}

Results are given as mean ± standard deviation. Lowercase letters indicate differences between concentrations according to Duncan's multiple comparison test, capital letters indicate differences between ultrasound powers, and numbers indicate differences between immersion times ($P<0.05$).

3.6. Sensory evaluation results of dried pears

The sensory evaluation results of pear samples dried after different pretreatments are given in Table 6. In the maltodextrin applied samples, the panelists gave the highest scores on the appearance, color, hardness, and chewiness scales to the pear slices that were not exposed to ultrasound and immersed in the 15°Bx maltodextrin solution for 5 minutes. This sample also received the highest

general appreciation of 7.33. In general, it was concluded that the panelists preferred the samples immersed in the 15 °Bx maltodextrin solution, which had a sweeter taste, compared to the samples applied with 7 °Bx maltodextrin. In the samples immersed in ascorbic acid, the highest general appreciation score was obtained with 7.46 in the samples immersed in 0.5% ascorbic acid solution at 50% ultrasound power for 10 minutes. These samples also received the highest score for appearance, hardness, and chewiness. The difference between the concentrations on color scores in both immersion solutions was found to be statistically significant ($P<0.05$). The difference between the immersion times on the appearance and general appreciation scores in maltodextrin application was found to be statistically significant ($P<0.05$). Also, the difference between the ultrasound powers and immersion times on the hardness appreciation scores in ascorbic acid samples was found to be statistically significant ($P<0.05$). In a study where pomegranate juice was mixed with maltodextrin powder of 7 and 18 DE and dried, it was stated that the pomegranate powders dried using 18 DE were lighter in color and the panelists did not prefer these light-colored pomegranate powders (Yaşar, 2008). In our study, the color of the pears became slightly lighter as the maltodextrin to 18 DE, but these samples received higher general appreciation scores from the panelists.

Table 6. Sensory evaluation results of dry samples

	°Bx	Ultrasound (%)	Time (min.)	Appearance	Color	Smell	Hardness degree	Hardness appreciation	Chewiness	Taste	General appreciation
Maltodextrin	7	0	1	5.26±1.05^{aA1}	5.20±0.34^{aA1}	5.80±1.07^{aA1}	6.33±0.83^{aA1}	6.40±0.79^{aA1}	6.80±0.90^{aA1}	6.60±0.93^{aA1}	6.26±0.88^{aA1}
			5	5.93±1.03^{aA2}	6.13±1.10^{aA1}	6.40±1.22^{aA1}	5.06±0.73^{aA1}	5.66±1.16^{aA1}	5.86±0.70^{aA1}	6.33±1.12^{aA1}	6.46±1.05^{aA2}
			10	6.33±0.99^{aA12}	6.26±1.11^{aA1}	6.00±0.75^{aA1}	6.00±0.77^{aA1}	6.53±0.56^{aA1}	6.80±0.89^{aA1}	6.86±0.49^{aA1}	6.86±0.91^{aA12}
		50	1	4.46±1.00^{aA1}	4.53±0.75^{aA1}	6.13±1.12^{aA1}	5.40±0.69^{aA1}	5.66±1.27^{aA1}	5.66±1.21^{aA1}	5.60±0.82^{aA1}	5.73±0.42^{aA1}
			5	5.33±0.76^{aA2}	5.60±0.6^{aA1}	6.53±0.78^{aA1}	6.53±1.11^{aA1}	6.26±0.95^{aA1}	5.20±0.68^{aA1}	5.86±0.81^{aA1}	5.86±0.79^{aA2}
			10	6.66±0.83^{aA12}	6.66±1.27^{aA1}	6.46±0.65^{aA1}	6.66±0.92^{aA1}	6.60±0.85^{aA1}	7.13±0.99^{aA1}	7.53±0.91^{aA1}	7.10±0.91^{aA12}
		100	1	5.73±1.09^{aA1}	5.60±1.26^{aA1}	6.40±0.88^{aA1}	6.20±0.67^{aA1}	6.66±0.86^{aA1}	6.53±1.00^{aA1}	5.66±0.74^{aA1}	6.00±1.25^{aA1}
			5	6.86±1.11^{aA2}	7.13±1.03^{aA1}	6.73±1.01^{aA1}	5.33±0.74^{aA1}	6.06±0.77^{aA1}	6.06±0.83^{aA1}	6.33±0.75^{aA1}	6.33±0.51^{aA2}
			10	5.86±0.54^{aA12}	5.93±0.91^{aA1}	6.13±1.10^{aA1}	6.33±0.75^{aA1}	6.40±0.95^{aA1}	6.46±1.05^{aA1}	6.80±1.04^{aA1}	6.46±1.05^{aA12}
	15	0	1	5.93±0.81^{bA1}	6.20±0.96^{bA1}	6.60±0.94^{aA1}	6.66±0.63^{aA1}	6.80±0.65^{aA1}	6.86±0.75^{aA1}	6.66±1.03^{aA1}	6.60±0.96^{aA1}
			5	7.06±1.14^{bA2}	7.20±0.99^{bA1}	6.53±1.13^{aA1}	6.46±0.98^{aA1}	6.96±0.82^{aA1}	7.25±0.86^{aA1}	7.20±0.60^{aA1}	7.33±0.79^{aA2}
			10	5.60±0.58^{bA12}	5.40±0.67^{bA1}	6.13±1.03^{aA1}	6.53±1.15^{aA1}	6.46±1.13^{aA1}	6.66±0.99^{aA1}	6.06±0.88^{aA1}	6.06±1.04^{aA12}
		50	1	6.33±1.25^{bA1}	6.13±0.70^{bA1}	6.46±0.99^{aA1}	5.86±0.98^{aA1}	6.53±0.78^{aA1}	6.06±0.81 ^{A1}	6.06±1.19^{aA1}	6.13±1.08^{aA1}
			5	6.06±1.07^{bA2}	6.53±1.34^{bA1}	6.46±1.12^{aA1}	7.40±0.92^{aA1}	6.80±0.83^{aA1}	6.80±0.87^{aA1}	7.40±0.93^{aA1}	7.20±0.69^{aA2}
			10	6.00±0.84^{bA12}	6.20±0.74^{bA1}	6.40±0.91^{aA1}	6.33±0.85^{aA1}	6.46±0.93^{aA1}	6.33±0.79^{aA1}	6.53±0.99^{aA1}	6.66±0.82^{aA12}
		100	1	5.46±0.68^{bA1}	5.80±0.86^{bA1}	5.80±0.93^{aA1}	7.26±0.96^{aA1}	5.20±1.06^{aA1}	6.13±0.75^{aA1}	6.20±0.80^{aA1}	5.86±0.92^{aA1}
			5	6.53±1.16^{bA2}	6.73±1.13^{bA1}	6.13±1.16^{aA1}	7.06±0.78^{aA1}	6.93±0.98^{aA1}	7.20±0.86^{aA1}	7.13±0.74^{aA1}	7.26±0.94^{aA2}
			10	6.40±0.86^{bA12}	6.66±0.72^{bA1}	6.26±0.94^{aA1}	5.66±0.74^{aA1}	6.60±0.81^{aA1}	6.40±1.10^{aA1}	7.13±0.65^{aA1}	6.73±0.84^{aA12}
Ascorbic Acid	0.5	0	1	6.20±1.02 ^{aA1}	6.00±1.28^{aA1}	6.60±0.80^{aA1}	6.20±0.78^{aA1}	7.13±0.83^{aA1}	7.40±0.82^{aA1}	6.93±0.86^{aA1}	7.06±1.32^{aA1}
			5	5.00±0.95^{aA1}	4.60±0.81^{aA1}	6.20±0.99^{aA1}	5.20±0.99^{aA1}	6.06±1.17^{aA2}	6.00±0.91^{aA1}	6.40±0.72^{aA1}	6.26±1.09^{aA1}
			10	5.46±1.14^{aA1}	5.86±0.77^{aA1}	6.33±0.81^{aA1}	6.33±0.63^{aA1}	6.00±0.94^{aA12}	6.40±0.70^{aA1}	6.66±0.82^{aA1}	6.66±0.91^{aA1}
		50	1	5.53±0.69^{aA1}	6.06±1.00^{aA1}	6.46±1.23^{aA1}	6.46±0.98^{aA1}	6.46±1.12^{aB1}	6.46±1.15^{aA1}	6.53±0.99^{aA1}	6.60±1.00^{aA1}
			5	6.13±0.96^{aA1}	6.20±0.78^{aA1}	6.20±1.14^{aA1}	6.60±0.91^{aA1}	6.73±0.75^{aB2}	6.46±0.56^{aA1}	6.53±1.16^{aA1}	6.53±0.92^{aA1}
			10	6.80±0.69^{aA1}	6.73±0.96^{aA1}	6.61±0.98^{aA1}	6.00±1.12^{aA1}	7.23±0.65^{aB12}	7.60±0.72^{aA1}	7.20±0.93^{aA1}	7.46±0.85^{aA1}
		100	1	6.26±1.18^{aA1}	6.26±1.03^{aA1}	6.40±0.96^{aA1}	5.46±0.67^{aA1}	6.00±0.81^{aB1}	5.60±0.78^{aA1}	6.73±1.00^{aA1}	6.53±1.35^{aA1}
			5	5.20±0.59^{aA1}	5.33±0.67^{aA1}	6.53±0.87^{aA1}	5.26±0.79^{aA1}	6.06±0.68^{aB2}	6.20±1.05^{aA1}	6.13±1.16^{aA1}	6.33±0.77^{aA1}
			10	6.40±0.75^{aA1}	6.26±0.60^{aA1}	6.46±0.82^{aA1}	6.20±0.70^{aA1}	6.73±0.94^{aB12}	7.53±0.85^{aA1}	7.33±0.77^{aA1}	7.00±0.66^{aA1}
	1.0	0	1	6.26±1.23^{aA1}	6.66±1.26^{bA1}	6.26±1.13^{aA1}	6.46±1.24^{aA1}	6.46±0.96^{aA1}	6.46±1.05^{bA1}	6.86±1.13^{aA1}	6.93±1.18^{aA1}
			5	6.73±0.76^{aA1}	6.93±0.79^{bA1}	6.53±0.90^{aA1}	5.06±0.86^{aA1}	6.20±0.98^{aA2}	7.06±1.04^{bA1}	7.40±0.72^{aA1}	7.20±1.02^{aA1}
			10	6.40±0.71^{aA1}	6.53±0.75^{bA1}	6.20±1.03^{aA1}	5.66±0.88^{aA1}	6.80±0.85^{aA12}	7.00±0.97^{bA1}	6.80±0.79^{aA1}	6.86±0.82^{aA1}
		50	1	6.00±1.00^{aA1}	6.46±0.94^{bA1}	5.80±0.57^{aA1}	6.46±0.92^{aA1}	7.20±1.31^{aB1}	6.80±1.18^{bA1}	6.73±1.06^{aA1}	7.13±1.29^{aA1}
			5	6.13±0.78^{aA1}	6.06±0.66^{bA1}	6.40±1.12^{aA1}	5.33±0.83^{aA1}	6.93±1.22^{aB2}	7.46±0.63^{bA1}	7.13±0.83^{aA1}	7.13±0.^{91aA1}
			10	6.13±0.78^{aA1}	6.33±0.95^{bA1}	6.20±0.85^{aA1}	6.40±0.72^{aA1}	6.93±0.70^{aB12}	7.06±0.70^{bA1}	6.93±0.81^{aA1}	7.06±0.68^{aA1}
		100	1	5.66±0.58^{aA1}	6.46±0.75^{bA1}	6.06±0.61^{aA1}	6.20±0.78^{aA1}	6.20±0.94^{aB1}	6.26±1.12^{bA1}	6.40±1.08^{aA1}	6.80±1.27^{aA1}
			5	6.13±0.80^{aA1}	5.80±0.74^{bA1}	6.53±1.12^{aA1}	5.60±0.63^{aA1}	6.26±0.79^{aB2}	6.53±0.57^{bA1}	6.73±0.47^{aA1}	6.73±0.88^{aA1}
			10	6.20±0.91^{aA1}	6.20±0.53^{bA1}	6.60±0.97^{aA1}	6.46±1.07^{aA1}	6.20±0.20^{aB12}	6.86±0.96^{bA1}	6.93±1.05^{aA1}	6.80±0.96^{aA1}

Results are given as mean ± standard deviation. Lowercase letters indicate differences between concentrations according to Duncan's multiple comparison test, capital letters indicate differences between ultrasound powers, and numbers indicate differences between immersion times ($P<0.05$).

Conclusion

In this study, the effects of different solutions, solution concentrations, ultrasound power, and duration applied as pretreatment on some quality and nutritional properties of pears dried with a vacuum-assisted two-way infrared dryer were determined. With the increase in the application time of the pre-treatments, the RR values increased and had a positive effect on the quality in this respect. Maltodextrin solution facilitates drying due to its applicability in high concentrations however, it increases the sugar content in the sample. In addition, Maillard reactions develop more in products with maltodextrin pretreatment, and the amount of HMF, which is an intermediate product, is formed more. Ascorbic acid application improves the pH and acidity of the products. In contrast to the use of maltodextrin, the use of ascorbic acid was found to reduce the amount of HMF. Therefore, ascorbic acid can be evaluated as an agent that prevents HMF formation, in addition to its benefits such as color protection and oxidation prevention in drying systems that cause low HMF formation. In terms of phenolic compound content, ascorbic acid protects phenolic compounds better and supports antioxidant activity, especially at high concentrations such as 1%. On the other hand, the application of ultrasound and the increase in ultrasound power levels led to the disintegration of phenolic compounds, resulting in a slight reduction in the phenolic content and antioxidant activity of pear slices. However, the increase in the ultrasound application time causes a slight increase in the presence of phenolic compounds and antioxidant activity due to the release of bound phenolics rather than the degradation of phenolic compounds. In the sensory evaluation, the panelists gave the highest score to the sample treated for 10 minutes at 50% ultrasound amplitude at 0.5% ascorbic acid concentration. As a result, it has been shown that the pretreatments will increase the quality of pears dried with a vacuum-assisted two-way dryer and will produce healthier and more appreciated products.

Ethical Statement

Ethical approval is not required for this study because the pears used in the study were collected free of charge and were not protected pears.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Author Contributions

Fatma Coşkun: Conducting laboratory analyses; investigation; methodology; visualization. Serdar Uğurlu: Conducting laboratory analyses, Emre Bakkalbaşı: Methodology; supervisor.

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Research Article

Comparative Analysis of Nutritional Attributes in Karaerik and Kabuğu Yufka Grapevine Leaves Under Varying Bud Load

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Abstract: This study, investigated the effects of variety selection (*Vitis vinifera* cv. Karaerik and Kabugu Yufka) and bud load management (24, 36, 48, and 60 buds per vine) on the physiological and nutritional parameters of grapevine leaves over a two-year period. The research examined various leaf characteristics including dry matter content, ash content, acidity, pH, dietary fiber, vitamins C and E, total phenolic content, and macro and micromineral composition. The findings revealed significant differences among varieties across multiple parameters. Karaerik consistently demonstrated higher dry matter content, ash content, acidity, vitamin C levels, and generally superior macro and micronutrient concentrations. Conversely, Kabugu Yufka exhibited higher vitamin E content. Bud load treatments had varying effects, with some parameters, such as dry matter content and leaf acidity in the second year, showing significant responses to increased bud load. The study also observed year-to-year variations, with notable changes in vitamin C, total phenolic content, and mineral compositions between the two growing seasons. These findings' consistent superiority of Karaerik in several nutritional aspects suggests its potential for producing higher quality grape leaves for culinary or nutraceutical purposes. However, the higher vitamin E content in Kabugu Yufka leaves indicates that variety choice may depend on specific nutritional targets. The varied responses to bud load treatments highlight the importance of tailored management strategies for each variety to maximize desired leaf characteristics.

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1. Introduction

Grapes are among the few fruits with a remarkably diverse range of culinary applications, being processed into various products that play a significant role in both traditional and modern cuisine.

Examples of such grape-derived products include grape juice, pestil, bastık, molasses, köme, vinegar, rakı, wine, and köfter, each representing a distinct method of utilization. Beyond these products, vine leaves also hold considerable culinary value and can be consumed either fresh or vine leaves preserved (Cangi and Yagcı, 2017). In recent years, vine leaf production has developed into a distinct commercial sector within viticulture, expanding beyond conventional grape cultivation to include the specialized production of leaves for commercial purposes. This transformation has been driven, in part, by socio-economic changes, particularly the increasing participation of women in the workforce, which has led to a greater demand for convenient, ready-to-eat food products, including pickled vine leaves. Consequently, viticulture for leaf production has emerged as a profitable industry, further strengthened by expanding export opportunities that enhance its economic appeal (Yagcı et al., 2012). Moreover, in regions where viticultural practices are limited by specific climatic constraints, such as late spring frosts, critical temperature thresholds, and the increased frequency of extreme weather events, the cultivation of preserved vine leaves is considered a more sustainable and economically viable alternative to traditional grape production (Cangi and Yagcı, 2012). In addition, the increasing emphasis on health-conscious nutrition and functional food products has intensified interest in grapevine leaves, which are recognized as an excellent source of bioactive molecules, particularly phenolic compounds (Schoedl et al., 2012; Aguilar et al., 2016; Moldovan et al., 2020; Banjanin et al., 2021; Goicoechea et al., 2021; Maia et al., 2021; Yildiz et al., 2024). The growing recognition of their nutritional and health benefits has further underscored their potential as an alternative agricultural product, particularly in regions where ecological constraints pose challenges to grape cultivation.

This study systematically analyzed the effects of different bud load treatments on key quality parameters in the leaves of Karaerik and Kabugu Yufka grape varieties. The evaluated quality parameters included dry matter and ash content, titratable acidity, pH, leaf color, vitamin E and C content, dietary fiber content, total phenolic content, and the concentrations of various macro and microelements. The research was conducted in accordance with the agronomic and economic findings of previous studies carried out in the region (Kalkan et al., 2024a and 2024b). The primary objective was to provide a complementary assessment by examining the qualitative characteristics of the leaves of the selected cultivars. Furthermore, the integration of these qualitative assessments with agronomic performance and economic feasibility aimed to offer a more comprehensive understanding of vine leaf production in the region, thereby enhancing its potential as a sustainable and economically viable agricultural practice.

2. Material and Methods

2.1. Material

The study utilized two grapevine varieties, Karaerik and Kabugu Yufka, as plant material. Karaerik is characterized by thin, slightly wavy, five-lobed leaves with a bright green color, sparse pubescence, and prominent serrations. The petiole is of medium thickness, and the petiolar sinus is U- or V-shaped. Kabugu Yufka leaves are five-lobed, yellowish green in color, with sparse woolly pubescence. The petiole is of medium thickness, and the petiolar sinus is closed (Kalkan ve ark., 2024a) (Figure 1a and 1b).

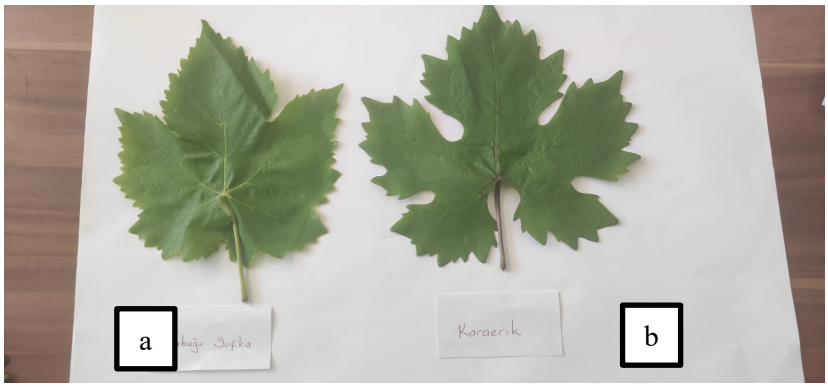

Figure 1. (a,b) Leaf images belong to Kabugu Yufka and Karaerik grape varieties.

2.2. Method

2.2.1. Data collection and analyses

Leaf harvesting for analysis commenced in the first week of June and concluded in the last week of July in both years. Leaves selected for sampling were the 4th, 5th, and 6th leaves on shoots that had reached two thirds of the size of a fully mature leaf from the tip, following the method described by Kılıç (2007) (Figure 2). A total of 25 leaves were collected for each treatment.

Figure 2. Leaves collected for pickling purposes (Photo: Kılıc, 2007).

2.2.2. Dry matter and ash content (%)

Dry matter content was determined by drying leaf samples in an oven at 105 °C until a constant weight was achieved. Ash content was measured by incinerating leaf samples in a muffle furnace at 500-600 °C until white ash was obtained (Dokuzlu, 2004).

2.2.3. Titratable acidity (%)

Titratable acidity was determined using the pH-metric method on an aqueous extract obtained from fresh leaves processed in a blender (Cemeroglu, 1992).

2.2.4. pH

Leaf samples were blended with a small amount of distilled water to form a puree. The pH was then measured by immersing a pH meter electrode in the puree (Cemeroglu, 1992).

2.2.5. Leaf color

Leaf color was measured using a Minolta colorimeter (Model CR-300) calibrated with a white standard plate (Y=92.40, x=0.3137, y=0.3195). Hunter color measurement parameters (L-brightness, a-red/green, b-yellow/blue) were recorded.

2.2.6. Vitamin C content

Ascorbic acid (Vitamin C) content was determined in fresh grape leaves using a modified method of Karhan et al. (2004) with High-Performance Liquid Chromatography (HPLC).

2.2.7. Vitamin E content

Vitamin E content was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) following the method described by Fiorentino et al. (2009).

2.2.8. Dietary fiber

Dietary fiber was determined using the AOAC Official Method 991.43 (Total, Soluble, and Insoluble Dietary Fiber in Foods, Enzymatic-Gravimetric Method, MES-TRIS Buffer, First Action 1991, Final Action 1994).

2.2.9. Mineral content

Concentrations of Ca, Fe, Mg, Mn, P, K, Zn, Cu, and Se were measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Vitamin E and mineral content analyses were conducted at the Chemistry Department, Faculty of Arts and Sciences, Erzincan Binali Yildirim University.

2.2.10. Total phenolic content

Total phenolic content (mg GAE/100 g) was determined using the Folin-Ciocalteu colorimetric method as described by Singleton and Rossi (1965).

2.3. Statistical analysis

Data were subjected to analysis of variance (ANOVA) using JUMP statistical software (version 7.0.1). Means were compared using the Least Significant Difference (LSD) test at a 5% significance level.

3. Results

3.1. Dry matter content in leaves of grape varieties

The effects of cultivar and bud load treatments on dry matter (DM) are given in Table 1. The effect of cultivars on % dry matter content was statistically significant (P<0.05) in both years. In the second year, treatments and variety x treatment interactions were found significant.

Table 1. Effect of variety and bud load on % dry matter in leaves

Year	Variety	Applications (bud/vine)				The main effect of variety
		24	36	48	60	
2021	Karaerik	28.20 ^{ns}	28.17	28.16	28.02	28.17 a
	Kabugu Yufka	28.00	27.70	27.15	26.48	27.38 b
	The effect of the treatment	28.10 ^{ns}	27.94	27.65	27.25	
2022	Karaerik	27.22 a	26.86 b	26.79 b	26.61 b	26.87 a
	Kabugu Yufka	26.13 c	26.10 c	26.03 c	25.18 d	25.86 b
	The effect of the treatment	26.67 a	26.48 ab	26.41 b	25.90 c	

The differences between the means indicated by different letters in the same column are statistically significant (p < 0.05). ns: not significant.

3.2. Leaf ash content in grape varieties

The effects of cultivar and bud load treatments on ash content are given in Table 2. While the effect of cultivars on ash content was found statistically significant (P<0.05), the difference between treatments was found significant in the second year.

Table 2. Effect of variety and bud load treatments on % ash in leaves

Year	Variety	Applications (bud/vine)				The main effect of variety
		24	36	48	60	
2021	Karaerik	1.58 ^{ns}	1.58	1.61	1.68	1.61 a
	Kabugu Yufka	1.45	1.50	1.55	1.63	1.53 b
	The effect of the treatment	1.52 ^{ns}	1.54	1.58	1.65	
2022	Karaerik	1.76 ^{ns}	1.70	1.69	1.66	1.70 a
	Kabugu Yufka	1.65	1.60	1.65	1.49	1.60 b
	The effect of the treatment	1.71 a	1.68 a	1.65 ab	1.57 b	

The differences between the means indicated by different letters in the same column are statistically significant (p < 0.05). ns: not significant.

3.3. Leaf acid content in grape varieties

The effects of cultivar and bud load treatments on acid content are given in Table 3. The effects of cultivar and bud load treatments on leaf acid content were statistically significant in both years. The effect of treatments was significant in the second year (P<0.05).

Table 3. Effect of variety and bud load treatments on % acid content in fresh leaves

Year	Variety	Applications (bud/vine)				The main effect of variety
		24	36	48	60	
2021	Karaerik	1.42 ns	1.50	1.46	1.54	1.46 a
	Kabugu Yufka	1.30	1.28	1.27	1.24	1.27 b
	The effect of the treatment	1.36 ns	1.39	1.34	1.39	
2022	Karaerik	1.23 ns	1.34	1.32	1.40	1.32 a
	Kabugu Yufka	1.24	1.20	1.37	1.38	1.29 b
	The effect of the treatment	1.24 c	1.27 bc	1.35 ab	1.39 a	

The differences between the means indicated by different letters in the same column are statistically significant (p < 0.05). ns: not significant.

3.4. Leaf pH value in grape varieties

The effects of cultivar and bud load treatments on pH values are given in Table 4. The pH values between varieties were statistically significant only in the first year (P<0.05). The differences in treatments and interactions were not statistically significant in both years.

Table 4. Effect of variety and bud load treatments on leaf pH in 2021 and 2022

Year	Variety	Applications (bud/vine)				The main effect of variety
		24	36	48	60	
2021	Karaerik	3.08	3.10	3.07	3.10	3.09 a
	Kabugu Yufka	3.06	3.06	3.06	3.04	3.06 b
	The effect of the treatment	3.07	3.08	3.07	3.07	
2022	Karaerik	3.32	3.30	3.28	3.28	3.30 ns
	Kabugu Yufka	3.29	3.33	3.30	3.30	3.30
	The effect of the treatment	3.30 ns	3.31	3.29	3.29	

The differences between the means indicated by different letters in the same column are statistically significant (p < 0.05). ns: not significant.

3.5. Leaf dietary fiber content in grape varieties

The results of the effect of cultivar and bud load on dietary fiber content are given in Table 5. The effect of cultivar and bud load treatments on leaf dietary fiber content in the first year was found statistically significant among cultivars, mean values, and cultivar x bud load interaction. In the second year, only the effect of cultivars was found statistically significant (P<0.05).

Table 5. Effects of bud load and variety on dietary fiber content (g 100 g^{-1}) in leaves

Year	Variety	Applications (bud/vine)				The main effect of variety
		24	36	48	60	
2021	Karaerik	11.03 d	11.55 b	11.25 c	11.90 a	11.43 a
	Kabugu Yufka	10.42 h	10.82 f	10.60 g	10.95e	10.70 b
	The effect of the treatment	10.73 d	11.19 b	10.93 c	11.43 a	
2022	Karaerik	13.97 ns	13.88	13.68	13.13	13.67 a
	Kabugu Yufka	11.79	11.68	12.28	10.65	11.60 b
	The effect of the treatment	12.89 ns	12.78	12.98	11.89	

The differences between the means indicated by different letters in the same column are statistically significant (p < 0.05). ns: not significant.

3.6. Vitamin C content in leaves of grape varieties

The effects of cultivar and bud load treatments on vitamin C content in leaves are given in Table 6. The effects of cultivar and bud load treatments on the amount of vitamin C in fresh leaves were found to be statistically significant among cultivars in both years (P<0.05). The mean values were not statistically significant in the cultivar x bud load interaction.

Table 6. Effect of variety and bud load on vitamin C content (mg 100 g^{-1}) in fresh leaves

Year	Variety	Applications (bud/vine)				The main effect of variety
		24	36	48	60	
2021	Karaerik	105.4 ns	97.50	117.8	106.5	106.79 a
	Kabugu Yufka	92.70	84.48	87.42	94.74	89.84 b
	The effect of the treatment	99.05 ns	90.99	102.6	100.6	
2022	Karaerik	130.47 ns	131.67	134.64	132.50	132.32 a
	Kabugu Yufka	112.73	116.76	117.49	121.38	117.09 b
	The effect of the treatment	121.60 ns	124.22	126.07	126.94	

The differences between the means indicated by different letters in the same column are statistically significant (p < 0.05). ns: not significant.

3.7. Vitamin E content in leaves of grape varieties

The data obtained for the effects of cultivar and bud load on vitamin E content in fresh leaves are given in Table 7. The effects of cultivar and bud load treatments on vitamin E content in leaves were found to be statistically significant in both years, among cultivars and in the cultivar x bud load interaction (P<0.05).

Table 7. Effect of variety and bud load on vitamin E content (mg 100 g^{-1}) in fresh leaves

Year	Variety	Applications (bud/vine)				The main effect of variety
		24	36	48	60	
2021	Karaerik	7.32c	8.69c	9.17c	7.30c	8.12 b
	Kabugu Yufka	21.82 a	17.03 b	19.27 ab	17.18 b	18.82 a
	The effect of the treatment	14.57 ns	12.86	14.22	12.24	
2022	Karaerik	15.38 c	15.73 c	12.14 c	13.98 c	14.31 b
	Kabugu Yufka	20.80 b	20.53 b	24.99 a	20.10 b	21.69 a
	The effect of the treatment	18.09 ns	18.13	18.57	17.04	

The differences between the means indicated by different letters in the same column are statistically significant (p < 0.05). ns: not significant.

3.8. Total phenolic content in grapevine leaves

The data obtained regarding the effects of cultivar and bud load treatments on total phenolic matter contents are given in Table 8. In the first year, total phenolic matter content between varieties was found to be statistically significant (P<0.05). There was no difference in the second year.

Table 8. Effect of variety and bud load on total phenolic matter content in leaves (mg GAE 100 g^{-1} DM)

Year	Variety	Applications (bud/vine)				The main effect of variety
		24	36	48	60	
2021	Karaerik	4093.6 ns	4002.9	3864.5	3930.7	3972.9 a
	Kabugu Yufka	3632.4	3340.3	3726.8	3744.8	3611.0 b
	The effect of the treatment	3863.0 ns	3671.6	3795.7	3837.7	
2022	Karaerik	3222.9 ns	3212.7	3019.1	3068.4	3130.8 ns
	Kabugu Yufka	2825.6	2815.3	2848.5	2912.4	2850.5
	The effect of the treatment	3024.26 ns	3014.0	2933.7	2990.4	

The differences between the means indicated by different letters in the same column are statistically significant (p < 0.05). ns: not significant.

3.9. Some macro mineral composition of grapevine leaves (K, Mg, P, Ca)

The effects of cultivar and bud load treatments on some macronutrients are given in Table 9. In terms of potassium content, cultivar x treatment interaction in the first year and differences between cultivars in the second year were found to be statistically significant. In terms of magnesium content, the differences between cultivars were found significant in both years. In terms of calcium content, only the differences between treatments were statistically significant ($P<0.05$). In terms of phosphorus content, the effect of cultivar and bud load treatments on phosphorus content in leaves was statistically significant between cultivars in the first year, but no difference was found in the second year.

Table 9. Effects of variety and bud load on some macro element amounts (ppm) in leaves

	Year	Variety	Applications (bud/vine)				The main effect of variety
			24	36	48	60	
Potassium	2021	Karaerik	2706.4 bc	2796.9 a-c	2810.7 a-c	2552.4 c	2716.5 ^{ns}
		Kabugu Yufka	2683.4 bc	2932.2ab	2581.1c	3002.0 a	2797.4
		The effect of the treatment	2694.7 ^{ns}	2695.9	2860.0	2777.2	
	2022	Karaerik	9196.9 ^{ns}	9189.7	8705.3	10050.9	9196.9 a
		Kabugu Yufka	8516.4	8698.3	8307.3	7948.8	8516.5 b
		The effect of the treatment	8856.6 ^{ns}	8944.0	8506.3	8999.9	
Magnesium	2021	Karaerik	2035.3 ^{ns}	2073.0	2297.0	2377.4	2195.6 b
		Kabugu Yufka	2783.5	2802.5	2736.4	2848.6	2793.0 a
		The effect of the treatment	2409.4 ^{ns}	2437.7	2516.6	2613.0	
	2022	Karaerik	2339.9 ^{ns}	2249.9	2636.0	2499.1	2339.9 b
		Kabugu Yufka	2746.1	2834.7	3029.1	2923.6	2746.1 a
		The effect of the treatment	2543.0 ^{ns}	2542.9	2832.6	2711.4	
Calcium	2021	Karaerik	1046.4 ^{ns}	1095.8	1030.2	1126.9	1074.8 ^{ns}
		Kabugu Yufka	1063.5	1049.2	1077.3	1085.7	1068.9
		The effect of the treatment	1055.0 ^{ns}	1072.5	1053.7	1106.3	
	2022	Karaerik	999.19 ^{ns}	905.8	1095.48	846.27	961.70 a
		Kabugu Yufka	850.97	805.03	791.90	818.76	850.97 b
		The effect of the treatment	925.08 ab	865.45 bc	943.69 a	832.51c	
Phosphorus	2021	Karaerik	3627.7 ^{ns}	3676.2	3884.3	3753.6	3735.5 a
		Kabugu Yufka	3202.2	3554.1	3369.7	3538.1	3416.0 b
		The effect of the treatment	3415.0 ^{ns}	3615.1	3627.0	3646.0	
	2022	Karaerik	3616.9 ^{ns}	3484.0	3666.1	3646.1	3603.2 ^{ns}
		Kabugu Yufka	3602.3	3209.0	3406.9	3553.3	3442.9
		The effect of the treatment	3609.6 ^{ns}	3346.5	3536.5	3599.7	

The differences between the means indicated by different letters in the same column are statistically significant ($p < 0.05$). ns: not significant.

3.10. Some micromineral composition of grapevine leaves (Fe, Zn, Mn, Cu, Se)

The effects of cultivar and bud load treatments on some micronutrient amounts are given in Table 10. The effects of cultivar and bud load treatments on iron content in leaves were found to be statistically significant in the first year, while zinc content was found to be statistically significant between cultivars in both years ($P<0.05$). In terms of manganese content, it was found statistically significant only for the cultivars in the first year, but there was no difference in the second year. The effect on copper content was not statistically significant. On the other hand, the effect on selenium content was not found to be statistically significant ($P<0.05$) in the first year, while it was found to be significant in the second year among the varieties and treatments.

Table 10. Effects of variety and bud load on some microelement amounts (ppm) in leaves

	Year	Variety	Applications (bud/vine)				The main effect of variety
			24	36	48	60	
Iron	2021	Karaerik	71.89 ns	61.73	67.57	62.52	65.92 a
		Kabugu Yufka	53.55	58.63	60.07	52.69	56.24 b
		The effect of the treatment	62.72 ns	60.18	63.82	57.61	
	2022	Karaerik	146.51 ns	167.41	150.19	131.14	148.814 ns
		Kabugu Yufka	127.77	126.96	159.91	138.34	138.2
		The effect of the treatment	137.14 ns	147.19	155.05	134.74	
Zinc	2021	Karaerik	129.8 ns	130.1	129.9	120.7	127.6 a
		Kabugu Yufka	117.2	105.7	104.2	115.3	110.6 b
		The effect of the treatment	123.5 ns	117.9	117.0	118.0	
	2022	Karaerik	113.38 ns	113.33	103.95	99.64	107.58 a
		Kabugu Yufka	97.51	94.23	89.61	76.24	89.40 b
		The effect of the treatment	105.45 ns	103.78	96.78	87.94	
Manganese	2021	Karaerik	30.59 ns	30.88	30.20	30.90	30.44 a
		Kabugu Yufka	24.40	26.Şub	28.27	30.39	27.27 b
		The effect of the treatment	27.50 ns	28.45	29.24	30.24	
	2022	Karaerik	38.22 ns	31.50	38.61	35.01	35.84 ns
		Kabugu Yufka	33.44	32.77	31.36	31.54	32.28
		The effect of the treatment	35.83 ns	32.14	34.98	33.27	
Copper	2021	Karaerik	9.36 ns	Eki.51	Ağu.41	9.Eyl	9.33 ns
		Kabugu Yufka	Ağu.75	Eyl.49	Ağu.83	Eki.32	Eyl.34
		The effect of the treatment	9.06 ns	10.0	Ağu.62	Eyl.70	
	2022	Karaerik	15.73 ns	14.79	14.76	17.00	15.57 ns
		Kabugu Yufka	15.34	16.Ağu	16.Ağu	14.61	15.53
		The effect of the treatment	15.54n s	15.44	15.42	15.81	
Selenium	2021	Karaerik	0.059 ns	0.057	0.054	0.059	0.057 ns
		Kabugu Yufka	0.057	0.049	0.055	0.057	0.055
		The effect of the treatment	0.058 ns	0.053	0.054	0.053	
	2022	Karaerik	0.072 ns	0.057	0.057	0.061	0.062 a
		Kabugu Yufka	0.055	0.069	0.061	0.039	0.056 b
		The effect of the treatment	0.064a	0.063a	0.059b	0.050c	

The differences between the means indicated by different letters in the same column are statistically significant ($p < 0.05$). ns: not significant.

4. Discussion

The results provided valuable insights into the effects of variety selection and bud load management on certain chemical parameters in grapevine leaves. The findings demonstrated significant variety-dependent variations and, in some cases, bud load-induced changes in leaf composition across two consecutive growing seasons. Regarding dry matter content, our results indicated that Karaerik consistently exhibited higher dry matter content compared to Kabugu Yufka across both years, with values ranging from 26.61% to 28.20% for Karaerik and 25.18% to 28.00% for Kabugu Yufka (Table 1). Kılıc (2007) reported that the lowest and highest dry matter content varied between 28.01-29.49% (8 buds/vine) in the goblet system and 26.22- 27.03% (24-16 buds/vine) in the cord system in terms of bud load and training systems. Gulcu and Demirci (2011) reported that the dry matter content of the Narince variety was 24.09%, the Yapıncak variety was 18.48% and the Tekirdağ Cekirdeksiz variety was 24.18%; Coban (2023) reported that the total dry matter content of Sultani Cekirdeksiz grape variety was 26.1%. The differences are thought to be due to factors such as variety-specific characteristics, environmental conditions, and viticulture practices (Koundouras et al., 2006; Kepenekci, 2007).

On the other hand, the ash content in our study ranged from 1.58% to 1.76% for Karaerik and 1.45% to 1.65% for Kabugu Yufka (Table 2). Sat et al. (2002) reported ash content in different grape varieties between 1.52 (Karaerik) and 2.15 (Hacıtesbihi). Coban (2023) reported that ash content varied in fresh grapevine leaves in May and July. Ovayurt and Soylemezoglu (2023) determined the amount of ash in different grape varieties between 0.48% (Yapıncak) and 1.57% (Tekirdağ Cekirdeksizi). Kılıc

(2007) reported that training systems and bud load applications affected the ash content of grapevine leaves. In our study, ash content in leaves decreased in the second year in parallel with the increase in bud load. The data obtained regarding the ash content of the varieties are generally similar to the literature. It is thought that the differences are due to the variety, bud load, year, and leaf removal times depending on the reports of the above researchers.

In our study, the % acidity values determined in fresh leaves were between 1.23-1.54 in the Karaerik grape variety and between 1.20-1.38 in the Kabugu Yufka variety depending on the year and treatments, which is similar to the previous studies. As a matter of fact, Basoglu et al. (2004) determined % acidity between 1.20 and 1.50 in fresh leaves of the Sultani Cekirdeksiz grape variety, and Kılıc (2007) determined % acidity between 1.29 and 1.62 in Narince variety according to the bud load applications. The researcher reported that acidity values were low at low bud load in the first year of the study and acidity increased with high bud load. Ovayurt and Soylemezoglu (2023) determined % acidity between 1.92 (Narince) and 2.08 (Yapincak) in fresh leaves of different varieties. On the other hand, researchers have reported that elevation (Koundouras et al., 2006) cultivation system (Kepenekci, 2007), variety (Sensoy and Balta, 2010; Kamiloglu and Ustun, 2014; Celik and Ates, 2025), soil, climate, topographical features (Demiray, 2006; Bayram et al., 2016) and cultural treatments (Bahar et al., 2018) affect acidity.

The pH values in our study ranged from 3.07 to 3.32 for Karaerik and 3.04 to 3.33 for Kabugu Yufka (Table 4). Academic studies on pH values in fresh grapevine leaves are quite limited. In the studies, the findings of pH values are mostly related to the leaves processed in brine. Sat et al. (2002) determined the pH values of fresh grapevine leaves as 3.39 in the Hacı Tesbihi variety, 3.31 in the Kabugu Yufka variety, 3.43 in the Agrazaki variety and 3.46 in Karaerik variety. Ovayurt and Soylemezoglu (2023) reported that pH values in fresh leaves of different grape varieties were determined between 3.1 (Emir and Narince) and 3.24 (Sultani Cekirdeksiz). In general, it can be said that the treatments did not have a stable effect on pH values. Similarly, in a study conducted by Kılıc (2007), it was determined that there was no statistical difference between bud load and pH.

On the other hand, our study found dietary fiber content ranging from 11.03% to 13.97% for Karaerik and 10.42% to 12.28% for Kabugu Yufka (Table 5). These results are in agreement with the findings of Celik (2014), who reported 11 g of dietary fiber per 100 g of fresh grapevine leaves, and Cangi et al. (2019) who found 10.5 g 100 g^{-1} in Narince leaves. Our values are also comparable to those reported by Ovayurt and Soylemezoglu (2023) for various varieties (12.06 to 14.01 g 100 g^{-1}). The consistency of our findings with previous studies suggests that dietary fiber content in grapevine leaves may be relatively stable across different varieties and growing conditions.

The vitamin C content in our study ranged from 97.5 to 132.5 mg 100 g^{-1} for Karaerik and 87.42 to 121.38 mg 100 g^{-1} for Kabugu Yufka (Table 6). Sat et al. (2002) determined vitamin C in fresh grapevine leaves as 54.00 mg/100g in the Hacıtesbihi variety, 100.29 mg/100g in the Karaerik variety, 61.75 mg 100 g^{-1} in the Kabugu Yufka variety and 77.08 mg 100 g^{-1} in Agrazaki grape variety. Vitamin C content in the leaves of the varieties may vary depending on whether the leaves are fresh or pickled. As a matter of fact, Sat et al. (2002) reported that the vitamin C levels of pickled leaves were significantly lower compared to fresh leaves and this decrease may be due to the processing technique. In addition, Franke et al. (2004) reported that vitamin C contents may vary with species, cultivar, and part analyzed.

Our study also found vitamin E content ranging from 7.30 to 15.38 mg 100 g^{-1} for Karaerik and 17.03 to 24.90 mg 100 g^{-1} for Kabugu Yufka (Table 7). Studies on the determination of vitamin E content in grapevines are mostly limited to wine and berry contents. Studies on vitamin E content in fresh grapevine leaves are almost nonexistent. Cangi et al. (2019) determined the vitamin E content in pickled Narince grapevine leaves as 6.96±3.37 mg 100 g^{-1}. Vitamin E contents in fresh grapevine leaves are higher than the findings of Cangi et al. (2019). This shows that vitamin E values are different in fresh and pickled grapevine leaves.

On the other side, the total phenolic content in our study ranged from 3019.1 to 4093.6 mg 100 g^{-1} for Karaerik and 2815.3 to 3744.8 mg 100 g^{-1} for Kabugu Yufka (Table 8). These values are generally higher than those reported by Ovayurt and Soylemezoglu (2023) for various varieties (1780 to 3130 mg GAE 100 g^{-1}). The higher phenolic content observed in Karaerik (a black variety) compared to Kabugu Yufka (a white variety) is consistent with the findings of Yang and Xiao (2013) who reported higher phenolic content in black grape varieties. As noted by Nadal and Arola (1995), De La Orts et al. (2005) and Sonmez Yildiz et al. (2023) factors such as variety, ecological conditions, maturity levels, and

cultural practices can influence phenolic content in grapevines. Our findings support this notion and provide additional evidence for variety-specific differences in phenolic content.

Macronutrient contents in leaves of Karaerik and Kabugu Yufka cultivars varied under different bud load treatments across cultivars, years, and, in some cases, in response to bud load management treatments (Table 9). The potassium content in the Karaerik grape variety ranges from 2552.4 to 10050.9 ppm, while in the Kabugu Yufka variety, it ranges from 2581.1 to 8698.3 ppm. The magnesium content in the Kabugu Yufka variety is between 2736.4 and 3029.1 ppm, whereas in the Karaerik variety, it ranges from 2035.3 to 2636 ppm. The calcium content in the Kabugu Yufka variety is between 2736.4 and 3029.1 ppm, while in the Karaerik variety, it ranges from 2035.3 to 2636 ppm. The phosphorus content in the Karaerik grape variety is between 3484 and 3884.3 ppm, while in the Kabugu Yufka grape variety, it ranges from 3202.2 to 3666.1 ppm. In general, our study found that phosphorus, potassium, and calcium levels were higher in the Karaerik variety, whereas magnesium content was higher in the Kabugu Yufka variety (Table 9). Aydin et al. (2005) determined the phosphorus content to be 0.11%, potassium content to be 0.54%, calcium content to be 2.02%, and magnesium content to be 0.33% in the leaf blade during the berry set period of the Yuvarlak Cekirdeksiz grape variety. Kara and Bacevli (2012) identified the phosphorus content in the fresh leaves of rootstock cuttings as ranging from 2594.5 ppm (41 B) to 3702.7 ppm (140 Ru); potassium content from 5523.3 ppm (140 Ru) to 7859.5 ppm (110 R); calcium content from 8098.7 ppm (110 R) to 11593.3 ppm (41 B); and magnesium content from 1991.4 ppm (110 R) to 3216.2 ppm (99 R). Tangolar et al. (2019) found the phosphorus content in the Early Sweet variety during full bloom to range between 0.26% and 0.38%, potassium content between 0.50% and 0.55%, calcium content between 1.06% and 1.48%, and magnesium content between 0.13% and 0.19%. Esetlili et al. (2020) reported the phosphorus content in the fresh leaves during the flowering period of the Cabernet Sauvignon grape variety to range between 0.19% and 0.34%, potassium content between 0.98% and 1.44%, calcium content between 2.14% and 3.11%, and magnesium content between 0.44% and 0.61%. Coban (2023) found the phosphorus content to be 0.22%, potassium content to be 1.36%, calcium content to be 2.3%, and magnesium content to be 0.55% in the fresh leaves of the Sultani Cekirdeksiz grape variety during May. The findings for the fresh leaves of the Karaerik and Kabugu Yufka varieties in this study are generally consistent with the results reported by Aydin et al. (2005), Coban (2023), Tangolar et al. (2019), and Esetlili et al. (2020).

Micronutrient contents in leaves of Karaerik and Kabugu Yufka cultivars varied under different bud load treatments across cultivars, years, and, in some cases, in response to bud load management treatments (Table 10). The iron content in the leaves of the Karaerik grape variety ranged from 61.73 to 138.34 ppm, while in the Kabugu Yufka grape variety, it ranged from 52.69 to 159.91 ppm. The zinc content in the Karaerik variety ranged from 99.64 to 130.1 ppm, and in the Kabugu Yufka variety, it ranged from 76.24 to 117.2 ppm. Manganese levels in the Karaerik variety ranged from 30.20 to 38.61 ppm, while in the Kabugu Yufka variety, it ranged from 24.40 to 33.33 ppm. Copper content in the Karaerik variety was between 8.41 and 17 ppm, and in the Kabugu Yufka variety, it was between 8.75 and 16.08 ppm. The selenium content in the leaves of the Karaerik variety ranged from 0.054 to 0.072 ppm, and in the Kabugu Yufka variety, it ranged from 0.039 to 0.069 ppm. In general, the Karaerik variety exhibited higher levels of iron, zinc, manganese, and selenium. Under low bud load conditions, selenium and zinc levels were higher, whereas these values decreased with an increase in bud load (Table 10). Aydin et al. (2005) reported that the manganese content in the leaf blades of the Yuvarlak Cekirdeksiz variety during the fruit-setting period was 48 ppm, iron content was 217 ppm, and copper content was 29 ppm. Kara and Bacevli (2012) determined the copper content in the fresh leaves of rootstocks 41 B, 110 R, and 1103 P to range from 8.3 ppm (140 Ru) to 13.2 ppm (41 B), iron content from 580.7 ppm (41 B) to 1575.0 ppm (110 R), manganese content from 31.8 ppm (99 R) to 127.0 ppm (140 Ru), and zinc content from 15.2 ppm (140 Ru) to 28.2 ppm (99 R). Tangolar et al. (2019) found that in the Early Sweet variety at full bloom, the leaf samples contained iron levels ranging from 83.2 to 57.7 mg kg^{-1}, manganese levels from 82.6 to 63.4 mg kg^{-1}, and zinc levels from 16.9 to 14.9 mg kg^{-1}. Esetlili et al. (2020) reported the iron content in the Cabernet Sauvignon variety to range from 144 to 242 mg L^{-1}, zinc from 33 to 44 mg L^{-1}, manganese from 87 to 140 mg L^{-1}, and copper from 20 to 38 mg L^{-1}. Licina et al. (1997) measured selenium content in vine organs as 0.07 µg/g in roots, 0.12 µg g^{-1} in stems, shoots, and leaves, and 0.02 µg g^{-1} in grapes. Liu et al. (2019) determined selenium content in grapevine plants as 0.935±0.014 g in roots, 0.426±0.009 g in stems, 1.193±0.061 g in leaves, and 1.081±0.055 g in shoots. The micronutrient levels in the leaves of the grape varieties in this study are

somewhat similar to the findings of the aforementioned researchers, but generally, the element levels were either lower or higher. These differences are likely attributed to various factors, including soil, grape variety, timing of leaf sampling, and the cultural practices applied. Indeed, Kacar and Katkat (2010) reported that numerous factors, such as the plant species, age, root growth, the physical, chemical, and biological properties of the soil, the types and quantities of available elements in the soil, agricultural practices, and climatic conditions, significantly influence the nutrient content of plants.

Conclusion

The Karaerik grape variety has stood out in terms of dry matter content, with the 24 and 36-bud vine training systems being the most prominent among the applied treatments. The Karaerik variety has also shown higher levels of ash content and acidity. In general, it was found that the applications had no significant stable effect on pH values. The dietary fiber and vitamin C contents were higher in the Karaerik variety. Although no statistical differences were found between the treatments, the 48 and 60 bud/vine systems yielded higher vitamin C values. The vitamin E content in fresh leaves was found to be higher in the Kabugu Yufka variety, while the Karaerik variety was noted for its higher total phenolic content. Overall, iron, zinc, manganese, and selenium levels were higher in the Karaerik variety. In treatments with lower bud load, selenium and zinc levels were higher, whereas these values decreased as the bud load increased.

Ethical Statement

Ethical approval is not required for this study as it is not a retrospective study.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Author Contributions

N.N.K, O.T.A and Z.K: Chemical analyses, A.B: Manuscript writing, R.C: Statistical analysis, S.A: Disease control, B.K and D.K: Literature review.

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Research Article

Genetic Relationships of Native *Phalaenopsis* Orchids from the South Kalimantan (Borneo), Indonesia: A Morphological and Molecular Approaches

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Abstract: *Phalaenopsis* (Orchidaceae) is the world's most popular and essential ornamental plant. Consequently, it is unsurprising that this orchid has high economic value and is a promising export commodity. Unfortunately, due to monetary value and other factors, like deforestation and habitat destruction, many *Phalaenopsis* species have suffered losses and are becoming rare in their natural habitat. This study aims to determine the genetic diversity and relationship of *Phalaenopsis* orchids natively from South Kalimantan, Indonesia, by using flower morphology and DNA polymorphism (RAPD) markers. A total of eight samples of *Phalaenopsis* were used in this study. The diversity was determined using the Shannon diversity index (H'). In contrast, the clustering and reconstruction of genetic relationships were performed using an unweighted pair group of arithmetic means (UPGMA) and principal component analysis (PCA). Following morphological traits, *Phalaenopsis* has a high diversity, averaging 0.71. The molecular markers used (RAPD) also show high genetic diversity. In the study, *Phalaenopsis* showed a genetic polymorphism of 95.46%. The UPGMA revealed the closest relationship of *P. cornu-cervi* and *P. sumatrana* at a genetic distance of 0.878 (for morphological markers) and *P. deliciosa* and *P. modesta* at 0.715 (for RAPD markers). In contrast, the furthest relationship was shown by *P. amabilis* with *P. sumatrana*, both for morphological (at coef. 0.434) and molecular (0.489) markers. Thus, our results are valuable in supporting the conservation and breeding efforts of *Phalaenopsis*, locally and globally.

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1. Introduction

Phalaenopsis (Orchidaceae), known as the moth or moon orchid, is the world's most popular and essential ornamental plant (Hsu et al., 2018). Its popularity strongly correlates to its flower characteristics, such as form, size, color, scent, and texture (Zhang et al., 2019). Also, based on other traits, e.g., fast-growing and fast-flowering or a relatively short early vegetative (juvenile) period (Paradiso and De Pascale, 2014). Consequently, this orchid has a significant trade value, with annual sales of more than 4 billion USD (Zhang et al., 2018). Large-scale potted *Phalaenopsis* is found in

several countries, particularly the Netherlands, Germany, the United States, China, and Japan (Zhang et al., 2019).

Nowadays, *Phalaenopsis* consists of about 66 formal species distributed mainly in Asia (Liu et al., 2016; Hinsley et al., 2018), including a restricted area of Australia (Tsai, 2011). However, the highest *Phalaenopsis* diversity is present in Indonesia, with more than 20 species (Deng et al., 2015). According to Rahayu et al. (2015), this orchid is spread in several islands, i.e., Sumatra, Java, Kalimantan, Sulawesi, Maluku, Nusa Tenggara, and Papua. Unfortunately, due to economic value and other factors, like deforestation and habitat destruction, many *Phalaenopsis* species have suffered losses and are becoming rare in their natural habitat (Zhang et al., 2018). As a result, conservation and breeding efforts, including genetic diversity analysis, must be performed urgently (Zahara and Win, 2019).

For years, analysis of orchids' genetic diversity has been conducted by morphological traits (Kwon et al., 2017). While these traits are environmentally influenced, they are commonly used in evaluating germplasm in general (Mursyidin et al., 2021). In contrast, several molecular markers have been employed to study the genetic diversity of *Phalaenopsis*, particularly AFLP (Chang et al., 2009), RAPD (Niknejad et al., 2009; Mursyidin et al., 2022b), and SSR (Tsai et al., 2015b; Ko et al., 2017). While these markers also have certain disadvantages, a combination of morphological and molecular ones may represent a comprehensive aspect of the genetic diversity of germplasm (Rocha et al., 2020).

This study aimed to determine the genetic diversity and relationship of *Phalaenopsis* orchids native to South Kalimantan, Indonesia, by using flower morphology and DNA polymorphism (RAPD) markers. Thus, our results are valuable in supporting the conservation and breeding efforts of native *Phalaenopsis* in the future.

2. Material and Methods

2.1. Plant samples

Eight samples of *Phalaenopsis* orchids were used in this study. All were obtained from several private collectors at Banjarbaru, Banjarmasin, and Tanah Laut regency of South Kalimantan, Indonesia (Table 1). In this case, samples were observed and documented directly at the sampling location for morphological analysis. Further, for molecular assay, orchid leaves were placed into a sealable container and brought with silica gel.

Table 1. Eight samples of *Phalaenopsis* orchids were used in this study

Name of species	Code	Common Name	Genetic Status
P. amabilis	A	Moth orchid	Native
P. stuartiana	B	Stuart's *Phalaenopsis*	Introduction*
P. deliciosa	C	Delicate *Phalaenopsis*	Native
P. modesta	D	Modest *Phalaenopsis*	Native
P. gigantea	E	Gigantic/giant *Phalaenopsis*	Native
P. maculata	F	Spotted *Phalaenopsis*	Native
P. sumatrana	G	Sumatran *Phalaenopsis*	Native
P. cornu-cervi	H	Deer antlered *Phalaenopsis*	Native

Note: *from Mindanao, Philippines.

2.2. Morphological analysis

Analysis of morphological characteristics was referred to in the guidebook of Christenson (2001), with soft modifications. It includes only flower morphological (qualitative and quantitative) characters, such as flower shape (petals; sepals), colors, motifs, and diameter (Table 3). In this case, the color trait was determined by the Royal Horticultural Society (RHS) color chart, whereas the measurement of flower diameter was made using a digital caliper. All characters were tabulated and converted into multivariate data.

2.3. RAPD analysis

RAPD analysis initially began with the DNA extraction of *Phalaenopsis* leaves using the DNAzol kit protocol (Molecular Research Center Inc., USA). A UV-Vis spectrophotometer quantified

the concentration and purity of DNA. The DNAs were amplified using ten selected RAPD primers (Table 2), with a total volume of 25 µl, consisting of 17.5 µl master mix PCR (MyTaq HS Red mix, Bioline, Meridian Bioscience), 0.5 ng μL^{-1} of each primer (10 nmoles), 2 µL of template DNA, and 4 µL of nuclease-free water. It was performed by Thermal Cycler PCR (Techne, TC3000G, USA) with a cycling condition referred to as Mursyidin and Daryono (2016). The amplicon was separated by 2% agarose gel electrophoresis in 1X TBE buffer (pH 8.0). The gel was stained using GelRed solution (Biotium, USA) and observed on a UV transilluminator with a 100 bp DNA ladder (Vivantis Technologies, Malaysia). Then, it was documented by a digital camera.

Table 2. RAPD primers used in this study

Primers	Sequences (5'-3')	GC content (%)
OPA-02	TGCCGAGCTG	70
OPA-04	AATCGGGCTG	60
OPA-09	GGGTAACGCC	70
OPA-10	GTGATCGCAG	60
OPB-01	GTTTCGCTCC	60
OPB-05	GATGACCGCC	70
OPB-06	TGCTCTGCCC	70
OPB-07	GGTGACGCAG	70
OPB-10	CTGCTGGGAC	70
OPS-12	CTGGGTGAGT	60

Source: Mursyidin et al. (2022b).

2.4. Data analysis

Data was tabulated, standardized, and analyzed for morphological traits using a multivariate method, with the assistance of the MVSP ver. 3.1 (Kovach, 2007). Diversity was determined using the Shannon diversity index (H'), based on the criteria of Mursyidin et al. (2022a). For the molecular (RAPD) marker, data in the form of DNA fragments present on an electrophoresis gel lane were tabulated, scored, and converted into binary matrix values: one (1) for showing DNA fragments and zero (0) for not present. The clustering and reconstruction of genetic relationships were performed using an unweighted pair group of arithmetic means (UPGMA) and principal component analysis (PCA). Dendrograms were evaluated with the statistical bootstrap method for 1000 replicates (Hampl et al., 2001).

3. Results and Discussion

3.1. Flower characteristics

For decades, *Phalaenopsis* has been crossed and produced a variety of offspring with charming flower characteristics. In this study, *Phalaenopsis* shows unique differences in shapes, colors, and shades or motifs of flowers (Figure 1). In general, *Phalaenopsis* from South Kalimantan, Indonesia, exhibits rhomboidal flower shapes, such as by *P. amabilis* and *P. stuartiana*; nearly rounded to rounded by *P. deliciosa* and *P. gigantea*, respectively; and star-like (Figure 1, and Table 3).

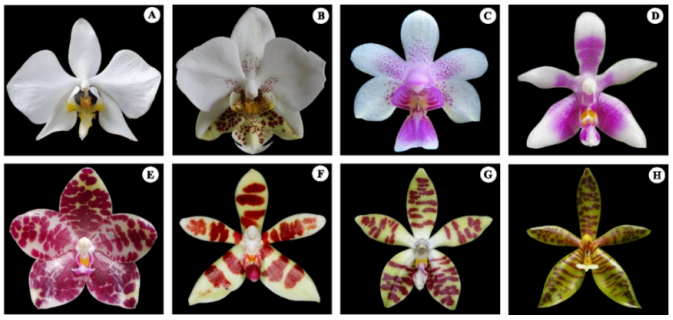

Figure 1. *Phalaenopsis* orchids used in this study show different characteristics of flower morphology. Note: A = *P. amabilis*; B = *P. stuartiana*; C = *P. deliciosa*; D = *P. modesta*; E = *P. gigantea*; F = *P. maculata*; G = *P. sumatrana*; H = *P. cornu-cervi.*

Table 3. General characteristics of *Phalaenopsis* flower observed morphologically

Observed traits	**Species**							
	P. amabilis	***P. stuartiana***	***P. deliciosa***	***P. modesta***	***P. gigantea***	***P. maculata***	***P. sumatrana***	***P. cornu-cervi***
Floral color	Pure white	White	White	White	Creamy-white	Creamy-white	Light yellow green	Greenish yellow
Floral shape	Rhomboidal	Rhomboidal	Nearly rounded	Star-like	Rounded	Star-like	Star-like	Star-like
Petal shape	Rhomboid	Rhomboid	Spathulate	Lanceolate	Elliptical	Spathulate	Oblanceolate	Oblanceolate
Sepal shape	Lanceolate-oblanceolate	Elliptical	Ovate-spathulate	Spathulate-	Elliptical	Oblanceolate-spathulate	Oblanceolate	Oblanceolate
Petal-sepal positions	Semi-overlap	Semi-overlap	Semi-overlap	Separate	Overlap	Separate	Separate	Separate
Motif on petal	None	Heavy small red-purple spotted on the base	Purple spotted and scattered along the half	Purple marks and spotting close to the base	Raised red-brown (concentric) markings	Transversal red-brown markings (thick stripped)	Transversal red-brown markings (medium stripped)	Medium intense red-brown marking
Motif on sepal	None	Inner half-pale yellow with widish heavy red-purple markings	Purple scattered spots	Purple marks and spotting close to the base	Raised red-brown (concentric) markings	Transversal red-brown markings (thick stripped)	Transversal red-brown markings (medium stripped)	Transversal red-brown markings (thin stripped) with spotted
Motif on labellum (lip)	Yellow with small brown-red-markings	Yellow with slight heavy red-purple marking	Massive purple with a line pattern	Yellow with purple markings	Yellow with purple markings	Bright red middle lobe to the entire labellum	Yellow-white with purple markings	Yellow-to bright yellow without markings
Flower type	Membranous	Membranous	Semi-transparent	Fleshy	Waxy	Fleshy	Fleshy	Semi-transparent
Flower diameter (cm)	10.2 ± 0.02	7.6 ± 0.20	2.1 ± 0.01	3.8 ± 0.21	5.6 ± 0.02	6.3 ± 0.01	6.5 ± 0.12	4.5 ± 0.15

For the primary color of flowers, *Phalaenopsis* is divided into three color classes, namely white (*P. amabilis*, *P. stuartiana*, *P. deliciosa*, and *P. modesta*); white-creamy (*P. gigantea* and *P. maculata*), as well as greenish-yellow (*P. sumatrana* and *P. cornu-cervi*). Meanwhile, concerning the motifs or patterns formed, this orchid generally has spot categories (in almost all species) and strips (in *P. maculata*, *P. sumatrana*, and *P. cornu-cervi*). In contrast, the flower diameter is more diverse, ranging from 2.1 to 10.2 cm. More details about the characteristics of *Phalaenopsis* flowers can be seen in Table 3.

3.2. Genetic diversity

Besides flower characteristics, information on genetic diversity is indispensable. According to Lloyd et al. (2016), genetic diversity is needed for rapid environmental changes. In this context, populations that have high (extensive) genetic diversity tend to be able to survive or adapt well to environmental changes. In contrast, populations with low genetic diversity tend to go extinct faster. In other words, genetic diversity plays a vital role in the direction of evolution, especially in producing future founder populations that include natural adaptive selection (Govindaraj et al., 2015a).

In this case, *Phalaenopsis* is highly diverse based on morphological traits, averaging 0.73 (Table 4). Petal and sepal motifs show the highest genetic diversity, amounting to 0.96 each, while the lowest genetic diversity is by petal positions (0.39). Meanwhile, the molecular markers (RAPD) also show high genetic diversity. *Phalaenopsis* showed a genetic polymorphism of 95.46% (Table 5). Based on these markers (Table 5), almost all markers used indicate perfect diversity (100%), except OPA-04, which generated only 54.55%.

Table 4. Diversity index of *Phalaenopsis* based on flower morphological characteristics

Observed traits	Code	H Index	Criteria
Floral color	A	0.73	High
Floral shape	B	0.81	High
Petal shape	C	0.78	High
Sepal shape	D	-	-
Petal-sepal positions	E	0.39	Low
Motif on petal	F	0.96	High
The color motif on the petal	G	-	-
Motif on sepal	H	0.96	High
The color motif on sepal	I	-	-
Motif on labellum (lip)	J	0.58	Medium
Color motif on labellum (lip)	K	0.58	Medium
Flower type	L	0.61	High
Flower diameter	M	0.94	High
Average		0.73	High

Table 5. Polymorphism degree of *Phalaenopsis* spp., generated by RAPD markers

Primer	Range of amplified DNA fragment (bp)	Total of DNA fragment (loci)	Number of polymorphic DNA	Polymorphism (%)
OPA-02	160-1000	11	11	100.00
OPA-04	160-1230	11	6	54.55
OPB-01	190-1250	10	10	100.00
OPB-06	270-1200	10	10	100.00
OPB-07	100-1500	18	18	100.00
OPS-12	300-1300	5	5	100.00
OPA-09	190-1000	18	18	100.00
OPA-10	100-1230	20	20	100.00
OPB-05	130-1200	18	18	100.00
OPB-10	100-1000	16	16	100.00
Average		13.70	13.20	95.46

This high polymorphism is closely related to the polymorphic fragments produced by each RAPD marker, as shown in Figure 2. Figure 2 shows several specific DNA fragments appearing in some

orchid samples. For example, in orchid samples 7 (*P. maculata*) and 8 (*P. amabilis*), specifically the OPA-10 marker, one specific fragment measures about 860 bp and 800 bp, respectively. According to Table 5, the range of most distantly amplified DNA fragments was recorded between 100-1500 bp by OPB-07. Meanwhile, the highest number of polymorphic DNA fragments (20 fragments) was produced by OPA-10. Interestingly, although OPS-12 generates the least number of bands, only five are polymorphic.

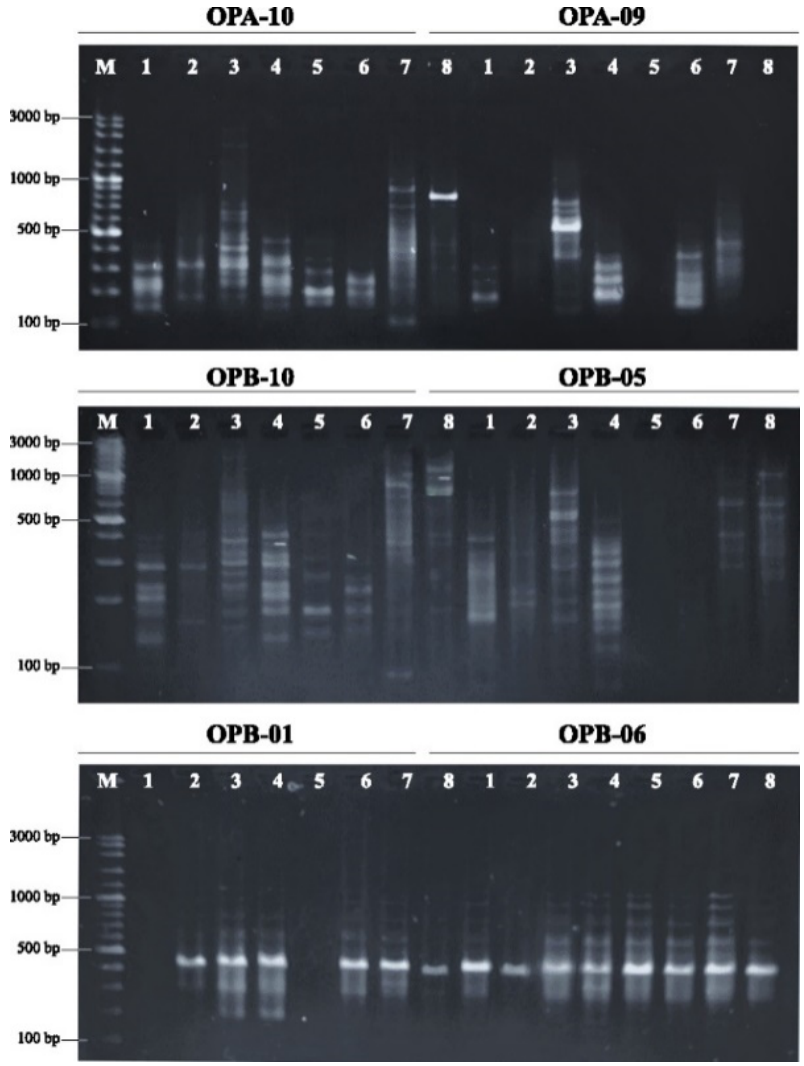

Figure 2. DNA profile of *Phalaenopsis* spp., generated by RAPD markers. 1 = *P. cornu-cervi*; 2 = *P. modesta*; 3 = *P. sumatrana*; 4 = *P. gigantea*; 5 = *P. stuartiana*; 6 = *P. deliciosa*; 7 = *P. maculata*; 8 = *P. amabilis*; M = DNA marker (100 bp).

According to Yusop et al. (2022), high genetic diversity is closely related to mutations in the genome. Because mutations cannot be determined in this study, it is significant to apply other molecular markers that are more accurate and specific. Referred to Kar et al. (2015) a population's high genetic diversity provides a high phylogenetic signal for resolving evolutionary relationships among plant germplasm at all taxonomic levels. Hence, it is beneficial for the conservation program, especially in promoting population survival and guaranteeing the adaptive potential of natural populations in the face of rapid environmental change (Teixeira and Huber, 2021). Further, genetic diversity is urgent for plant breeding, particularly in parental selection or selecting parents with genetically distinct (Aesomnuk et al., 2021; Wu et al., 2021) and developing new cultivars with desirable traits (Govindaraj et al., 2015b).

3.3. Genetic relationships

Furthermore, information on genetic relationships is also needed to guide breeding and conservation programs (Mursyidin et al., 2023). Generally, breeders and conservationists need this information when crossing individuals. For conservation, information on phylogenetic relationships can help resolve species delimitation, gene flow, and genetic differentiation, and infer species and their evolutionary history (Fernández-García, 2017). In addition, such information can also be used to resolve problems of previous evolutionary history, determine the genetic status of present species, and manage future species (Fernández-García, 2017). For the breeding program, the phylogenetic information can forecast progeny's genetic variety when individuals cross (Acquaah, 2015). If hybridized, distantly related individuals will produce offspring with high genetic diversity. In contrast, closely related individuals tend to have progeny with narrow genetic diversity (Koide et al., 2019).

Based on morphological markers (flowers), *Phalaenopsis* is separated into three main clusters, where *P. amabilis* and *P. stuartiana* belong to the same cluster, including *P. gigantea* and *P. deliciosa* (Figure 3A). The remaining four species belong to the first cluster (Figure 3A). Meanwhile, based on molecular markers (RAPD), this orchid genus is separated into five main clusters (Figure 3B), where clusters I and V consist of one species each, namely *P. maculata* and *P. cornu-cervi*. Meanwhile, three other clusters (II-IV) are membered by two species each, namely *P. amabilis* with *P. stuartiana*, *P. gigantea* with *P. sumatrana*, and *P. deliciosa* and *P. modesta*.

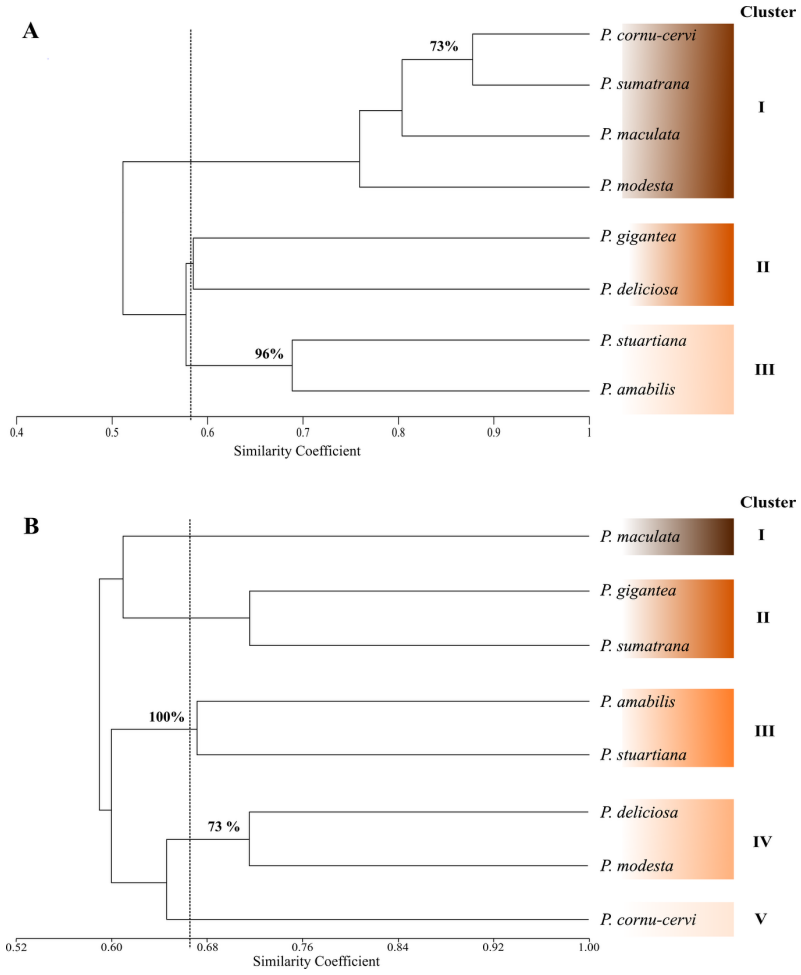

Figure 3. Genetic relationship of native *Phalaenopsis* orchids from South Kalimantan, Indonesia, by morphological (A) and molecular (B) markers. The percentages on nodes are bootstrap values for 1000 replicates.

However, the PCA results show a different grouping of *Phalaenopsis* (Figure 4). Based on morphological markers (Figure 4A), these orchids are divided into five groups spread over four

quadrants. Meanwhile, the RAPD marker was able to separate *Phalaenopsis* into six groups. However, it is spread over two quadrants only (Figure 4B). More details about interspecies relationships can be seen in Figure 5. Based on Figure 5, *P. cornu-cervi* and *P. sumatrana* showed the closest relationship at a genetic distance of 0.878 (for morphological markers), and *P. deliciosa* and *P. modesta* at 0.715 (for RAPD markers). In contrast, the furthest relationship was shown by *P. amabilis* with *P. sumatrana*, both for morphological (at coef. 0.434) and molecular (0.489) markers.

In this case, other studies also display a unique relationship of this germplasm. For example, using the maturase K (*mat*K) marker, Mursyidin et al. (2021) reported the closest relationship between *P. cornu-cervi* with *P. pantherina* and *P. gigantea*. In contrast, the furthest is shown by *P. amabilis* with *P. celebensis*. Moreover, using the *rbc*L and *trn*L-F markers, the neighborhood between *P. amabilis* and *P. celebensis* was also reported (Mursyidin et al., 2021). However, the different relationships of *Phalaenopsis* are shown by the ITS (internal transcribed spacers) marker, where *P. amabilis* and *P. sanderiana,* including *P. cornu-cervi* with *P. borneensis* have close relationships (Tsai et al., 2015) Furthermore, using microsatellite markers, Fatimah and Sukma (2011) revealed the closeness of *P. amabilis* to *P. fuscata*.

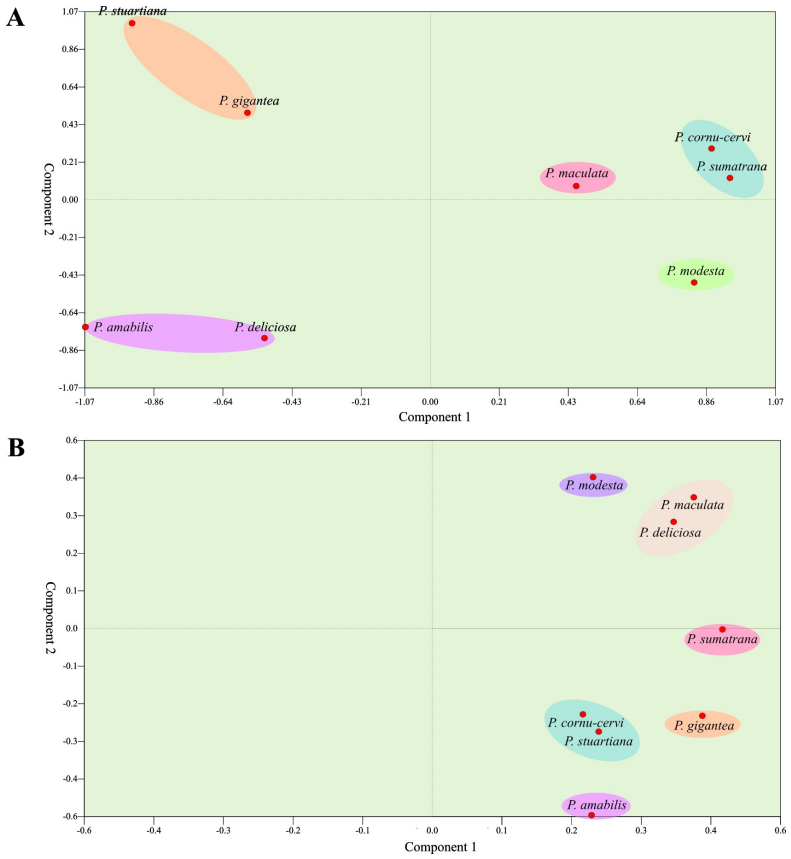

Figure 4. Grouping of native *Phalaenopsis* orchids from South Kalimantan, Indonesia, generated by PCA, for morphological (A) and molecular (B) traits.

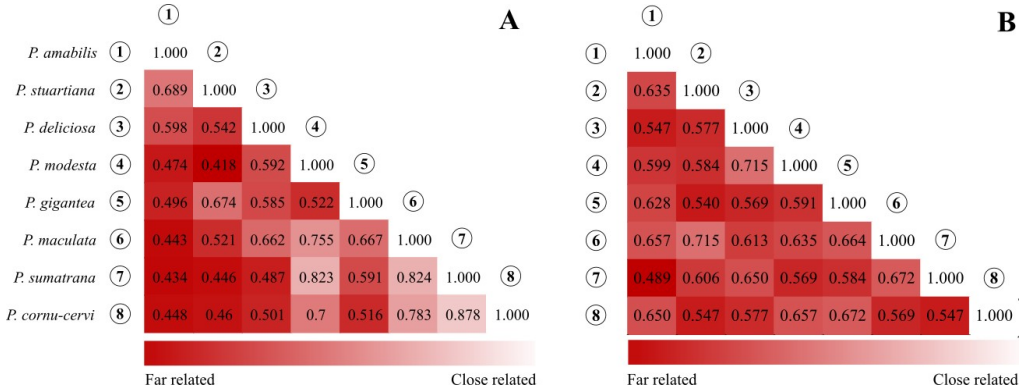

Figure 5. Genetic distance of native *Phalaenopsis* orchids from South Kalimantan, Indonesia, generated by morphological (A) and molecular (B) markers.

However, the improvement of orchid genetic diversity must be done in several ways, such as introgression, hybridization, and mutation (Allier et al., 2020). Given that genetic diversity can be affected by several factors, such as the breeding system, seed dormancy and dispersal mechanism, geographic variation, and environmental disturbance (Huang et al., 2016), the long-term goal of the orchid-breeding program could be directed through an outcrossing approach (Aesomnuk et al., 2021).

Apart from the importance of genetic diversity and relationships, this information is valuable in supporting the conservation and breeding efforts of native *Phalaenopsis* in the future. In other words, since the study on determining the genetic diversity and relationship of native *Phalaenopsis* from South Kalimantan, Indonesia, is limited, this information is also valuable for researchers and breeders in developing new orchid cultivars with particular interest.

Conclusion

Based on morphological and molecular markers, *Phalaenopsis* is highly diverse and shows unique relationships. The UPGMA revealed the closest relationship between *P. cornu-cervi* and *P. sumatrana*, including *P. deliciosa* and *P. modesta*. In contrast, the furthest relationship was shown by *P. amabilis* with *P. sumatrana*. While this information is valuable in supporting the future conservation and breeding efforts of *Phalaenopsis*, data have certain limitations, so further verification of its diversity and relationship using another molecular marker, especially SNP (single-nucleotide polymorphism), is necessary.

Ethical Statement

Ethical approval is not required for this study because this is not a protected germplasm.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Author Contributions

DHM conceptualized and designed the overall study, analyzing data and writing a final manuscript. MAH carried out sample collection and molecular analysis. The authors read and approved the final manuscript.

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Research Article

Assessment of Susceptibility to Downy Mildew Disease in Some Grape Varieties and Genotypes Using Marker-Assisted Selection

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Abstract: The leaves of the vine plant, like its fruits, are used in human nutrition and meals in many countries. Grape leaves serve as an important ingredient in traditional foods. In Türkiye, the leaves of Narince, Sultani Çekirdeksiz and Yapıncak grape varieties are preferred for stuffed grape leaf production. However, pesticide residues generate a serious problem for brined vine leaves. Fungicides with different active ingredients are used to combat downy mildew and powdery mildew diseases in viticulture. Improper use of these chemicals results in serious residue problems on product surfaces. Such cases pose serious threats to human health and the environment. This study used the marker-Assisted Selection (MAS) method to identify individuals containing genes resistant to *Plasmopara viticola* in grape genotypes obtained through hybridization. The presence of the Rpv3 gene in hybrid individuals was examined. Total nucleic acids were extracted from fresh leaves of the plants, and the regions related to the Rpv3 gene were amplified on the genomic DNA with GF18-06/GF18-08 primers. PCR products were visualized using an agarose gel electrophoresis system, and allele gene sizes were also determined by fragment analysis. MAS method yielded 27 genotypes with the Rpv3 gene. DNA sizes were also confirmed by fragment analysis. The promising genotypes were selected for future studies.

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1. Introduction

Grapes are utilized in several different ways. Besides grapes, vine leaves are also used as a foodstuff in various countries (Cangi and Yağcı, 2017). Vine leaves are low in calories and rich in dietary fiber, calcium, phosphorus, phenolic compounds, vitamin C and K1 (El Nehir et al., 1997). In Turkey, leaves of Narince, Sultani Cekirdeksiz, and Yapıncak grape cultivars are mainly used for stuffed grape leaves (Cangi and Yağcı, 2017). Vine leaves used as foodstuff are collected from the vineyards where grapes are produced. Leaves are generally collected between May and July. During this period, different contact or systemic fungicides and insecticides are used in vineyards against some pests and diseases (Eutyepa dieback, powdery mildew, downy mildew, leaf scab, etc.) (Yanar et al., 2017; Bakırcı

et al., 2019). When the pesticide application and leaf collection periods are not well adjusted, pesticide residues on vine leaves may generate a serious problem for human health (Cangi et al., 2014).

Hybrid breeding studies in viticulture have a history of about 200 years. The primary objective of hybridisation studies was initially to achieve high yield and quality varieties, but later on, resistance to biotic or abiotic stressors gained greater emphasis (Reynolds, 2015; Atak, 2024). Breeders largely apply the classical hybridisation method. In this method, genetic variation is created with appropriately selected parents and selection is made among the resultant genotypes. Classical breeders determine yield and quality parameters through field observations. In this method, the breeding period usually takes 25-30 years (Eibach and Töpfer, 2015). With the new techniques developed in recent years, new grape varieties, especially seedless, are being developed in a much shorter time (Doyğacı et al., 2024).

Scientists have made great progress in studies on grapevine genetics since the early 20th century (Vivier and Pretorius, 2000). With the introduction of polymerase chain reaction (PCR)-based DNA analyses, the first genetic map was created (Lodhi et al., 1995). Later on, QTL (Quantitative Trait Loci) were defined for some important traits such as powdery mildew and downy mildew (Fischer et al., 2004). Then, breeders went beyond classical methods and applied MAS (Marker Assisted Selection) methods (Zyprian et al., 2003; Eibach et al., 2007) and used molecular markers for identification of genetic resources. In this way, breeders achieved a time savings of 8-10 years in breeding processes (Verma et al., 2019).

Vitis vinifera species are susceptible to downy mildew (Krul and Mowbray, 1984). However, there are also species within the genus *Vitis* that are resistant to downy mildew. For instance, while North American vine species of *V. aestivalis* and *V. labrusca* are moderately susceptible, *V. cardifolia*, *V. rupestris* and *V. rotundifolia* are relatively resistant. Hybridisation studies were carried out between *V. vinifera* and North American species and new cultivars were obtained by combining downy mildew resistance and fruit quality (Wilcox et al., 2015). Among them, Baron, Cabernet blanc, Cabernet Carbon, Cabertin, Piroso, Rondo, Sauvignac and Monarch varieties with Rpv3 locus were published in the Vitis International Variety Catalogue (VIVC) (https://www.vivc.de/). Regent is also among these varieties. QTLs specifically providing downy mildew resistance were identified in Regent variety (Welter et al., 2007).

Several studies were conducted to determine downy mildew resistance through marker-assisted selection in genotypes obtained from Regent variety and hybrid combinations in which this variety was used as parents (Fischer et al., 2004; Akkurt et al., 2022; Polat and Suluhan, 2024). However, there are no studies on the allele sizes associated with the Rpv3 locus in Isabella variety that are tolerant to both powdery mildew and downy mildew (Atak et al., 2017; Yıldırım et al., 2019; Doğu et al., 2023), in Kishmish Vatkana variety that are tolerant to powdery mildew (Kozma et al., 2006; Hoffmann et al., 2008; Coleman et al., 2009; Bozkurt et al., 2023) and in genotypes obtained from hybridisations in which these varieties were used as parents.

The primary objective of this study is to detect downy mildew tolerant genotypes with high leaf quality at an early stage through marker-assisted selection to reduce the negative effects of pesticide use.

2. Material and Methods

2.1. Material

In 2019, classical hybridisation studies were conducted on the combinations of Narince × Isabella (NVL), Narince × Regent (NRG), and Narince × Kishmish Vatkana, resulting in the production of 447 hybrids. In the years 2020 and 2021, these genotypes were evaluated for their suitability in terms of lob number, leaf sinus depth, leaf hair density, and vein thickness for brined vine leaves, and it was generally determined that they were suitable genotypes (Bozkurt, 2023; Bozkurt and Yağcı, 2024).

Narince grape variety is a wine variety originating from Turkey (Anonymous, 2023) and its leaves are also brined (Cangi and Yağcı, 2017) (Figure 1). Regent variety is obtained as a result of interspecies hybridisation. It has Ren3, Ren9 and Rpv3.1 loci. Regent variety is highly tolerant to downy mildew and powdery mildew diseases (Figure 2), (VIVC, 2020). Kishmish Vatkana grape variety is originated from Uzbekistan (VIVC, 2020) and is tolerant to vineyard powdery mildew (Kozma et al., 2006; Hoffmann et al., 2008) (Figure 3). Isabella (*V. labrusca*) grape variety is tolerant to powdery

mildew and downy mildew (Figure 4) (Yıldırım et al., 2019). These three varieties constitute an important genetic resource for resistance breeding studies (Atak et al., 2017).

Figure 1. Narince (VIVC, 2020).

Figure 2. Regent (VIVC, 2020).

Figure 3. Kishmish vatkana (VIVC, 2020).

Figure 4. Isabella (VIVC, 2020).

2.2. Methods

2.2.1. Total genomic DNA isolation

Scions were collected from the genotypes during pruning and subsequently planted in 0.85-liter pots filled with a peat and perlite mixture. The genotypes were cultivated under standard conditions in a climate chamber. Shoots, approximately 2-3 cm in length, were harvested from the plants and used as a DNA source. Molecular analyses were conducted at the Advanced Technology Research and Application Center of Sivas Cumhuriyet University in 2022. About 150 mg of tissue was taken from the shoot tips of F1 plants, transferred to sterile Eppendorf tubes and stored at -80°C until DNA isolation stage. DNA isolation from these tissues was performed in accordance with the protocol of Piccolo et al. (2012).

2.2.2. Polymerase Chain Reaction (PCR)

The Rpv3 gene region was selected to determine whether the DNA samples of 33 genotypes were resistant to downy mildew (Di Gaspero et al., 2012). The 'GF18-06' and 'GF18-08' primers, which were developed from the 12X reference map of grapevine and determined to be related to the Rpv3.1 gene region, were used (Schwander et al., 2012; Zyprian et al., 2016). Information about the primer pairs, fragment length and annealing temperatures is provided in Table 1.

Table 1. Primers and standard characteristics

Gene	Primer	Forward / Reverse primer	Reference	Fragment Length (bp)	Annealing Temperature (T_A)
Rpv3	GF 18-06F	GGTCTCCTAGAAAGCCAAGCAA	Di Gaspero et al., 2012	389	60
	GF 18-06R	TCCCTTTTCCCCTTGTTCTCG			
	GF 18-08F	GACAATAGCGAGAGAGAATGGG		399	
	GF 18-08R	AGTTGGCTAAAACCCTAGAGGC			

All PCR reactions were prepared in 25 µl volume. The 25 µl reaction volume was composed of: 0.125 U Taq DNA polymerase (Fermentas), 2.5 µl reaction buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl and 0.8% Nonidet P-40), 1 µl of each primer at 10 pmol, 2.5 µl of 2.5 mM dNTP (MBI Fermentas), 2.5 µl of 25 Mm $MgCl_2$ and 1 µl of 100-500 ng template DNA. Final volume was achieved with the use of 25 µl dH_2O. The PCR reactions are enumerated in Table 2 and were performed on a Blue-Ray Biotech thermocycler.

Table 2. PCR reactions

Initial denaturation	at 94 °C for 5 min	
Denaturation	at 94 °C for 30 sec	
Annealing	at 60 °C for 30 sec	30 cycles
Extension	at 72 °C for 2 min	
Final extension	at 72 °C for 10 min	

To determine downy mildew resistant/susceptible genotypes, PCR amplifications of 33 samples were performed with two SSR markers (GF18-06 and GF18-08) specific to the Rpv3.1 gene. Amplification products were run on 1/1.5% agarose gel electrophoresis containing ethidium bromide (2 µg ml^{-1}) and imaged with a UV transilluminator.

Relevant allele sizes of PCR-amplified samples were determined with the use of a Bioanalyzer Qsep100 fragment analyzer. The genotypes for which allele sizes were determined were then assessed as resistant and/or sensitive.

3. Results

Agarose gel electrophoresis revealed that 33 amplified PCR products yielded a PCR product of 389 bp for GF18-06 (Figure 5) and 399 bp for GF18-08 (Figure 6).

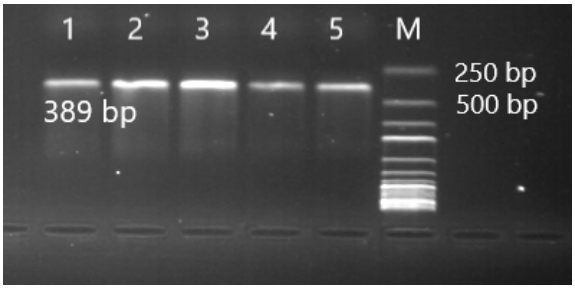

Figure 5. PCR image of Rpv3 gene (GF 18-06).

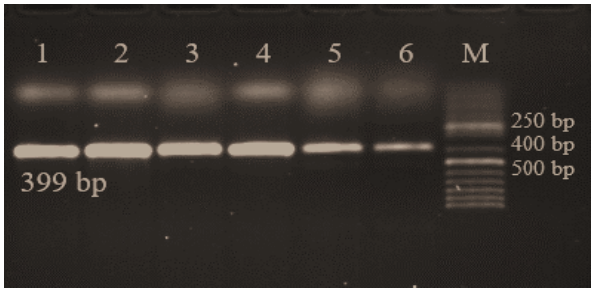

Figure 6. PCR image of Rpv3 gene (GF 18-08).

Allele size images for GF18-06 and GF18-08 primers obtained with a fragment analyzer are shown in Figure 7 and Figure 8.

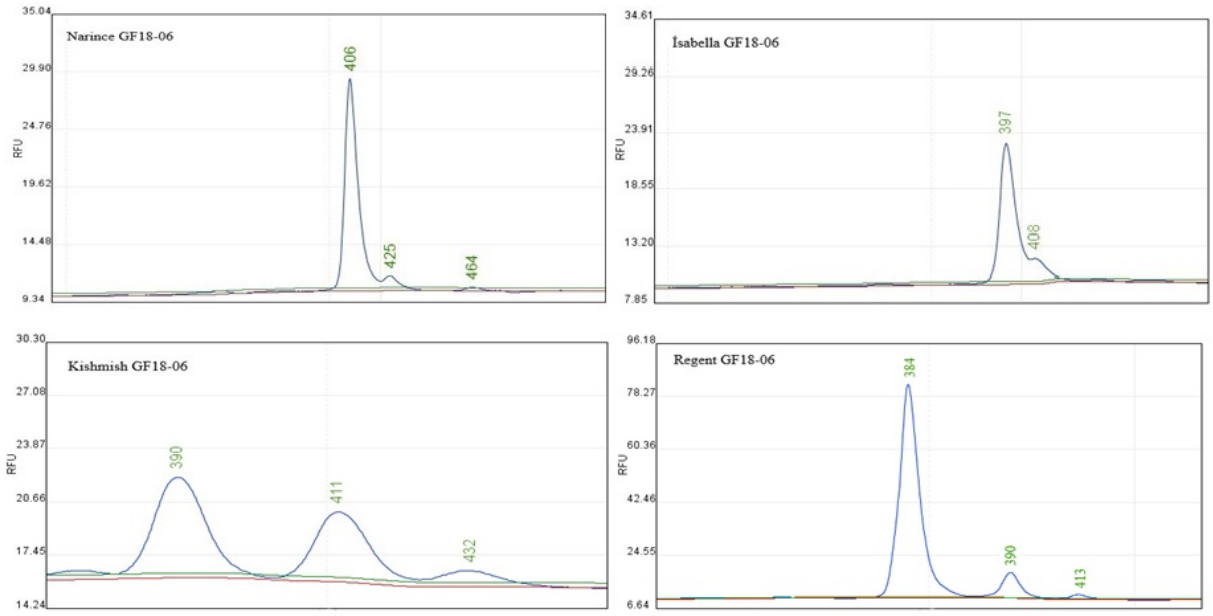

Figure 7. Allele size image for GF18-06 primer to Narince, Isabella, Kishmish, and Regent.

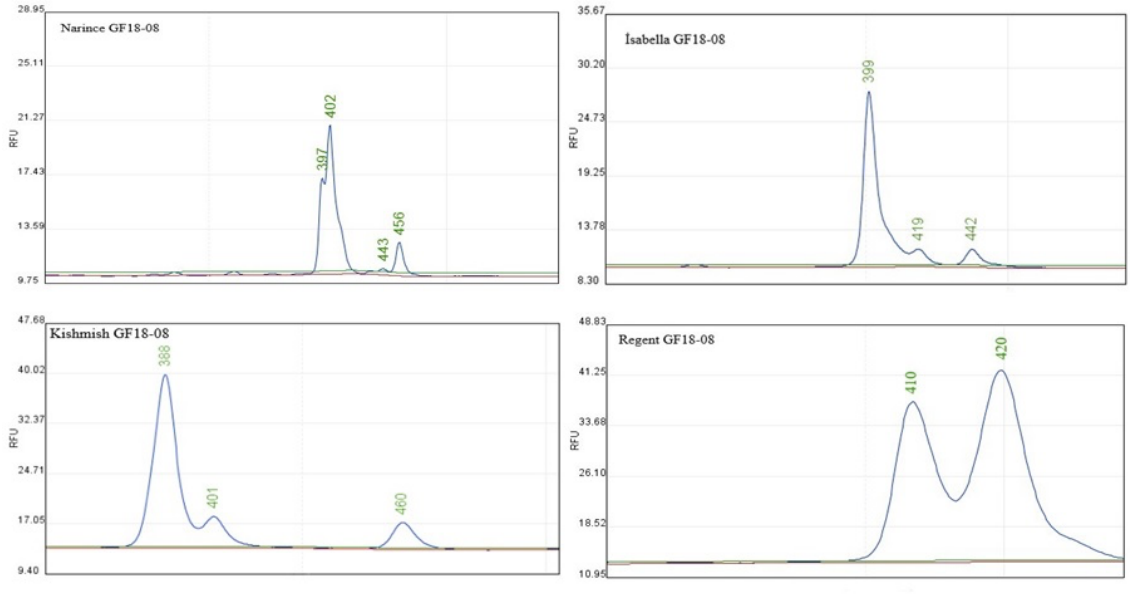

Figure 8. Allele size image for GF18-08 primer to Narince, Isabella, Kishmish, and Regent.

Genetically tested varieties and genotypes all yielded PCR product bands for both primers. With the GF 18-06 primer, allele sizes of 384-390-413 bp were determined in Regent, 390-411-432 bp in Kishmish Vatkana, 406-425-464 bp in Narince and 397-408 bp in Isabella. With the GF 18-06 primer, allele sizes of 410-420 bp were determined in Regent, 388-401-460 bp in Kishmish Vatkana, 397-402-443-456 bp in Narince and 399-419-442 bp in Isabella. Genotypes exhibited bands in the direction of allele sizes derived from the maternal and paternal parents.

In Regent variety with the Rpv3.1 locus, allele sizes of the GF18-06 and GF18-08 primers were taken as reference. Allele sizes of the parents and genotypes are provided in Table 3 and Figure 9, 10, 11, 12, 13, 14 and 15.

Considering the allele sizes of the reference variety Regent, 384-390-413 bp with the GF-18-06 primer and 410-420 bp with the GF 18-08 primer, it was seen that the NRG genotypes carried at least one allele from both primers in the Regent variety. Among them, NRG-147 (2 alleles for the GF18-06 primer, 1 allele for the GF18-08 primer) and NRG-64 (1 allele for the GF18-06 primer and 2 alleles for the GF18-08 primer) were found to be prominent genotypes since they were the same as the Regent variety. The K. Vatkana variety had also 1 allele of the same size (390 bp) in the GF18-06 primer of the Regent variety (Table 3).

The Isabella variety exhibited allele sizes of 397-408 bp with the GF18-06 primer. An allele size of 397 bp was detected in 20 genotypes belonging to the NVL combination; 408 bp in 3 genotypes (NVL-77, NVL-34 and NVL-154). When the allele sizes were examined for the GF18-08 primer, it was determined that 21 genotypes, except for NVL-145, NVL-43 and NVL-186 in the NVL hybrid group and Isabella variety, carried 399 bp allele (Table 3).

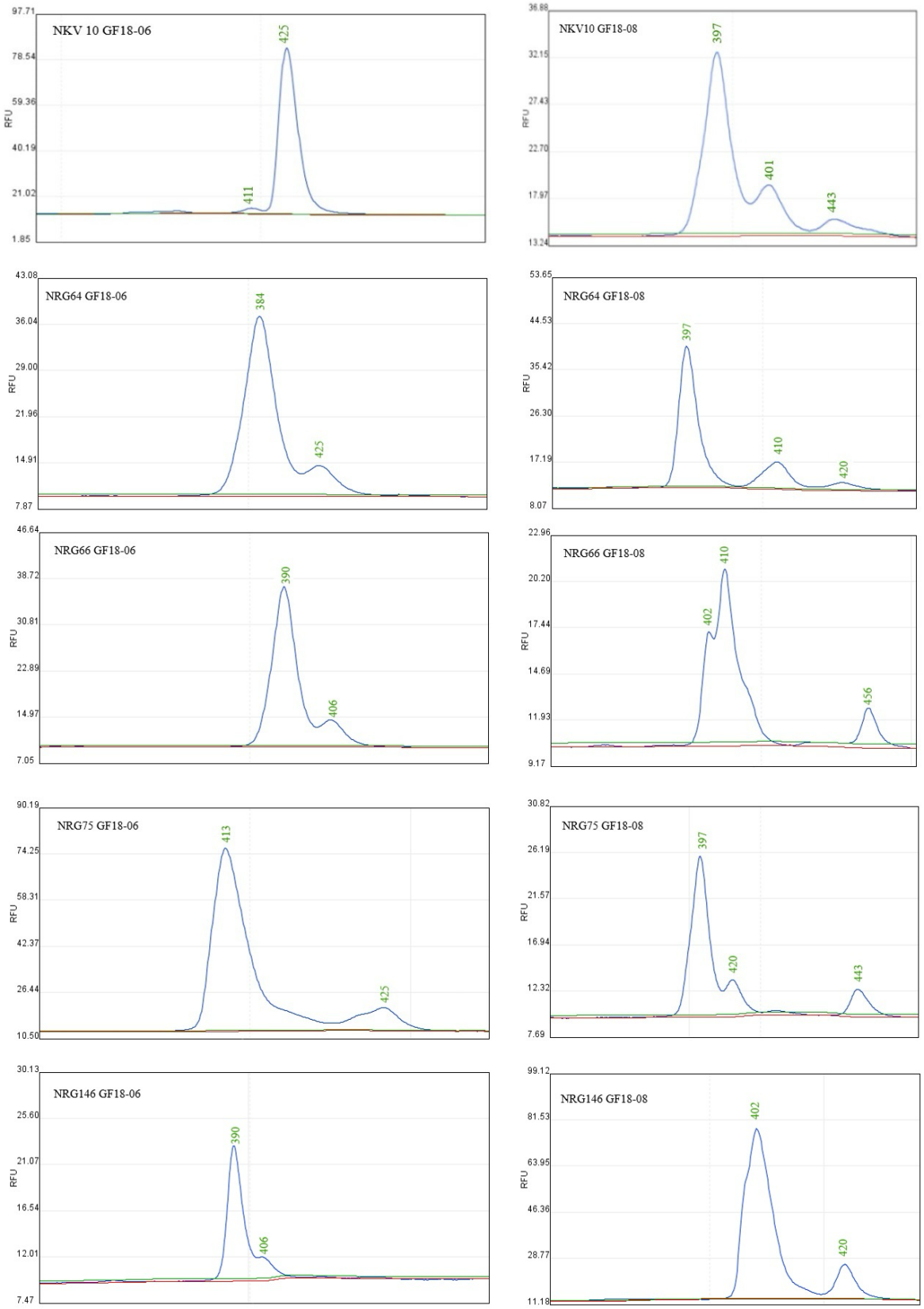

Figure 9. Allele sizes of GF18-06 and GF18-08 primers to NKV-10, NRG-64, NRG-66, NRG-75, and NRG-146.

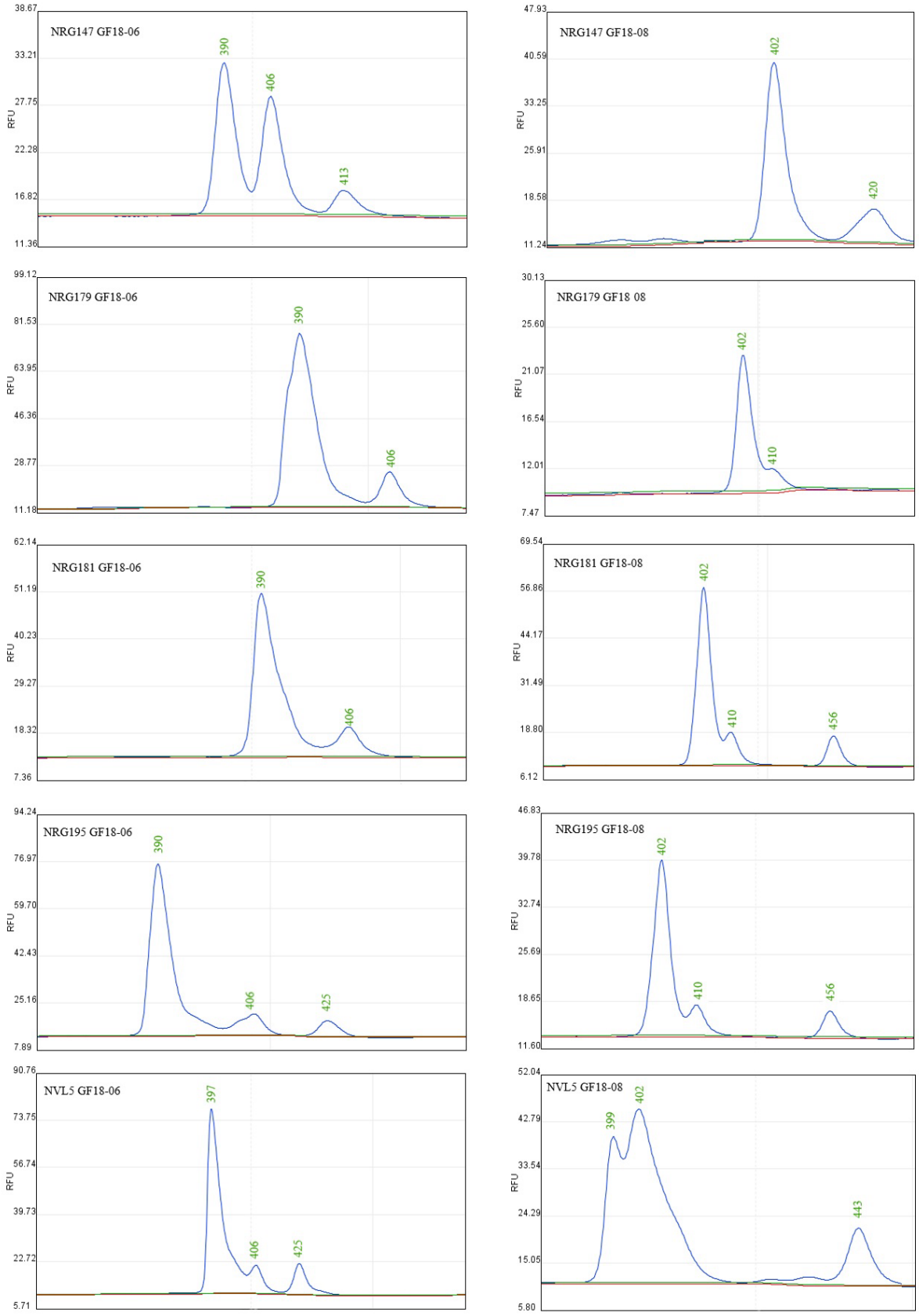

Figure 10. Allele sizes of GF18-06 and GF18-08 primers to NRG-147, NRG-179, NRG-181, NRG195, and NVL-5.

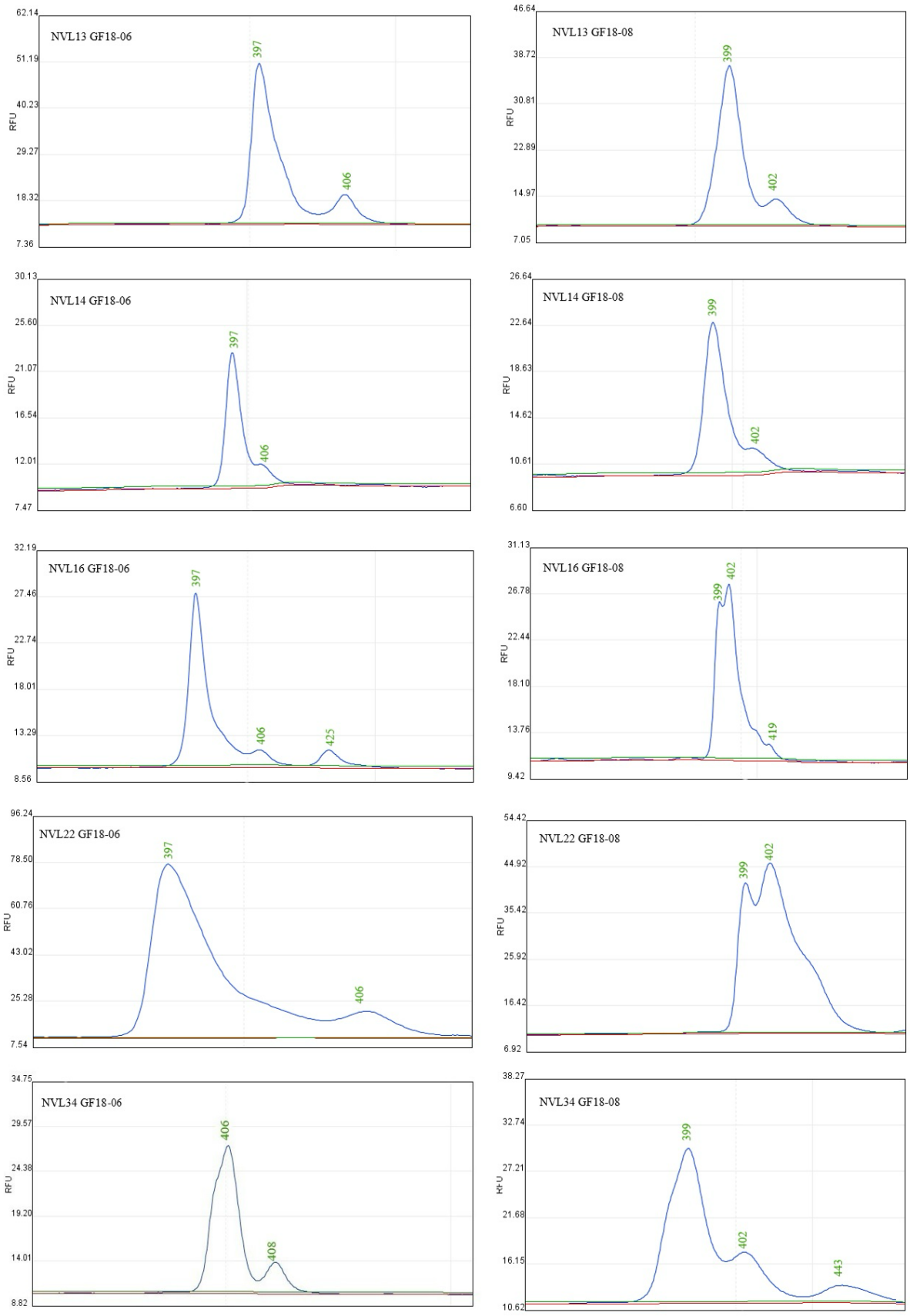

Figure 11. Allele sizes of GF18-06 and GF18-08 primers to NVL-13, NVL-14, NVL-16, NVL-22, and NVL-34.

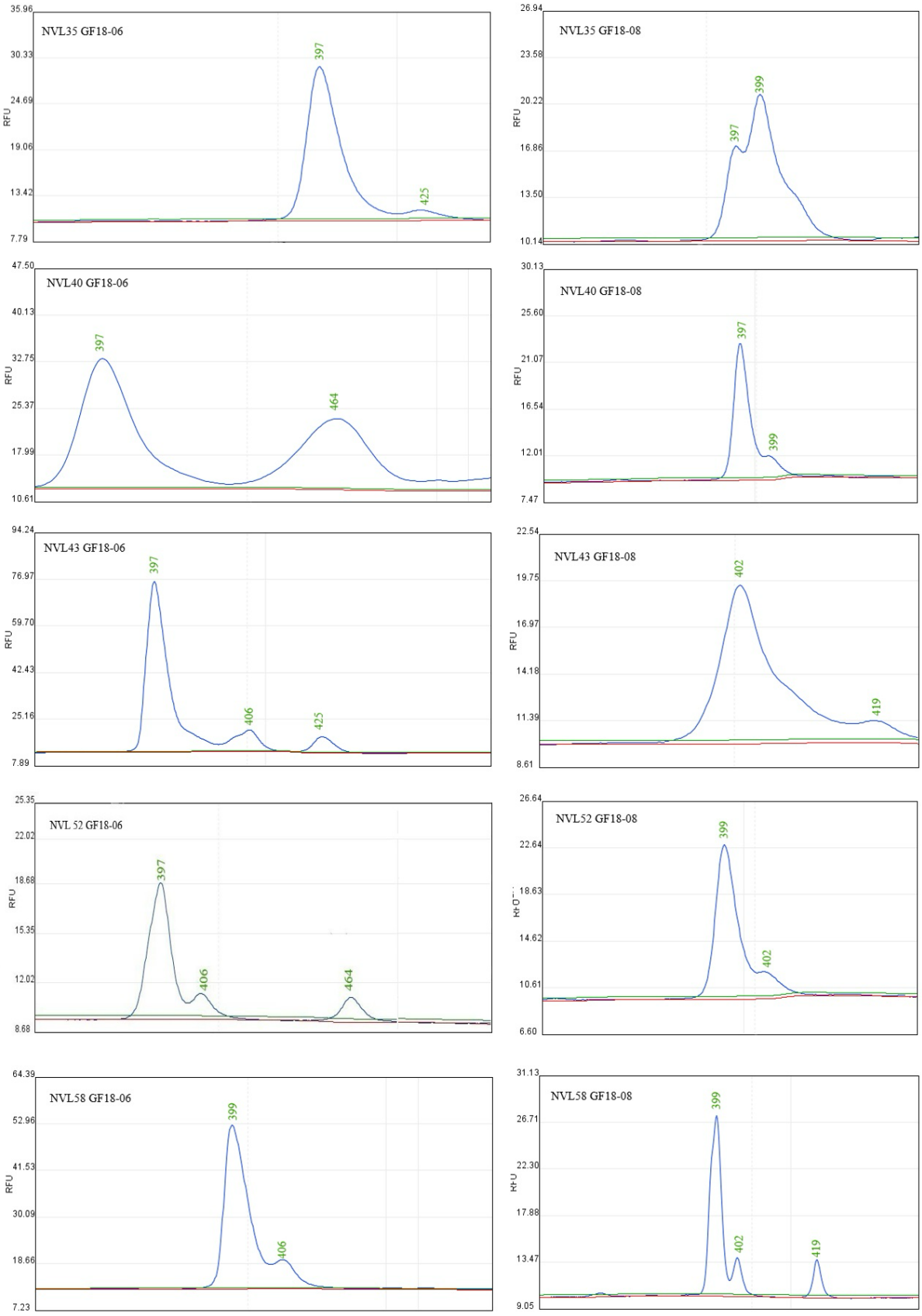

Figure 12. Allele sizes of GF18-06 and GF18-08 primers to NVL-35, NVL-40, NVL-43, NVL-52, and NVL-58.

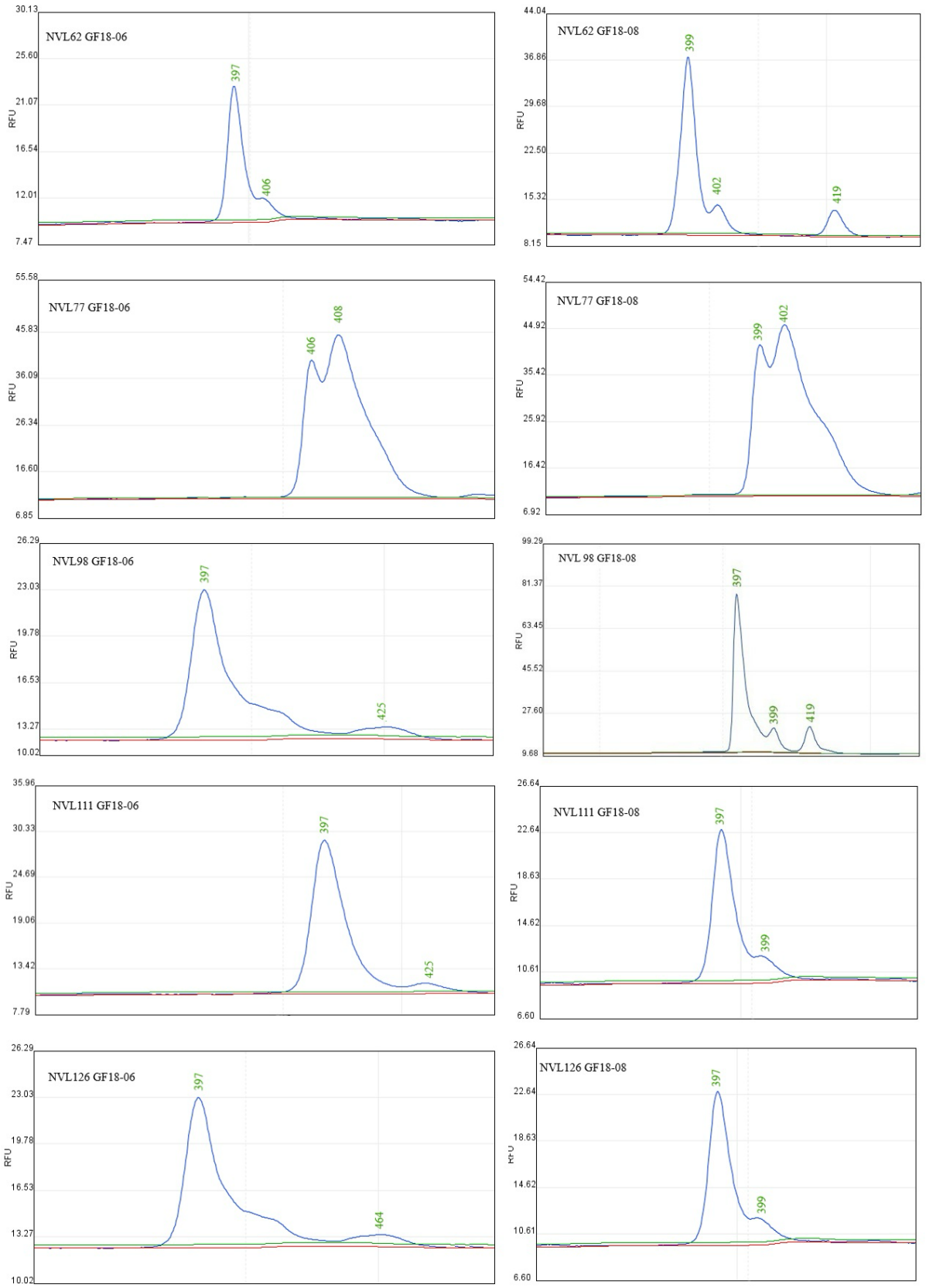

Figure 13. Allele sizes of GF18-06 and GF18-08 primers to NVL-62, NVL-77, NVL-98, NVL-111, and NVL-126.

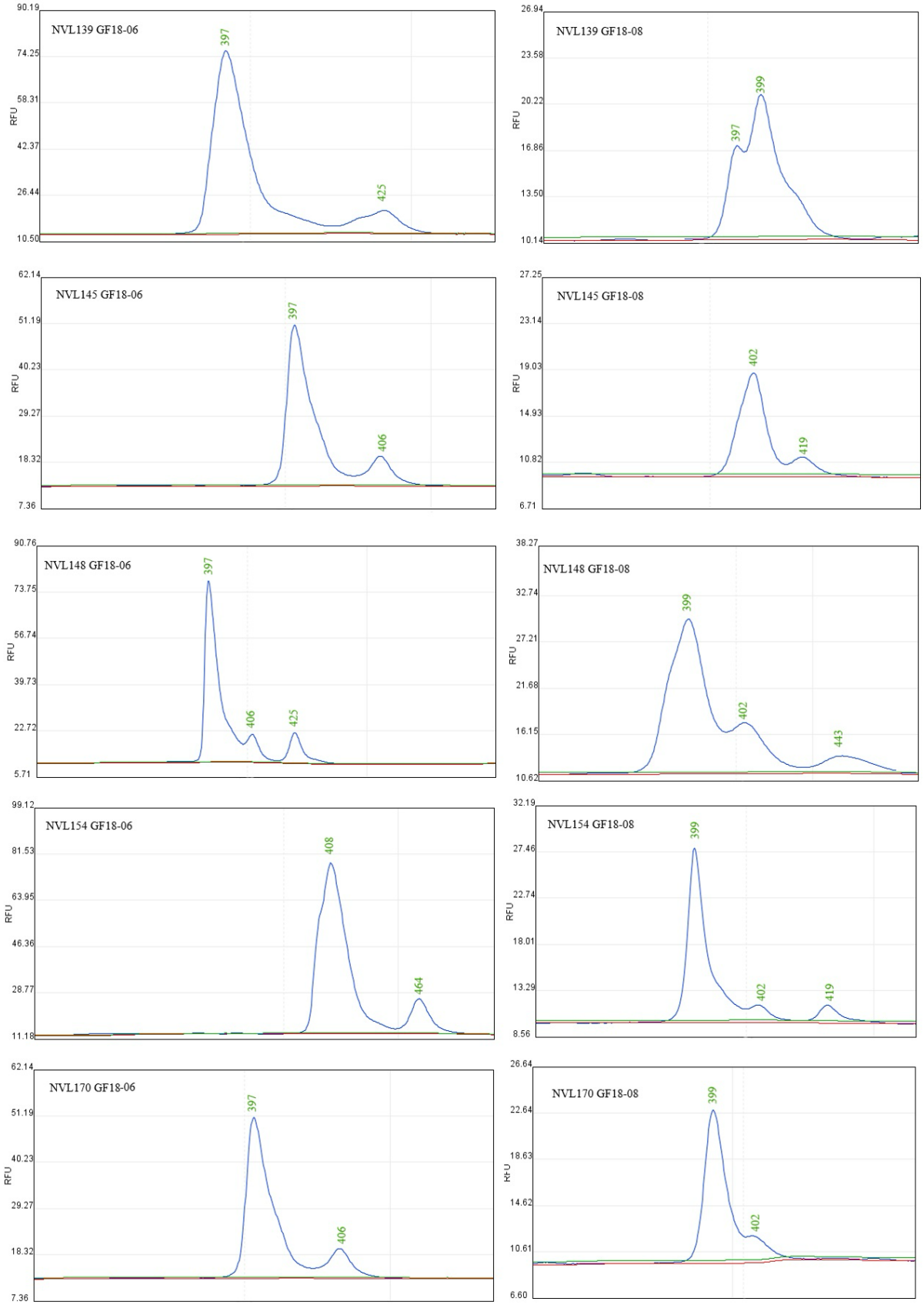

Figure 14. Allele sizes of GF18-06 and GF18-08 primers to NVL-139, NVL-145, NVL-148, NVL-154, and NVL-170.

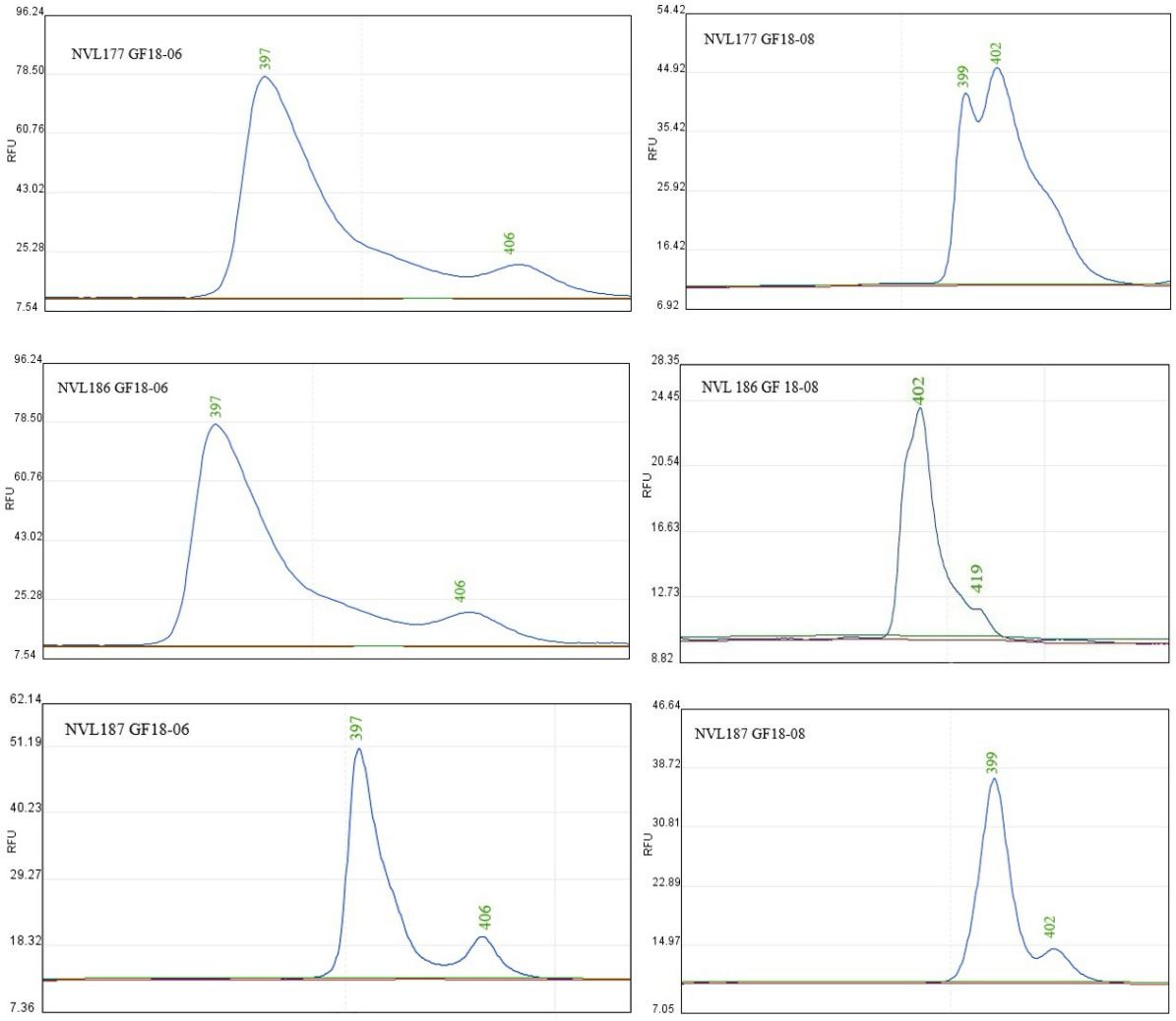

Figure 15. Allele sizes of GF18-06 and GF18-08 primers to NVL-177, NVL-186, and NVL-187.

Table 3. Allele sizes of GF18-06 and GF18-08 primers

Sample	Allele sizes of GF18-06 primer			Allele sizes of GF18-08 primer			
Isabella	397	408		399	419	442	
K. vatakana	390	411	432	388	401	460	
Regent	384	390	413	410	420		
Narince	406	425	464	397	402	443	456
NKV-010	411	425		397	443	401	
NRG-064	384	425		397	410	420	
NRG-066	390	406		402	456	410	
NRG-075	413	425		397	443	420	
NRG-146	390	406		402	420		
NRG-147	390	406	413	402	420		
NRG-179	390	406		402	410		
NRG-181	390	406		402	410	456	
NRG-195	406	425	390	402	410	456	
NVL-005	406	425	397	399	402	443	
NVL-013	397	406		399	402		
NVL-014	397	406		399	402		
NVL-016	406	425	397	399	402	419	
NVL-022	397	406		399	402		
NVL-034	406	408		399	402	443	

Table 3. Allele sizes of GF18-06 and GF18-08 primers (continued)

Sample	Allele sizes of GF18-06 primer			Allele sizes of GF18-08 primer		
NVL-035	397	425		397	399	
NVL-040	397	464		397	399	
NVL-043	397	406	425	402	419	
NVL-052	397	406	464	399	402	
NVL-058	399	406		399	402	419
NVL-062	397	406		399	402	419
NVL-077	406	408		399	402	
NVL-098	397	425		399	397	419
NVL-111	397	425		399	397	
NVL-126	397	464		399	397	
NVL-139	397	425		399	397	
NVL-145	397	406		402	419	
NVL-148	397	406	425	399	402	443
NVL-154	408	464		399	402	419
NVL-170	397	406	425	399	402	
NVL-177	397	406		399	402	
NVL-186	397	406		402	419	
NVL-187	397	406		399	402	

4. Discussion

The results obtained from our study were found to be consistent in many respects when compared with similar studies (Shidfar et al., 2019; Yıldırım et al., 2019; Akkurt et al., 2022). In the present study, the MAS method offered a reliable tool for early selection of resistant genotypes. As it was previously stated in Foria et al. (2018), the present findings confirmed that the response to downy mildew in plants screened for the Rpv3.1 differed based on the genetic background of the plants.

Genotypes with allele sizes of 385-390-407 bp (Akkurt et al., 2022) in relation to Rpv3.1 in the Regent variety with the GF18-06 primer were assessed as resistant candidates. In the present study, an allele size of 390 bp was detected in all genotypes belonging to the NRG hybrid group and these genotypes were assessed as resistant to downy mildew. Detection of an allele size of 390 bp in the Kishmish Vatkana variety suggested that this variety might also be tolerant to downy mildew. The Present findings indicated that the Kishmish Vatkana variety, resistant to powdery mildew (Kozma et al., 2006; Hoffmann et al., 2008; Coleman et al., 2009; Bozkurt et al., 2023), may offer a valuable source also for downy mildew. A similar case is valid for the Isabella variety. The Isabella variety exhibited allele sizes of 397-408 bp with the GF18-06 primer. An allele size of 397 bp in 20 genotypes belonging to the NVL combination and 408 bp in 2 genotypes (NVL-77 and NVL-34) (Table 3). Such a case suggests that the allele size of 397 bp in the Isabela variety might be related to downy mildew resistance. It has been indicated in previous studies where artificial downy mildew tests were conducted on the Isabella variety under both field and laboratory conditions that this variety was resistant to downy mildew (Atak et al., 2017; Yıldırım et al., 2019; Doğu et al., 2023). Unlike the present findings, Zyprian et al. (2016) reported only 387 bp allele associated with downy mildew resistance in the QTL map obtained from the hybrid combination 'GF.GA-47-42' × 'Villard Blanc' with the GF 18-06 marker. In the present study, although 387 bp alleles were not detected, it was thought that 390 bp 'Regent' alleles could provide a source for hybridisation populations for breeding resistance to downy mildew.

Previous studies revealed that downy mildew-resistant Regent variety yielded allele sizes between 399-410-420 bp with the use of GF18-08 primer associated with Rpv3.1 locus and GF18-08 marker had a strong relationship with downy mildew resistance (Uzun et al., 2018; Akkurt et al., 2022). In the present study, NRG-181, NRG-66, NRG-179, NRG-64 and NRG-195 genotypes of Narince x Regent combination yielded an allele size of 410 bp and other genotypes exhibited an allele size of 420 bp. In a similar study, Akkurt et al. (2022) identified 113 of a total of 145 genotypes that gave

amplification products with GF 18-08 marker as resistant. It was particularly indicated that GF 18-08/410 bp 'Regent' allele was strongly associated with downy mildew resistance. In the present study, with GF18-08 primer, an allele size of 399 bp was determined in 21 genotypes belonging to Isabella variety and Narince × Isabella combination. The Present findings suggest that allele size of 399 bp in Isabella variety may be related to downy mildew resistance. Zyprian et al. (2016) used GF18-08 marker in downy mildew resistant varieties Regent, Suberux, Villard Blanc and Seibel 6468 and assessed "399 bp" allele as related to resistance. In a similar study, Possamai et al. (2020) screened 26 different grapevine genotypes, which are Raboso Piave (RP), Kozma 20-3 (K), Solaris (S), Chardonnay and Glera parent grapevine varieties and their hybrids, for specific segregating Rpv loci with the use of SSR markers. It was indicated that 110 different RP x K genotypes carried resistance alleles of Rpv3.1, Rpv12 or both and 255 different RP x S genotypes had resistance alleles of Rpv3.3, Rpv10 or both. It was also indicated that hybrids carrying Rpv3.1 and Rpv12 loci showed the strongest resistance response (low sporulation and necrosis), while those carrying Rpv3.3 locus showed the highest levels of necrosis and the genotypes carrying Rpv10 represented intermediate levels of both sporulation and necrosis. In another study, it was determined that Rpv3.1-mediated resistance identified in Villard Blanc variety was associated with a defense mechanism that resulted in inhibition of pathogen growth and development through triggering the synthesis of stilbenes and HR toxic to fungi (Eisenmann et al., 2019). Additionally, transient co-expression of TIR-NB-LRR gene pairs from Rpv3 locus of *V. vinifera* leaves activated pathogen-induced HR and sporulation was reduced as compared to leaves of non-transformed plants (Foria et al., 2020).

Conclusion

Based on the results obtained in this study, several important conclusions can be drawn regarding the resistance of grapevine genotypes to downy mildew. The molecular analysis indicated that certain genotypes, particularly those from the Narince × Regent (NRG) hybrid group, exhibited allele sizes corresponding to known resistance markers for downy mildew, specifically the 390 bp allele with the GF18-06 primer, which is linked to the Rpv3.1 locus in Regent variety. These genotypes, including NRG-147 and NRG-64, were identified as potential candidates for downy mildew resistance. Additionally, the Kishmish Vatkana variety showed the presence of the 390 bp allele, suggesting a possible tolerance to downy mildew. The Isabella variety also demonstrated promising results, with allele sizes of 397-408 bp with the GF18-06 primer, which were consistent with previously reported downy mildew resistance. The presence of the 399 bp allele in 21 genotypes from the NVL hybrid group further supported this potential resistance. Moreover, the GF18-08 primer revealed strong associations between the 410 bp and 420 bp alleles in NRG genotypes, with the 410 bp allele particularly linked to resistance in previous studies. These findings align with the results of previous research, such as those by Akkurt et al. (2022), and highlight the effectiveness of molecular markers, particularly the GF18-06 and GF18-08 primers, in identifying resistant genotypes. Overall, this study supports the use of molecular marker-assisted selection (MAS) as a reliable tool for early selection of grapevine genotypes resistant to downy mildew. It is recommended that these promising genotypes be further evaluated under field conditions to confirm their resistance profiles and their potential for use in breeding programs aimed at developing downy mildew-resistant grapevine cultivars.

Ethical Statement

This article does not require ethical approval because it does not contain any studies with human or animal subjects.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

The authors of the study contributed equally to all stages of the research.

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Research Article

Pesticide Residues in Raisin and Health Risk Assessment

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Abstract: This study aimed to determine pesticide residues in raisin samples from the Besni and Gölbaşı districts of Adıyaman province, located in the Southeastern Anatolia region of Türkiye. Method validation was carried out for parameters including linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, precision (repeatability and in-laboratory reproducibility), and measurement uncertainty. The results met the criteria outlined in SANTE/11312/2021. A total of 260 pesticides were analyzed, with pesticide residues detected in 95 out of 100 samples. Among these, 42 samples contained a single pesticide, while 53 samples had two or more residues. The insecticides cypermethrin, indoxacarb, and malathion, along with the fungicides boscalid, flubendiamide, fluopyram, pyrimethanil, and spiroxamine, were identified. All detected pesticide residues were within the LOQ and maximum residue limit (MRL), with no residues exceeding the MRL. According to the analysis, eight different pesticides were identified in the samples. The study confirms that pesticide residues in dried grape samples comply with the MRLs, suggesting minimal health risks for consumers, as both long-term and short-term dietary risks were found to be negligible. However, the presence of multiple pesticide residues underscores the need for ongoing monitoring and stringent regulatory measures to ensure food safety and maintain compliance. These findings provide valuable insights into improving sustainable agricultural practices in grape production and establishing a more effective monitoring system for pesticide residues in raisins.

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1. Introduction

Grapes (*Vitis genus*, Vitaceae family) are among the oldest cultivated fruit species, dating back to 3500 B.C. This long history of cultivation has significantly shaped their global economic and cultural importance, particularly in the development of diverse grape-based industries like wine and raisin production. With over 15 000 varieties worldwide, including more than 1 200 in Anatolia, grapes represent significant varietal diversity (Güçer et al., 2021). Approximately 6% of the world's grape cultivation areas are located in Türkiye, the sixth-largest grape producer globally (FAO, 2024). Grape production is important not only for fresh consumption but also for processed products like raisins. Grapes play a significant role in global agriculture, serving as vital raw material for processed products like raisins, which hold considerable economic and nutritional importance.

Türkiye has a prominent role in the global grape industry. Between 2010 and 2021, 51% of Türkiye's total grape production was dedicated to table grapes. According to 2019 statistics, 7% of global grape production was processed into dried grapes. Türkiye ranks first in global raisin exports with a share of approximately 33%. In the 2020/21 season, the leading producers of dried grapes were Türkiye, the United States, Iran, and India, collectively accounting for over 60% of global dried grape production (Demiray and Hatırlı, 2021).

Türkiye holds a significant position as a major producer and exporter of dried grapes worldwide. Most seedless dried grapes are cultivated in the Aegean region. Additionally, viticulture has a longstanding history in Besni, a district of Adıyaman province. The variety known as "Peygamber üzümü" when fresh and the "Besni grape" when dried is predominantly grown in this area and is widely consumed as a dried product (Demiray and Hatırlı, 2021). Raisins are rich in natural sugars and serve as an excellent source of dietary fiber, essential vitamins, minerals, and antioxidants. Raisins are rich in natural sugars, dietary fiber, essential vitamins such as B-complex and K, minerals like potassium, calcium, iron, and antioxidants. The dehydration process concentrates these nutrients, making raisins a healthy and convenient snack. They are also free of fat and cholesterol (Rahimi et al., 2021).

Grape production is challenged by numerous pests and diseases, including insects like *Lobesia botrana*, mites such as *Tetranychus urticae*, and fungi including *Botrytis cinerea* and *Plasmopara viticola*, which require frequent management practices (Balkan and Kara, 2023). Additionally, vineyards are affected by various weed species from different families, necessitating continuous control measure. To produce high-quality, high-yield grapes, producers frequently resort to pesticide applications. These chemicals are preferred to their rapid action, ease of access, and simple application. However, excessive pesticide results in environmental pollution, health risks, resistance development, and residue issues (Polat and Tiryaki, 2022; Duman and Tiryaki, 2023).

The widespread use of pesticides in agriculture has raised significant concerns regarding their potential negative impacts on human health and the environment (Balkan and Yılmaz, 2022). Consequently, monitoring pesticide residues in various matrices, including soil and agricultural products, has become critically important (Cebeci, 2020). This surveillance is essential to understand, control, and regulate pesticide exposure in both agricultural produce and the environment. Such measures play a vital role in protecting consumer health and ensuring the environmental sustainability of agriculture. Monitoring pesticide residues provides a scientific basis for making agricultural practices safer and more sustainable.

In developed countries, the detection and monitoring of pesticide residues is a top priority to safeguard both consumer health and the environment.. The European Union (EU) has implemented the Rapid Alert System for Food and Feed (RASFF), which facilitates swift and coordinated responses to health threats arising from food or feed. Issues related to residues are reported to RASFF contact points and subsequently communicated to the European Commission. This framework enables member states to take necessary precautions or stay informed. In this context, an analysis of RASFF notifications for pesticide residues in grapes an essential export commodity for Türkiye between 2021 and 2024 reveals that active substances such as acetamiprid, cypermethrin, dithiocarbamates, iprodione, lambda-cyhalothrin, metalaxyl, triadimenol, and pyriproxyfen exceeded the Maximum Residue Limits (MRLs) (RASFF, 2024). Additionally, previous studies have reported the presence of various pesticide residues in raisins and grapes, underscoring ongoing food safety concerns (Turgut et al., 2011; Shaber et al., 2017; Nalcı et al., 2018; Yakar, 2018; Constantinou et al., 2021; Mahdavi et al., 2022; Farshidi et al., 2023; Kanbolat et al., 2023; Zhang et al.,2024).

In recent years, consumer sensitivity towards accessing safe food has increased significantly (Nerpagar et al., 2023). The rejection of exported plant-based products at customs due to residue concerns and the resulting negative impact on a country's image have become widely discussed issues. To minimize these challenges, it is essential to disclose agricultural production processes and outcomes transparently. Therefore, on-site residue monitoring across agricultural locations is crucial. Given the rising demand for safe and high-quality agricultural products, the need for systematic residue analysis is more critical than ever. This study aims to analyze pesticide residues in dried grape samples (raisins) and evaluate the potential health risks associated with them.

2. Material and Methods

2.1. Reagents and chemicals

Pesticide reference materials were purchased from Dr. Ehrenstorfer Laboratories GmbH (Augsburg, Germany). Acetonitrile (MeCN > 99% purity), methanol (MeOH > 99% purity), magnesium sulfate anhydrous ($MgSO_4 \geq 99\%$ purity), ammonium formate (NH_4HCO_2 with 99.0% purity) and acetic acid (AcOH) were obtained from Millipore. PSA (Primary Secondary Amine, 40 µm particle size) was supplied from Supelco Analytical (Bellefonte, PA, USA).

2.2. Sampling procedure and sample preparation

The collected samples were homogenized, and 7.5 g of each sample was weighed into a 50 ml falcon tube, followed by the addition of 7.5 ml of distilled water. The subsequent steps are illustrated in Figure 1. The extraction and cleanup followed the QuEChERS AOAC Method 2007.01, as outlined by Lehotay, (2007). This method is particularly suitable for this analysis due to its simplicity, efficiency, and effectiveness in handling complex matrices like dried grapes while ensuring high recovery rates for various pesticides.

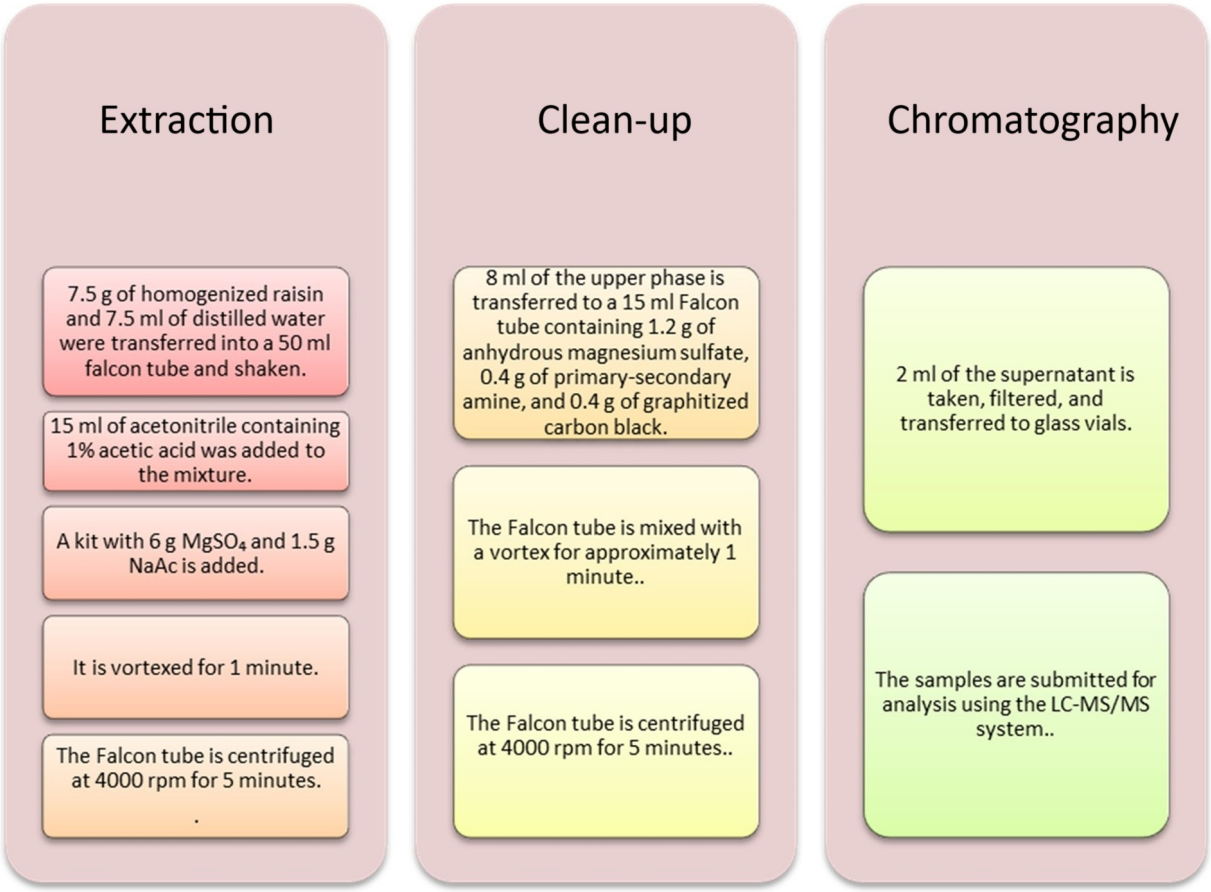

Figure 1. Analytical steps of the QuEChERS-AOAC Official Method 2007.01

2.3. Instrumentation and method validation

The analyses were conducted using the equipment and conditions specified in Table 1.

Table 1. Instruments and conditions

LC System and Conditions (Nexera X2)		**MS Conditions (LCMS-8050)**	
Pump	LC-30AD		
Autosampler	SIL-20A	**Detector**	MS/MS
Degasser	DGU-20A3R		
Column	Purospher STAR RP-18 endcapped (2.1 mm x 100 mm, 2 µm)	**Ionization mode**	ESI (+/ -)
Oven temp.	50 °C	**Desolvation line temp.**	250 ºC
Solvent A	10 mmol L^{-1} ammonium acetate/water	**Interface temp.**	300 ºC
Solvent B	Methanol	**Block heater temp.**	400 ºC
Gradient	25%B. (0.5 min)- 98%B. (10.5-13.0 min)-25%B. (13.1-16 min)	**Nebulizer gas flow**	3.0 L min^{-1}
Flow rate	0.4 mL min^{-1}	**Drying gas flow**	10.0 L min^{-1}
Injection vol.	10 µL	**Heating gas flow**	15.0 L min^{-1}
Rinse solution	R0: 50% methanol	**Dwell time**	1-33 msec

Chromatographic method validation/verification is crucial to quality assurance (QA) and quality control (QC). The QuEChERS method, widely used in well-equipped laboratories requires validation/verification under local laboratory conditions (Dülger and Tiryaki, 2021). Method performance criteria encompass linearity, limits of detection (LOD), limits of quantification (LOQ), accuracy, trueness, precision (repeatability and intra-laboratory reproducibility), and uncertainty. The method has been validated following the guidelines outlined in the "Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed" document (SANTE, 2021).

For the assessment of linearity, a seven-point calibration curve was constructed at concentrations of 0 (blank), 5, 10, 25, 50, 100, and 200 µg kg^{-1}, with three replicate injections at each level. Matrix-matched calibration was employed to minimize matrix effects. LOD and LOQ were determined by fortifying a pesticide-free matrix (dried grapes) with a working solution at a concentration of 10 µg kg^{-1}. Ten replicate analyses were performed, and the standard deviation (SD) of the results was used to calculate LOD and LOQ. LODs were calculated as three times the SD, while LOQs were defined as ten times the SD, in accordance with established guidelines (Magnusson and Örnemark, 2014). The recovery of pesticides from the matrix and the method's precision were evaluated through five replicate analyses of samples enriched at two different concentration levels (10 and 50 µg kg^{-1}). Repeatability (RSDr) was assessed on the same day by two different analysts, whereas intra-laboratory reproducibility (RSDwR) was evaluated over five consecutive weeks, also by two analysts. Precision was expressed as relative standard deviation (RSD), and %RSD values were calculated to ensure compliance with the ≤20% criterion. The accuracy of the data was assessed based on the recovery values obtained from both repeatability and reproducibility studies, ensuring compliance with the acceptable range of 70-120% as defined by (SANTE, 2021). In accordance with SANTE guidelines, the expanded measurement uncertainty (U') for all pesticides was estimated using Approach 2. Instrument data were processed using "LabSolution® software (version 5.97)".

2.4. Pesticide residues in raisins

A total of 100 besni raisin samples were analyzed. To detect pesticides of varying chemical structures following extraction and cleanup, a validated QuEChERS-LC-MS/MS method was employed. Three analytical portions were taken from each sample, and all analyses were performed in triplicate. Pesticide identification was based on two key criteria: retention time (RT) and ion ratio in accordance with established guidelines (SANTE, 2021). The detected residues were evaluated against the MRLs set by the European Union (EU-MRL, 2024).

2.5. Dietary risk assessment

Dietary pesticide exposure was evaluated by estimating both long-term and short-term intake, corresponding to chronic and acute health risk assessments.

2.5.1.Chronic Risk

The International Estimated Daily Intake (IEDI) (mg kg^{-1} bw) and the chronic exposure risk (HQc) for pesticide residues were calculated using the following equations:

$$\mathrm{IEDI} = (\mathrm{STMR} - \mathrm{P}\ x\ \mathrm{FC})/\mathrm{bw} \quad (1)$$

$$\mathrm{HQc} = \mathrm{IEDI}/\mathrm{ADI} \quad (2)$$

STMR-P (mg kg^{-1}) represents the supervised trials median residue (The median residue in raisins was calculated by multiplying the STMR in the raw commodity by a processing factor). FC (kg day^{-1}) denotes the average fruit consumption, bw (kg) refers to the average body weight, and ADI (mg kg^{-1} bw) represents the acceptable daily intake of pesticide. The ADI values for the pesticides analyzed were obtained from the EU Pesticides Database (EU-MRL, 2024). The average annual grape consumption per individual was 26.1 kg, corresponding to a daily intake of 0.072 kg for the general national population (TUIK, 2024). A body weight of 73.7 kg was assumed for adults (> 15 years) (TUIK, 2025).

2.5.2. Acute Risk

The assessment of pesticide residue exposure was conducted using a deterministic approach. Short-term intake calculations were carried out based on the International Estimation of Short-Term Intake (IESTI) methodology, as outlined by the Joint FAO/WHO Meeting on Pesticide Residues (FAO/WHO, 2018). IESTI (mg kg^{-1} bw) and acute exposure risk (HQa) were determined using the following equations:

$$\mathrm{IESTI} = (\mathrm{LP}\ x\ \mathrm{STMR} - \mathrm{P}\ )/\mathrm{bw} \quad (3)$$

$$\mathrm{HQa} = \mathrm{IESTI}/\mathrm{ARfD} \quad (4)$$

LP (kg) is the large portion of raisin consumption, ARfD (mg kg^{-1} bw) is the acute reference dose.

If HQ > 1, health risk was deemed unacceptable, while HQ < 1 indicated an acceptable health risk. The large portion consumption values (LP) were sourced from the WHO Food Consumption Database (WHO, 2024). The ARfD (acute reference dose) values for each pesticide were retrieved from WHO and EFSA databases (EU-MRL, 2024). The acute risk of boscalid could not be evaluated due to the unavailability of ARfD.

3. Results

3.1. Method validation

The method validation parameters for the pesticide in raisins are detailed in Table 2. The correlation coefficients (R^2) for the pesticide calibrations exceeded 0.9949. The LOD ranged from 0.85 to 2.65, while the LOQ were calculated to be between 2.83 and 8.85. Recovery values within the method's scope ranged from 79.05% to 114.87%, and relative standard deviation (RSD) values varied from 4.40% to 19.94% (Table 2). According to the SANTE guidelines, each active substance must achieve recovery rates between 70% and 120% and RSD values of ≤20% to meet the method performance criteria. The expanded measurement uncertainty (U') values remained below the 50% threshold established by SANTE. The detected pesticides met all these criteria (Table 2).

Table 2. Method validation parameters

Pesticide	RT (min)	Precursor ion, m z^{-1}	Product ions, m z^{-1} (CE, eV)	Correlation coefficient (R^2)	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Fortification (µg kg^{-1})	Measured (µg kg^{-1})	Recovery[1] (%)	RSD (%)	U (%)
Boscalid	6.797	342.90	307.00 (-21)	0.9949	2.65	8.84	10	9.48	94.83	10.20	30.17
			140.30 (-19)				50	41.58	83.16	6.81	
Cypermethrin	8.902	433.20	190.95 (-15)	0.9993	2.12	7.05	10	8.08	80.77	4.56	30.93
			192.85 (-15)				50	44.75	89.51	3.62	
Flubendiamide	7.436	680.90	254.20 (29)	0.9973	2.49	8.29	10	9.69	96.89	12.44	31.64
			272.10 (18)				50	55.07	110.15	11.60	
Fluopyram	6.060	397.00	145.00 (-39)	0.9953	1.56	5.22	10	9.94	99.37	10.63	19.23
			173.00 (-19)				50	48.39	96.78	6.01	
			208.00 (-18)								
Indoxacarb	8.193	528.10	203.00 (-25)	0.9927	0.94	3.12	10	9.75	97.51	12.27	32.12
			150.10 (-53)				50	57.43	114.87	9.54	
			218.00 (-30)								
Malathion	7.129	331.00	127.10 (-23)	0.9999	1.42	4.73	10	8.63	86.35	12.56	30.46
			99.00 (-23)				50	45.57	91.14	3.89	
Pyrimethanil	7.379	200.10	107.00 (-23)	0.9987	2.36	7.88	10	7.91	79.05	8.17	28.74
			82.10 (-26)				50	48.35	96.69	5.42	
Spiroxamine	8.150	298.00	144.10 (-30)	0.9997	0.85	2.83	10	8.58	85.85	5.77	23.73
			100.20 (-22)				50	46.52	93.03	4.83	

[1]Overall recovery of the method (accuracy of the method,%):93.50 (n = 320, RSD% =3.85).

3.2. Pesticide residue analyses in raisin

The results of the pesticide residue analysis conducted on 100 raisin samples are summarized in Table 3. The analysis identified eight pesticides, with residues detected in 95% of the samples. Among these, three insecticides (cypermethrin, indoxacarb, and malathion), while five were fungicides: boscalid, flubendiamide, fluopyram, pyrimethanil, and spiroxamine. Processing factors, which describe the ratio of pesticide residue levels in processed food to those in raw agricultural commodities, account for changes in residue concentrations due to food processing methods such as drying, washing, or cooking (Polat, 2021). After applying the processing factors, all detected residues were found to remain~~ed~~ below the MRLs (Table 3).

Table 3. Pesticide residues (mg kg^{-1}) in raisin

Pesticide	Frequency of detection	Pesticide residue	Processing factor	Number of samples >LOQ and percentage (%)	Number of samples >MRL	MRL[3] (mg kg^{-1})
Boscalid	19	0.013-0.123	2.4 [1]	95 (95)	-	5
Cypermethrin	90	0.010-0.219	3.3 [2]		-	0.5
Flubendiamide	2	0.031-0.121	0.3 [1]		-	2
Fluopyram	50	0.010-0.082	2.9 [1]		-	2
Indoxacarb	2	0.014-0.016	2.7 [1]		-	2
Malathion	1	0.012	-			0.02
Pyrimethanil	18	0.011-0.049	3.7 [1]		-	5
Spiroxamine	1	0.016	4.0 [1]		-	0.6

[1]Zincke et al., 2022, [2] Dinçay et al., 2017, [3]EU-MRL, 2024.

In the study, pesticide residues were detected in at least one sample of 95 out of 100 dried grape samples. Among these, one pesticide residue was found in 42 samples, while two or more residues were identified in 53 samples. All 200 pesticide residue values were within the range of the LOQ and the MRL. No residue values above the MRL were detected (Figure 2).

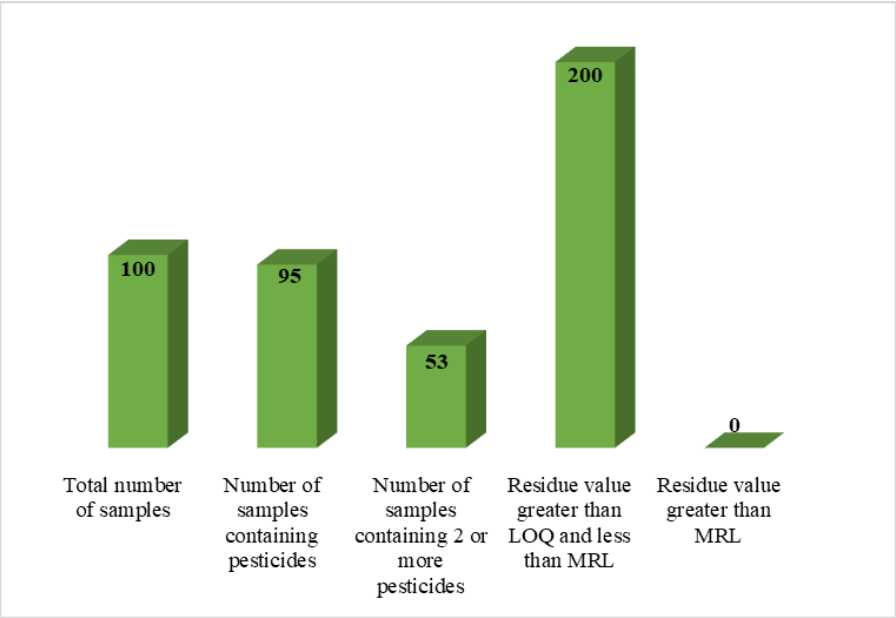

Figure 2. Pesticide residue levels in raisins.

The active ingredients detected in the dried grape samples are presented in Figure 2. Cypermethrin, fluopyram, boscalid, and pyrimethanil were the most frequently identified active substances. According to the Plant Protection Products (PPP) database, cypermethrin is registered at 200 g L^{-1} (preharvest interval, PHI: 7 days) for control of grapevine moth (*Lobesia botrana*); fluopyram is registered at 200 g L^{-1} Fluopyram + 200 g L^{-1} Tebuconazole (PHI: 14 days) for control of powdery mildew (*Erysiphe necator*); boscalid is registered at 200 g L^{-1} boscalid + 100 g L^{-1} kresoxim-methyl, with 50% boscalid (PHI: 28 days) for control of powdery mildew; and pyrimethanil is registered at 300 g L^{-1} pyrimethanil (PHI: 7 days) for control of gray mold (*Botrytis cinerea*) (PPP, 2023).

3.3. Health risk assessment

The long-term (chronic) and short-term (acute) dietary risk assessments for the pesticide residues detected in raisins are summarized in Table 4. Chronic dietary exposure was evaluated based on the ADI, while acute dietary exposure was assessed using the ARfD.

Table 4. Long-term and short-term risk assessment of pesticide residues in raisins

Pesticide	ADI (mg kg^{-1} bw)	Long-term risk assessment IEDI (mg kg^{-1} bw)	HQc	ARfD* (mg kg^{-1} bw)	Short-term risk assessment IESTI (mg kg^{-1} bw)	HQa
Boscalid	0.040	4.60E-05	1.15E-01	/	2.42E-05	-
Cypermethrin	0.050	5.82E-05	1.16E-01	0.20	2.23E-05	1.11E-04
Flubendiamide	0.017	7.37E-05	4.34E-01	0.10	3.10E-04	3.10E-03
Fluopyram	0.012	4.15E-05	3.46E-01	0.50	1.81E-05	3.62E-05
Indoxacarb	0.005	1.46E-05	2.91E-01	0.01	6.81E-06	1.36E-03
Malathion	0.030	1.16E-05	3.88E-02	0.30	1.47E-05	4.90E-05
Pyrimethanil	0.170	1.46E-05	8.56E-03	1.00	4.97E-06	4.97E-06
Spiroxamine	0.025	1.55E-05	6.21E-02	0.10	4.90E-06	4.90E-05

The highest HQc value is 0.35 for fluopyram, indicating that its long-term exposure is closest to the ADI but still within acceptable limits. Other pesticides have HQc values significantly below 1, suggesting no considerable long-term dietary risk. The highest acute intake estimate is for flubendiamide (0.003 mg kg^{-1} bw), which remains well below its ARfD (0.10 mg kg^{-1} bw). Indoxacarb (0.001 mg kg^{-1} bw) also shows a notable value but remains within safe limits. All HQa values are below 1, meaning none of the pesticides pose an acute dietary risk. The estimated daily intake for all pesticides is substantially lower than their respective ADI values, indicating no chronic risk. Similarly, IESTI values are well below the ARfD thresholds, confirming that there is no acute dietary risk for consumers.

4. Discussion

Pesticide residue studies on dried grapes in Türkiye and other countries indicate similar findings, particularly in detecting pesticides like boscalid, cypermethrin, and pyrimethanil.

Research on pesticide residues in raisins and grapes from Türkiye has revealed significant findings. Turgut et al. (2010) conducted a study on dried grapes from the Aegean region, identifying chlorpyrifos-ethyl, chlorpyrifos-methyl, deltamethrin, lambda-cyhalothrin, dichlofluanid, iprodione, and procymidone. Gazioğlu Şensoy et al. (2017) reported the presence of ten different pesticides in dried grape samples; however, the absence of national or international standard tolerance values for raisins prevented a comparative evaluation of their impact. Dinçay et al. (2017) identified boscalid, cypermethrin, fluopyram, indoxacarb, and pyrimethanil in dried grapes. In both studies from the Aegean region, lambda-cyhalothrin, chlorpyrifos ethyl, and methyl were reported to be above the MRL. The researchers indicated that pyrimethanil exceeded the MRL in dried grapes. Soydan et al. (2021) analyzed 3044 fruit and vegetable samples from the Aegean region, detecting 64 different pesticide residues, including chlorpyrifos, azoxystrobin, triadimenol, carbendazim, pyrimethanil, cyprodinil, fludioxonil, and boscalid, with 11.6% exceeding MRL limits. In reviewing previous pesticide residue studies on raisins and grapes in Türkiye, similar detections of boscalid, cypermethrin, and pyrimethanil were detected. Moreover, while some pesticides were reported to exceed the MRL in earlier studies, no such values were found in our study. The residue levels detected in the Adıyaman province were below the MRL, which may be attributed to the application of cypermethrin during the early stages of fruit development, both before and after flowering, and the early application of pyrimethanil. Agricultural practices in this area, such as the strategic timing of pesticide applications during the early growth stages and the relatively dry climate, which redeuces fungal pressure, play a significant role in maintaining low residue levels contrast, the higher humidity levels in the Aegean region lead to an increased frequency of pesticide applications. Nalcı et al. (2024) reported that pesticide residue levels in early, mid, and late-season grape varieties varied significantly based on agricultural practices and environmental conditions.

When examining pesticide residue studies conducted in other countries, several pesticides including pyrimethanil, cypermethrin, boscalid, and chlorpyrifos have been frequently detected. Constantinou et al. (2021), identified pyrimethanil, boscalid, cyprodinil, fludioxonil, tebuconazole, indoxacarb, fenhexamid, and imidacloprid in dried grape samples, noting that ten of these pesticides are not authorized in the EU. They reported that six pesticides (carbendazim, ethion, fenpropathrin, fenvalerate, iprodione, and phosmet) exceeded the maximum residue limits. Mahdavi et al. (2022) investigated ready-to-eat raisins from Iran, detecting 57 pesticide residues, including carbendazim, acetamiprid, thiodicarb, iprodione, and chlorpyrifos, with 23% of samples exceeding MRLs. Farshidi et al. (2023) recorded residues of azoxystrobin, bromopropylate, chlorpyrifos, cypermethrin, diazinon, difenoconazole, ethion, fenitrothion, fenpropathrin, fenvalerate, kresoxim-methyl, malathion, metalaxyl, penconazole, permethrin, phosalone, and piperonyl butoxide in dried grape samples. Zhang et al. (2024) conducted an extensive four-year analysis in South and Southwest China, identifying 40 different pesticide residues, including difenoconazole, cyhalothrin, and propiconazole, in 94.6% of grape samples, of which 5.7% exceeded MRLs. Compared with studies conducted in other countries, our study found similar active ingredients (boscalid, cypermethrin, indoxacarb, and pyrimethanil) present in the dried grape samples. Studies in Iran and Greece have also reported these compounds in dried grape samples, aligning with the current study's and highlighting common pesticide usage patterns across major grape-producing regions. Pesticide residues in dried grapes are a widespread issue across in various regions, with similar pesticide usage trends observed in multiple studies. The lower residue levels detected in our study suggest that regional agricultural practices and climatic conditions play a significant role in the dissipation of pesticide residues.

Conclusion

In this study, it was determined that raisin samples contained pesticide residues below the MRLs. The dietary risk assessment confirms that pesticide residues in dried grapes do not pose significant health risks, as all chronic HQc and acute HQa exposure values are below 1. Additionally, the IEDI values are significantly lower than the ADI and the IESTI values remain well below the ARfD. These findings have important implications for both domestic and international trade, as compliance

with MRLs can improve market access and strengthen consumer confidence in the safety of agricultural products. Routine monitoring and strict regulations are essential to ensuring food safety. Specific measures, such as increasing the frequency of random sample testing, implementing digital traceability systems for pesticide usage, and conducting farmer education programs on integrated pest management, could significantly improve the effectiveness of these efforts. The findings can also inform sustainable agricultural practices and improve residue monitoring in grape production. By encouraging policy changes that prioritize stricter residue regulations and adopting advanced technologies like precision agriculture tools and real-time residue detection systems, pesticide use can be minimized while maintaining compliance with safety standards.

Ethical Statement

Ethics committee approval is not required for the study.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Author Contributions

TB and ÖFÖ made significant contributions to the design and execution of the study. KK contributed to conduction of research. TB and ÖFÖ performed the experiments. TB calculated, analysed and interpreted data. TB and KK attented in designing and writing the manuscript. TB gave final confirmation for the submission of revised version. All authors read, approved final manuscript.

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Research Article

Characterization of *Euglena* sp. Amino Acids and Fatty Acid Methyl Ester (FAME) in Correlation to Ammonium Sulfate $(NH_4)_2SO_4$ Variation: Large-Scale Cultivation

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Abstract: Ammonium sulfate $(NH_4)_2SO_4$ as a source of N compounds in the growth medium of *Euglena* sp. has a major role in growth, vegetative cell formation, protein formation, lipid, and other organic compounds. *Euglena* sp. are microalgae that can grow in environments with acidic pH and high ammonia levels. In this study, variation of ammonium sulfate treatments at different concentrations includes low nitrogen level/F1 (0.5 g L^{-1}), control (1 g L^{-1}), and high nitrogen level/F2 (2 g L^{-1}). This study aimed to define and quantify the amount of FAME, amino acids, growth, and biomass of *Euglena* sp. cultured on a large scale over a period of 16 days. FAME components were tested using Gas Chromatography Flame-Ionization Detector (GC-FID) and amino acids were characterized using Ultra Performance Liquid Chromatography (UPLC). The highest lipid content in F1 (0.209±0.0133 mg mL^{-1}), while the highest protein content (23.1792±2.2607µg mL^{-1}), and the highest biomass content (0.1444±0.0406 mg mL^{-1}) found in F2. The SFA components in *Euglena* sp. elevated with low nitrogen treatment, with methyl arachidate (C20:0), methyl heneicosanoate (C21:0), and methyl palmitoleate (C16:1) with values of 18.76%; 13.54%; and 10.18%, respectively. The amino acid characterization generated 18 compounds, with the highest amino acid concentration of L-Arginine in the control group (15233.09 mg kg^{-1}), and typical amino acids obtained were L-Alanine, L-Tyrosine, L-Histidine, L-Tryptophan, L-Aspartic acid, and L-Serine (VIP score>1).

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1. Introduction

Microalgae have been widely used in numerous areas due to their capacity to swiftly and easily create biomass that is mass-produced. Moreover, microalgae represent a viable supply of diverse chemicals, several of which may be subject to further development (Haris et al., 2022). The nutrient composition of microalgal species varies widely, each of the main components are primarily composed of protein (25–40%), fat (10–30%), and carbs (5–30%) (Ahmed et al., 2023). Various studies and isolation of new strains have been widely carried out as one of the methods of microalgae cultivation to produce various macromolecular materials and products that have high quality (Irhamni et al., 2014).

To increase metabolite production and growth rate, it is necessary to pay attention to the right procedures in the cultivation process, including environmental conditions and the media used (Widiyanto et al., 2014). Production of large quantities of microalgae can be done with various cultivation techniques. One of them is by using an open pond system with a non-aseptic environment (Di Caprio, 2020). This cultivation technique is considered more efficient for producing high amounts of biomass than laboratory-scale cultivation but requires a larger volume of culture media. In addition, the influence of environmental parameters such as pH, temperature, light intensity, weather, and biotic elements originating from pollutants such as bacteria, fungi, etc. cannot be separated from large-scale cultivation (Molina et al., 2019).

Euglena sp. is one type of microalgae that is currently being cultivated on a large scale. This species has several advantages over other types of microalgae and its ability to produce various high-value products. *Euglena* sp. is a unicellular organism with a fast growth rate and can synthesize various bioactive chemicals through very complex metabolic pathways (Kottuparambil et al., 2019). This species produces important metabolites such as fatty acids, carbohydrates, proteins, pigments, and 59 essential nutrients needed by the body (Vieira et al., 2021).

Lipids and fatty acids in microalgae have important roles as storage products, membrane components, metabolites, as well as energy sources. Algal oil is now widely used in various applications due to its similar characteristics to vegetable oil and fish oil. Its application as an alternative energy source as a substitute for fossil oil products is starting to be widely applied. The fatty acid composition of microalgae varies depending on the species. *Euglena* sp. has a fatty acid composition dominated by C16 (42%) and C18 (50%) methyl esters. This composition has similarities with the composition of vegetable oils (Kottuparambil et al., 2019). In addition, *Euglena* also has significant palmitic, linolenic, and linoleic acid components, which are 46%, 23%, and 22%, respectively. This fatty acid composition in microalgae is related to its function as a biodiesel fuel (Amelia et al., 2023). The overall protein content in microalgal biomass is contingent upon the specific microalgal species and may attain up to 70% of the dry weight (Andreeva et al., 2021). According to Villegas et al. (2015), the protein found in microalgae can be used as fuel, chemicals for industry, human food, and animal feed. Essential amino acids such glutamic acid, asparagine, arginine, leucine, and lysine are found in *Euglena* sp. (Ahmed et al., 2023).

Biomass production, especially lipid and protein content will be affected by the composition and concentration of nutrients in the growth medium. One method that can be applied to increase growth as well as the quantity and quality of metabolites in microalgae is through regulating the level of nitrogen (Yaakob et al., 2021). Nitrogen is one of the macronutrients that has a major effect on microalgae cell growth. Jung et al. (2021) stated that large amounts of biomass will be produced under ideal growth conditions. This theory has been tested previously through experiments by Tossavainen et al. (2019), where the availability of high N content in *Euglena gracilis* culture will produce high protein, chlorophyll, and carotenoid content. Meanwhile, limited nitrogen in the culture leads to the production of lipids in the microalgae. Under low nitrogen conditions in the culture, microalgae will accumulate lipids produced in the cells. This can occur because, under stressful conditions, cell division is inhibited, which does not affect the production of microalgal lipids (Ulya et al., 2018).

Microalgae can utilize nitrogen sources in the form of nitrate, ammonia or organic nitrogen such as urea. Nitrogen sources in the form of ammonia are the most preferred by microalgae and plants in general (Afifah et al., 2021). This is because the energy used during the metabolic process to reduce ammonium to organic matter is lower than the energy for the reduction of other forms of nitrogen (Mandal et al., 2018). The use of ammonium will avoid energy consumption due to nitrate/nitrite reduction and energy from the production of nitrate reductase (NR) and nitrite reductase (NiR) enzymes

(Lachman et al., 2018). Apart from N-ammonia, microalgae also consume nitrogen in the form of N-nitrite and N-nitrate. In algal cells, N-nitrate and N-nitrite will undergo shrinkage and $N\text{-}NO_2^-$ will turn into $N\text{-}NH_4^+$. Therefore, microalgae more easily accumulates nitrogen sources in the form of ammonia.

Different medium compositions will affect variations in nutrient amounts that can significantly alter the biochemical composition of microalgae. Therefore, it is important to perform media optimization to achieve higher growth yields and value-added products from microalgae. This research will examine the effect of nitrogen variation on lipid and protein content, and determine the composition of fatty acid and amino acid content of *Euglena* sp. in large-scale cultivation, as well as to obtain the most optimal microalgae growth conditions.

2. Material and Methods

2.1. Materials

The research was done from December 2023 to February 2024. *Euglena* sp. was cultivated at Biodiversity Research Station II, Karanggayam. The culture of *Euglena* sp. was obtained from the IDN33 culture collection at the Laboratory of Biotechnology, Faculty of Biology, Universitas Gadjah Mada. Cultivation was carried out by growing *Euglena* sp. in Cramer & Myers (CM) modification medium for large-scale 500 L volume with composition of MKP Pak Tani PT. Saprotan Utama (P_2O_5 52% and K_2O 34%), ZA Fertilizer PT. Petrokimia Gresik [$(NH_4)_2SO_4$], $MgSO_4$, KCl, $FeSO_4.7H_2O$, $MnCl_2.4H_2O$, $CoSO_4.7H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_20$, $Na_2MoO_4.2H_2O$, vitamin B1, and vitamin B12.

2.2. *Euglena* sp. cultivation

The preparation of inoculant stock was carried out on laboratory scale with initial culture concentration (OD 680_{nm}) was 0.3. The starter culture was left for three weeks to equalize the age of *Euglena* sp. Then, stock culture was scaled up from laboratory scale to large-scale. The cultivation of mass-scale composition consisted of 100 L inoculant microalgae with pH 3.5 and 400 L of Cramer & Myers (CM) medium modification with a total volume of 500 L. The microalgae were grown in varying nitrogen of 1 g L^{-1} (control), 0.5 g L^{-1} (F1), and 2 g L^{-1} (F2). Nitrogen source that was used in the culture medium was ammonium sulfate. There were five repetitions in each culture group (n=5).

2.3. Measurement of the growth

The growth rate of the sample was determined daily during the research by measuring optical density (OD) and the number of cells. The optical density was measured using a Thermo Scientific Genesys 150s UV/Vis spectrophotometer to determine the absorbance at wavelengths of 680 nm. The number of cells were determined using a neubauer hemocytometer under a OLYMPUS CX22 microscope. The sample volume utilized for cell density computation was 900 µL, with the addition of 100 µL of 70% alcohol to the microtubes. The amount of cells number was then analyzed using the following formulas (Equation 1).

$$cell/mL = \frac{Counted\ cell\ quantity}{4} \text{ x } 10^4 \quad (1)$$

2.4. Growth kinetic modeling

The Logistic and Gompertz Models were employed to characterize the growth kinetics of *Euglena* sp. The logistic model has various parameters, with X denoting cell densities, X_0 indicating beginning cell densities, X_{max} signifying maximum cell density, and μ_{max} representing the specific growth rate per day during the exponential and stationary stages of microalgal growth. This model did not consider the mortality of microalgae following the stationary phase (Phukoetphim et al., 2017; Maghfiroh et al., 2023). The logistic model could be calculated using the following formulas (Equations 2 and 3).

$$\frac{dx}{dt} = \mu max \left(1 - \frac{X}{\mu max}\right) x \quad (2)$$

$$X = \frac{Xo.exp(\mu max\ t)}{1 - \left[\left(\frac{Xo}{xmax}\right)(1 - exp\ (\mu max\ t))\right]} \quad (3)$$

The Gompertz model was relatively more complex when compared to the Logistic model. This model has three parameters that could describe several characteristics of microalgae. The maximum cell production rate (rm) and time lag (tL) are variables in the Gompertz model (Phukoetphim et al., 2017; Maghfiroh et al., 2023). The Gompertz model was determined using the following formula (Equation 4).

$$x = Xo + [\ Xmax.exp\ [-exp\ ((\left(\frac{rm.exp\ (1)}{Xmax}\right)\ (tl - t) + 1] \quad (4)$$

The Logistic model and Gompertz model were determined using the residual sum of squares between the cell density and the calculated results, where SSR was the residual sum of squares and SST was the total sum of squares (Equation 5).

$$R^2\ = (1 - \frac{SSR}{SST}) \quad (5)$$

2.5. Biomass measurement

A sample of *Euglena* sp. mass culture, taken up to 50 mL in a conical tube and filtered using 47 mm Whatman GF/C filter paper with 1.5 µm. Buchner funnel was used to measure biomass or dry weight. The filter paper with the biomass was dried for approximately an hour at 100 °C in an oven incubator. The biomass was weighed using an AND GR-200 analytical balance after it had dried (Erfianti et al., 2023).

$$Dry\ weight\ (mg/mL) = \frac{Filter\ paper\ final\ weight\ -\ filter\ paper\ initial\ weight}{Sample\ volume} \quad (6)$$

2.6. Measurement of Lipid Content

The total lipid content of *Euglena* sp. was determined using the Bligh and Dyer (1959) method. This method was one of the standard procedures performed for the extraction and isolation of total fat fraction from biological matrices based on polar and non-polar solvent systems. Fifty milliliters of *Euglena* sp. culture was centrifuged in a Hettich Zentrifugen Universal 320R centrifuge, and the resultant biomass was combined with solvents including chloroform, methanol, and distilled water with a constant ratio of 2:2:1 (v/v/v). The solution was centrifuged at 4000 rpm until three layers formed. The yellow bottom layer was taken using a micropipette and transferred to a petri dish that had previously been weighed. The petri dish was put into the oven at 50 °C for 24 hours. Calculation of lipid content using the following formula (Equation 7):

$$Lipid\ content\ (mg/mL) = \frac{Petri\ dish\ final\ weight\ -\ petri\ dish\ initial\ weight}{Sample\ volume} \quad (7)$$

2.7. Protein extraction and quantification

Protein extraction of *Euglena* sp. used buffer lysis which contains 50 mM tris buffer pH 8,3; SDS 10% solution, protease inhibitor using PMSF (Fenilmetilsulfonil fluorida), glycerol, MilliQ (Anjos et al., 2022). Analysis of protein content began with protein extraction from *Euglena* sp. cells. 50 mL of sample from each treatment was centrifuged with a Hettich Zentrifugen Rotofix 32A centrifuge for 10 minutes at 3500 rpm. The pellet that settled on the tube was weighed and transferred into a 1.5 mL microtube. A total of 10 µl of buffer was added per 1 mg sample weight and then homogenized for 1 minute. The sample was extracted in a sonicator at a cold temperature for 2×10 min with a 1 min pause in between, then incubated in a dry block thermostat (bioSan Bio TDB-100) for 10 min at 95 °C. Next,

the sample was centrifuged in Hettich Zentrifugen Universal 320R at 13000 rpm for 5 minutes at 4 °C, and the supernatant formed can be transferred to a new microtube (Knoshaug et al., 2020). Protein quantification was determined by Bradford assay and standard solution of Bovine Serum Albumin (BSA) analyzed by BioTek ELX800 Microplate reader at 595 nm. The 96-well microplate was filled with 200 µl Bradford and added 8 µl of both standard solution and isolated samples, calculation of protein content by substituting the absorbance value into a linear regression equation so that the formula y = ax + b is obtained with y is the absorbance value (Roche, 2024).

2.8. Fatty Acids Methyl Ester (FAME) analysis

Fatty Acids Methyl Ester (FAME) concentration was analyzed through the direct transesterification method and Gas Chromatography Flame-Ionization Detector (GC FID-Agilent Technologies 7890B). Transesterification reaction is a reaction that occurs between oil (triglyceride) and alcohol (Amelia et al., 2023). The commonly used alcohol in this reaction was methanol because it is one of the stable short-chain alcohols. Biomass from *Euglena* sp. microalgae was harvested by centrifugation technique at 4000 rpm for 5 minutes. Samples from each treatment were taken on the last day of cultivation (day 16^{th}). The samples were then centrifuged for 5 minutes at 4000 rpm to separate the pellet and supernatant. The pellet was then washed using 50 mL of distilled water after the supernatant was discarded. After that, a second centrifugation was carried out followed by three rinses using distilled water. The sample was hydrolyzed using 10 mL of concentrated HCl, then heated for 3 hours at 80 °C, and then cooled at room temperature (25 °C). Next, the sample was extracted using 25 mL of diethyl ether and petroleum ether (1:1 v/v). The top layer of the sample was discarded, then the sample was evaporated with N_2 gas in a water bath. The sample was methylated by adding 0.5 mL of oil and 1.5 mL of sodium methanol solution, then heated at 60 °C for 10-15 minutes with stirring and cooled at room temperature (25 °C) in a small-closed test tube. After that, 2 mL of boron trifluoride methanol was added and incubated at room temperature (25 °C) after heating for 5-10 minutes at 60 °C. 1 mL of heptane and 1 mL of saturated NaCl were used for sample extraction. The top layer was put into a GC vial and 1 µL of sample was injected into the GC.

2.9. Amino acids characterization

The hydrochloric acid (HCl) hydrolysis method was used to characterize the amino acids from frozen biomass using 0.1–1 g of sample with standard solution (SIGMA-ALDRICH, AAS18–10X1ML, Lot# SLCL 2556). The hydrolysis products were mixed with distilled water in a 50 mL volumetric flask, filtered using a syringe filter, and then introduced into an Ultra Performance Liquid Chromatography (UPLC) apparatus. The C18 column, mobile phase (eluent accQ, ultra tag, aquabidest), column temperature (49 °C), and detector (PDA Ch1 260 nm) were the chromatographic conditions for this test (Liw et al., 2019).

2.10. Statistical analysis

The results were analyzed using Microsoft Excel and IBM Statistical Product and Service Solution software (Version 26, IBM Corporation, USA). A one-way ANOVA test with a data significance threshold determined by a *p*-value <0.05 at the 5% level and post-hoc analysis using the Duncan Multiple Range Test (DMRT) is used to examine the mean differences in each variable. The kinetics of growth models using the Anaconda Navigator application with the Logistics model and the Gompertz model. Data processing graph visualization was displayed using the Origin-Pro application. The results of fatty acids methyl ester and amino acid profiles are analyzed using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/).

3. Results

3.1. Cell growth characteristic of *Euglena* sp.

The augmentation of cell size and cell quantity is a determinant of the growth rate of microalgae in culture (Martinez-Ruiz et al., 2022). Growth properties of *Euglena* sp. can be determined through specific growth rate and doubling time. The specific growth rate (SGR) quantifies the rate of microalgal

cell proliferation over time, influencing nutrient production and the cell division mechanism. Doubling time refers to the duration necessary for microalgae cells to increase their population twofold (Nurafifah et al., 2023). The number of cells was measured using a hemocytometer.

Table 1. Specific growth rate (SGR) and doubling time (Td) of *Euglena* sp.

Treatment	SGR	*Doubling Time* (T_d)
Control (1 g L^{-1})	0.108 ± 0.008^{a}	6.416 ± 0.186^{c}
F1 (0.5 g L^{-1})	0.125 ± 0.007^{b}	5.541 ± 0.265^{a}
F2 (2 g L^{-1})	0.115 ± 0.004^{ab}	6.026 ± 0.089^{b}

Notes: Mean values followed by different letters indicate significant differences at the α = 5% test statistical level.

Based on table 1, it is known that the correlation between specific growth rate and division time is inversely proportional, where when the specific growth rate is high, the cell division time will be low and vice versa. In the table, F1 treatment has the highest specific growth rate value compared to the other two treatments which is 0.125 ± 0.007 with the lowest division time of 5.541 ± 0.265. While the lowest specific growth rate was obtained in the control culture with the highest division time. Through statistical analysis, it is known that the specific growth rate in each treatment shows a significant difference ($p<0.05$). While at doubling time, there was no significant difference in F1 and F2 treatments ($p>0.05$) but the control treatment was significantly different from the F1 treatment.

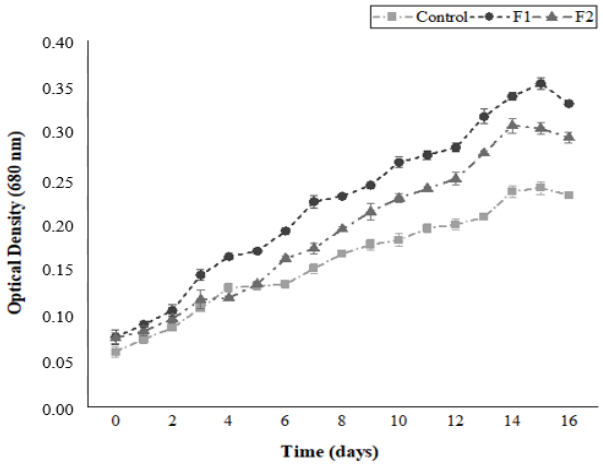

Figure 1. Cell density of *Euglena* sp. on various ammonium sulfate concentration treatments.

According to Figure 1, it can be seen that the cell density in *Euglena* sp. both in the control, F1, and F2 treatment cultures continued to increase until the end of the cultivation period. The highest cell density was obtained in the F1 treatment, while the culture with the lowest cell density was shown in the control. The highest cell density was found on the 14th day of cultivation in each treatment. The cell density pattern obtained is positively correlated with the cell growth rate, where the absorbance of cell density will increase if the number of *Euglena* sp. cells also increase. From day 11 to day 14, the growth of the control group reached a stationary phase, which was absent in treatments F1 and F2, as illustrated in Figure 1. The stationary phase commences when the high density of *Euglena* cells experiences diminished growth due to intercellular competition for limited food resources essential for metabolism. The wavelength of 680 nm was used to determine the cell density of *Euglena* sp., because this wavelength is commonly applied for measuring cell density in microalgae (Toyama et al., 2019).

3.2. Growth kinetic modeling

Based on Figure 2, it was found that through the Gompertz model, the high concentration ammonium sulfate treatment showed a maximum cell growth rate (rm) of 6.33 cells mL^{-1}. The lag time values (tL) sequentially for control, F1, and F2 were 0.333/day; -0.541day; 0.365/day. Using the Logistic model, the maximum specific growth rate (μ_{max}) in each treatment was 0.366/day (control);

0.3365/day (F1); and 0.338/day (F2). The highest R^2 error values for control, F1, and F2 were 0.955; 0.988; and 0.995.

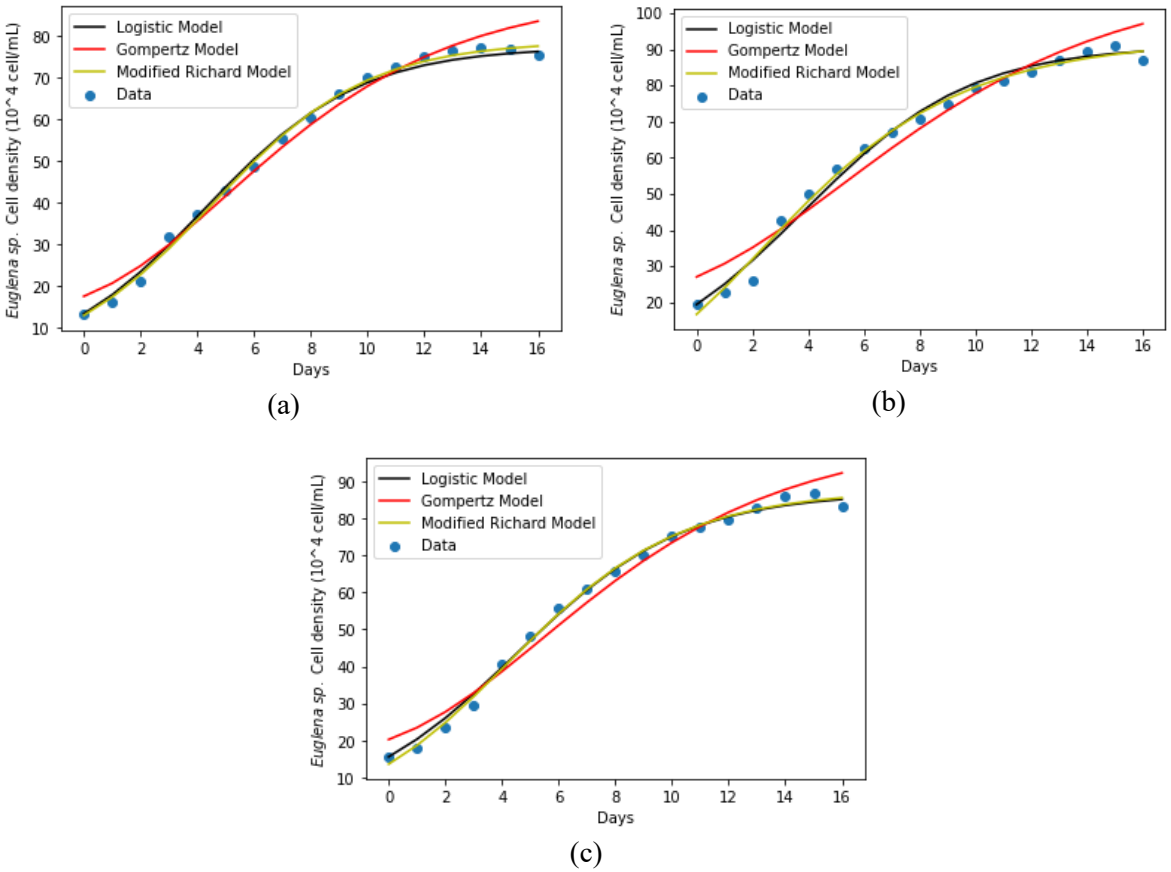

Figure 2. Fitting of Gompertz and Logistic Model (cell density) on the growth of *Euglena* sp. (a) control; (b) low ammonium sulfate concentration, (c) high ammonium sulfate.

3.3. The effect of variation nitrogen on biomass

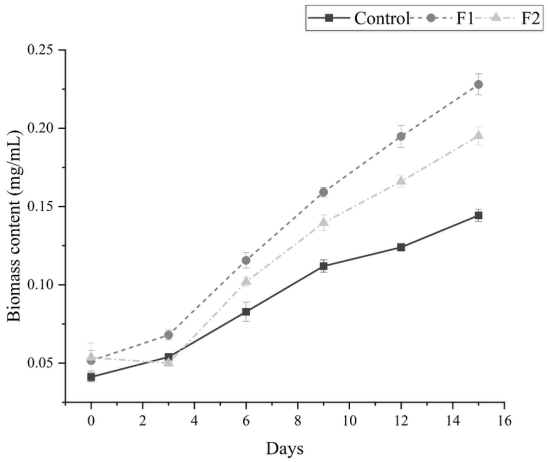

Figure 3. The content of *Euglena* sp. biomass under $(NH_4)_2SO_4$ variation treatment.

Based on the figure above, the daily biomass production rate of *Euglena* sp. aligns with the growth rate curve depicted in figure 5, based on optical density measurements. On the fifteenth day, the F1 group exhibited the highest average biomass value of 0.228±0.007 mg mL^{-1}, while the F2 group

recorded a biomass of 0.195±0.006 mg mL^{-1}, and the control group yielded a biomass of 0.144±0.004 mg mL^{-1}. In this experiment, the biomass production growth observed in each treatment group did not correspond with established theory and prior research, such as that by Erfianti et al. (2023), which indicated that medium variations with a nitrogen concentration of 1.5 g L^{-1} yielded greater biomass of *Euglena* sp. than media with nitrogen concentrations of 0.75 g L^{-1} and 0.375 g L^{-1}. The nitrogen source in this study, specifically ammonium sulfate, can degrade into ammonia.

3.4. Lipid content and fatty acids methyl ester of *Euglena* sp.

Figure 4 shows the lipid content produced by *Euglena* sp. during 16 days of cultivation and the productivity of lipids produced per day. The highest lipid content of *Euglena* sp. was produced by treatment F1 (low nitrogen content) at 0.209 mg mL^{-1}, followed by treatment F2 at 0.151 mg mL^{-1} and the lowest lipid content in the control treatment at 0.138 mg mL^{-1}. The same trend was shown in the productivity results, where the highest productivity was produced in the F1 treatment, followed by the F2 treatment and the lowest in the control, with values of 0.0165 mg mL^{-1}/day; 0.0123 mg mL^{-1}/day; and 0.0104 mg mL^{-1}/day, respectively. Through these results, it can be known that F1 treatment is more effective in increasing lipid content and productivity in *Euglena* sp. The above results also show that *Euglena* sp. lipid productivity is directly proportional to biomass productivity (Figure 4). When biomass productivity is high, lipid productivity will follow (Sobari et al., 2013). Based on the statistical test, it is known that each treatment has a significant difference ($p<0.05$).

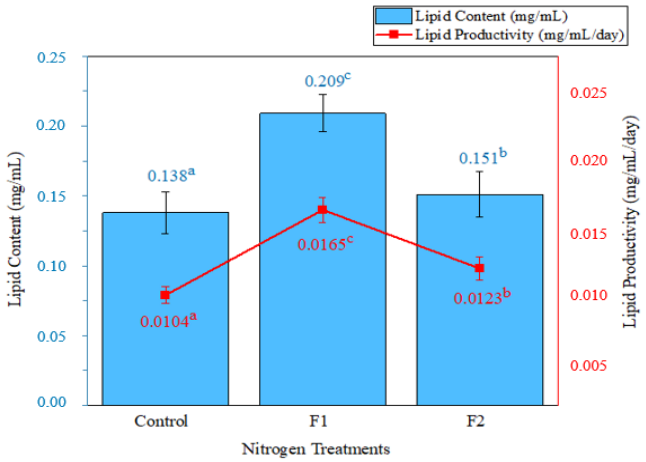

Figure 4. The content and productivity of *Euglena* sp. lipid under $(NH_4)_2SO_4$ variation treatment during mass-cultivation of. Data means ± SD (n=5), one-way ANOVA was used to compute significant differences between treatments, and the Duncan Multiple Range Test (DMRT) was used to confirm the results ($p<0.05$).

Table 2, shows the fatty acid methyl ester (FAME) components found in *Euglena* sp. with different nitrogen variation treatments. Based on the results obtained, it can be seen that the most compounds found in the three treatments are methyl heptadecanoate (C17:0), methyl arachidate (C20:0), methyl heneicosanoate (C21:0), and methyl palmitoleate (C16:1). In general, *Euglena* sp. in this study produced more saturated fatty acids and less unsaturated fatty acids. The F1 treatment with low N content produced the highest concentration of saturated fatty acids (43.24%), followed by the F2 treatment (42.44%) and the control (41.38%). The highest monounsaturated fatty acid was produced in treatment F1 (28.36%), followed by treatment F2 (26.44%), and the lowest in the control (25.18%). Meanwhile, polyunsaturated fatty acids (PUFA) were produced more in the control treatment (24.65%) compared to F2 (23.72%) and F1 (20.81%). Based on the above results, ammonium sulfate with low concentration can produce more saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in *Euglena* sp.

Table 2. Fatty acids methyl ester (FAME) on *Euglena* sp. grown in various nitrogen treatment

Parameters	C.Number	Control	Treatment 1/F1	Treatment 2/F2
Methyl laurate	C12:0	0.40	0.36	0.40
Methyl tridecanoate	C13:0	1.11	1.03	1.47
Methyl myristate	C14:0	6.33	5.77	6.15
Methyl palmitate	C16:0	1.77	1.90	1.84
Methyl heptadecanoate	C17:0	7.51	7.82	7.42
Methyl arachidate	C20:0	7.37	10.18	9.04
Methyl heneicosanoate	C21:0	13.51	13.04	13.04
Methyl lignocerate	C24:0	3.38	3.14	3.08
Methyl cis-10-pentadecenoate	C15:1	1.56	1.93	1.82
Methyl palmitoleate	C16:1	16.98	18.76	17.83
Methyl cis-10-heptadecanoate	C17:1	5.05	6.98	6.19
Methyl linolelaidate	C18:2	5.40	3.23	6.70
Methyl cis-8,11,14-eicosatrienoate	C20:3	1.37	1.36	1.33
Methyl erucate	C22:1	0.59	0.69	0.60
Methyl cis-5,8,11,14-eicosatetraenoate	C20:4	4.75	4.05	3.42
Methyl cis-5,8,11,14,17-eicosapentaenoate	C20:5	7.02	6.18	6.38
Methyl cis-4,7,10,13,16,19-docosahexaenoate	C22:6	6.11	5.99	5.89
SFA		41.38%	43.24%	42.44%
MUFA		25.18%	28.36%	26.44%
PUFA		24.65%	20.81%	23.72%
%Relative Area			100.00	

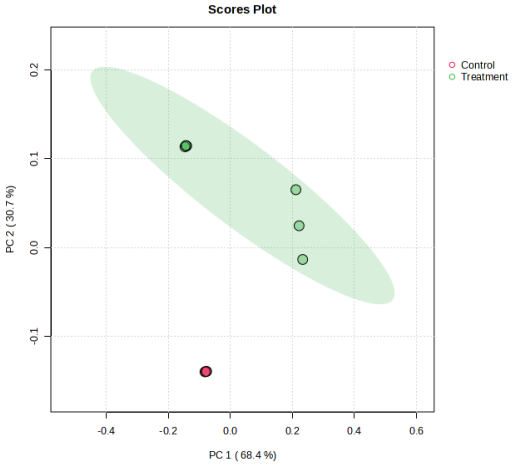

Figure 5. Principal component analysis (PCA) of the fatty acid methyl ester (FAME) *Euglena* sp. under nitrogen variation treatment.

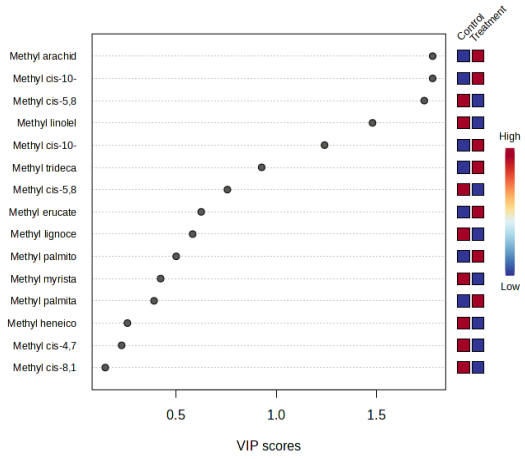

Figure 6. Importance features of the fatty acid methyl ester (FAME) *Euglena* sp. under nitrogen variation treatment.

Figure 5 shows the results of PCA analysis on fatty acids of *Euglena* sp. obtained from UPLC analysis. PC 1 explains 68.4% of the total variance while PC 2 accounts for 30.7% of the total variance. The PCA model in these results can be said to be good because the cumulative Q2 value obtained is the total of the sum of PC1 and PC2 >50%. The F1 and F2 treatments are in the same cluster (cluster 2), while the control is in a different cluster (cluster 1). Based on Figure 6, methyl arachid, methyl cis-10-heptadecenoate, and methyl cis-5,8,11,14,17-eicosapentaenoate are known to have VIP values greater than (VIP>1.5). Based on the results of significance features, methyl arachid is the most common and significant compound of *Euglena* sp. in nitrogen variation treatment.

3.5. Protein content and amino acids of *Euglena* sp.

Figure 7 shows the fluctuation in *Euglena* sp.'s protein content and productivity values treated with ammonium sulfate. The examination revealed that the F2 treatment group had a significantly higher protein content of *Euglena* sp. (23.1792±2.2607 µg mL^{-1}) than the control group. Furthermore, treatment group F2 produced the highest protein productivity of all treatments in this investigation, with a value of (0.4183±0.0975 µg mL^{-1}/day), as shown in Figure 7, which also displays the value of protein productivity daily.

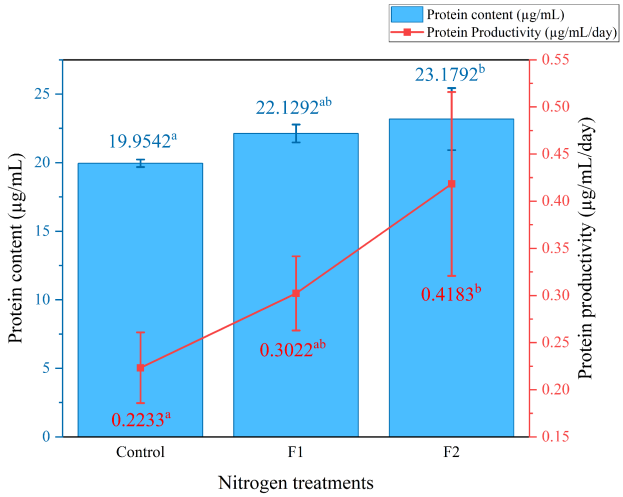

Figure 7. The content and productivity of *Euglena* sp. protein under $(NH_4)_2SO_4$ variation treatment during large-scale cultivation. Data means ± SD (n=5), one-way ANOVA was used to compute significant differences between treatments, and the Duncan Multiple Range Test (DMRT) was used to confirm the results ($p<0.05$).

Table 3. Amino acid on *Euglena* sp. grown in various nitrogen treatment

Name	Abbreviations	Amino acid content (mg kg^{-1})			Molecular formula
		Control	F1	F2	
L-Alanine	Ala	9474.67	7114.54	5908.90	$C_3H_7NO_2$
L-Arginine	Arg	15233.09	9919.30	13581.21	$C_6H_{14}N_4O_2$
L-Aspartic Acid	Asp	9837.21	7517.98	5939.41	$C_4H_7NO_4$
Glycine	Gly	6019.12	4384.87	4370.28	$C_2H_5NO_2$
L-Glutamic Acid	Glu	10655.58	8315.72	5394.30	$C_5H_9NO_4$
L-Histidine	His	1303.25	1144.76	1023.57	$C_6H_9N_3O_2$
L-Isoleucine	Ile	4263.35	2959.31	1405.57	$C_6H_{13}NO_2$
L-Cystine	Cys	7398.56	5362.21	6711.27	$C_6H_{12}N_2O_4S_2$
L-Leucine	Leu	8866.46	6623.70	4473.19	$C_6H_{13}NO_2$
L-Lysine	Lys	6369.82	5044.25	2886.28	$C_6H_{14}N_2O_2$
L-Methionine	Met	1446.25	1196.90	1412.52	$C_5H_{11}NO_2S$
L-Tryptophan	Trp	1494.03	563.08	1188.96	$C_{11}H_{12}N_2O_2$
L-Valine	Val	7044.66	5093.27	2736.61	$C_5H_{11}NO_2$
L-Phenylalanine	Phe	4043.01	3071.35	2395.63	$C_9H_{11}NO_2$
L-Proline	Pro	7979.34	5484.11	3545.70	$C_5H_9NO_2$
L-Serine	Ser	3379.44	2532.86	2163.26	$C_3H_7NO_3$
L-Threonine	The	3778.28	2885.57	2046.48	$C_4H_9NO_3$
L-Tyrosine	Tyr	2827.23	2874.68	2526.04	$C_9H_{11}NO_3$

The results of the amino acid characterization of *Euglena* sp. impacted by ammonium sulfate $(NH_4)_2SO_4$ are displayed in Table 3. It is evident that *Euglena* sp. under the control, F1, and F2 treatments, contains eighteen different amino acid molecules. The largest known amino acid is L-arginine ($C_6H_{14}N_4O_2$), which is found in the control (15233.09 mg kg^{-1}), F1 as much as (9919.30 mg kg^{-}

^{1}), and F2 (13581.21 mg kg^{-1}). In the F1 group, L-tryptophan ($C_{11}H_{12}N_2O_2$) was the amino acid with the lowest levels, whereas L-histidine ($C_6H_9N_3O_2$) was discovered at the lowest quantities in the control and F2 treatments.

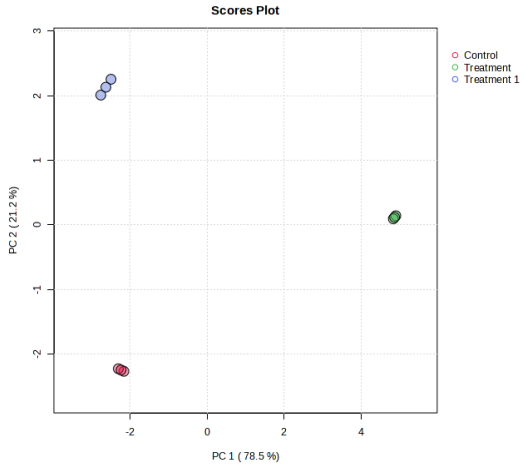

Figure 7. Principal component analysis (PCA) of *Euglena* sp. amino acid under nitrogen variation treatment.

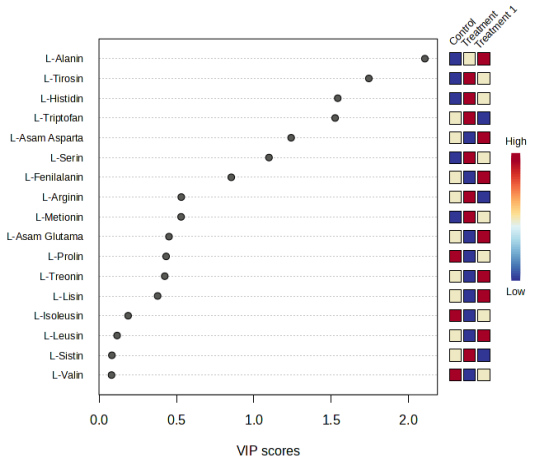

Figure 8. Importance features identified by PLS-DA of the *Euglena* sp. amino acid under nitrogen variation treatment.

The amino acid PCA analysis data for *Euglena* sp., obtained from UPLC analysis, is presented in Figure 7. Three distinct clusters, each comprising 18 compounds, have emerged without intersection, signifying that the chemicals in PC1 and PC2 are very well grouped, with their combined contribution exceeding 50%. L-Alanine, L-Tyrosine, L-Histidine, L-Tryptophan, L-Aspartic acid, and L-Serine are the six molecules depicted in Figure 8 that possess VIP scores over 1, VIP scores greater than 1 signify that certain amino acid molecules are distinctive or significant in *Euglena* sp. (Stoessel et al., 2018). The major feature results indicate that L-Alanine is the predominant and most significant chemical from *Euglena* sp. in addressing nitrogen variation.

4. Discussion

The success rate of a cultivation method can be characterized in the early phase of microalgae growth. This can be seen from the short lag phase or from the exponential phase where the ratio of the increase in the number of microalgae populations per unit time is very high due to rapid cell division (Hudaidah et al., 2013). In this study, the lag phase of *Euglena* sp. occurred very briefly, which is less than 24 hours from observation day 0 to day 1. This can be seen from the lag time value in each treatment based on the Gompertz growth model kinetics, where each treatment has a lag phase of 0.333/day (control), -0.541/day (F1), and 0.365/day (F2). On day 2, *Euglena* sp. cells in each treatment began to enter the exponential phase characterized by increased cell growth. The peak exponential phase occurred on day 14 in the control and day 15 in the F1 and F2 treatments. After that, cell growth began to stagnate and experienced a decrease in growth rate after the peak phase. Nutrients become a factor that limits cell growth in the growth decline phase, which results in decreased cell division (Muchammad et al., 2013). The results obtained show that the increase in growth patterns in each culture is directly proportional to the increase in cell density, whereas as cell density increases, the number of cells also increases.

Specific growth rate and doubling time are one of the growth characteristics of *Euglena* sp. Specific growth rate can be defined as the speed of microalgae cells to grow per unit of time and to determine the capacity of nutrients produced as well as the cell division process. While doubling time is the length of time for microalgae to double their number (Nurafifah et al., 2023). Based on table 1, the low ammonium sulfate concentration treatment (F1) produced the highest specific growth rate of 0.125 ± 0.007 with the shortest division time of 5.541 ± 0.265. The specific growth rate has an inversely proportional relationship to the division time, where when the division time is low, the specific growth rate will be high and vice versa. Microalgae cells will double when entering the logarithmic phase where

microalgae cells will actively divide at a rapid and continuous rate (Amelia et al., 2023). Microalgae species with short cell division times and high specific growth rates will be more efficient because the length of cultivation until harvest can be achieved with a shorter duration (Maghfiroh et al., 2023).

Based on the growth kinetics model in Figure 2, the Logistic model when applied to *Euglena* sp. with nitrogen variation treatment is closer to the microalgae growth curve than the Gompertz model. This suitability is determined based on the coefficient of determination R^2 in each model, where the Logistic model shows the highest R^2 value. Based on previous research conducted by Maghfiroh et al. (2023), the Logistic model and the Gompertz model are two non-linear models that are suitable for the growth of organism populations, especially microalgae. In addition, these two models are considered the simplest models and can be used to describe the growth rate of microalgae in general.

The treatment of low ammonium sulfate levels was able to produce the highest cell growth of *Euglena* sp. compared to the treatment of additional ammonium sulfate concentration. Research conducted by Khanra et al. (2020) states that many microalgae species prefer nitrogen in the form of ammonium and high ammonium concentrations in the growth medium can increase the ability of microalgae to absorb NH_4^+ ions. However, ammonium concentrations that are too high can inhibit the conversion of ammonium into amino acids rather than its entry into cells, which can reduce growth under conditions of high nitrogen concentration. This is also supported by the research of Bakku et al. (2023) that excessive NH_4^+ flux can inhibit ATP formation and photosynthesis regulation, causing ammonium poisoning.

Previous research by Rios et al. (2014) showed that the growth curve in *Desmodesmus* sp. with different nitrogen concentrations in each treatment produced similar growth behavior and there was an increase in the number of cells at 0% nitrogen until the 4th day of cultivation. Another study revealed that the growth rate of *C. vulgaris* was higher in nitrogen-limited media (Agirman and Cetin, 2017). In addition, N additional conditions in the mass cultivation of *E. gracilis* resulted in lower cell density. The N addition and N reduction treatments became similar as the cultivation period progressed and the biomass formed in the low nitrogen treatment became higher. This suggests that *E. gracilis* is able to adjust to the cyclic process of N acquisition under N^- conditions resulting in higher growth rates compared to N^+ conditions (Bakku et al., 2023).

The growth of *Euglena* sp. as shown in Figure 1 has a close connection with dry biomass formation. The biomass of *Euglena* sp. in the F1 treatment group was the highest when compared to other treatments. This treatment contains 0.5 g L^{-1} of ammonium sulfate, compared to 1 g L^{-1} in the control group. The high biomass of *Euglena* sp. in medium with low nitrogen levels is linked to its growth rate. According to Nigam et al. (2011), growth rate is directly related to microalgae biomass because healthy microalgae are more active in converting CO_2 into biomass, resulting in high biomass productivity. At the beginning of the culture phase, the amount of biomass is still low because microalgae are still in the adaptation stage with their environment. After that, the biomass will increase exponentially over time. An exponential increase in biomass implies that microalgae are in a growing phase. During the logarithmic phase, the number of cell divisions increases, resulting in higher cell counts and biomass content (Krishnan et al., 2015).

Previous research states that biomass production is influenced by the concentration of nitrogen given to the culture medium (Menegol et al., 2017). Figure 3 shows that the F1 treatment produced the highest amount of biomass compared to the F2 treatment and the control, so that the F1 treatment was effective in increasing the biomass of *Euglena* sp. According to Hudaidah et al. (2013), intensive environmental pressure, especially in media containing low nitrogen elements, can affect microalgae biomass. The results obtained in this study are supported by the research of Wang et al. (2018), that the algae species *Chlorella* sp. proved to accumulate starch granules and oil after three days in N-deficient conditions, and resulted in an increase in total biomass and energy yield. Biomass results in this study were positively correlated with density (cells/ml), meaning that reducing the concentration of ammonium sulfate has a positive effect on cell density which affects biomass production.

In this study, the lipids produced were influenced by the content of biomass produced, which is in line with research by Griffiths et al. (2011). Lipid productivity is also directly proportional to biomass productivity, where when there is an increase in biomass content, lipid content will also increase. This is evidenced by the results obtained in this study that treatment F1 with low ammonium sulfate concentration produced the highest total biomass so that the resulting lipid content in treatment F1 was also the highest compared to the other two treatments. Meanwhile, lipid productivity or the mass of

lipids produced per unit volume of microalgae per day is also influenced by the growth rate of microalgae (Chisti, 2007).

Lipid content can increase even under nitrogen deprivation conditions because nitrogen depletion causes inhibition of cell division without a gradual decrease in lipid production which results in the accumulation of lipids in cells. This suggests that microalgae are able to adapt metabolic pathways to store large amounts of lipids under nitrogen deprivation conditions (Li et al., 2015). *Euglena* sp. will store the end result of photosynthesis from the Calvin cycle in the form of carbohydrates (paramylon) in the cell as the main additional energy source of the cell which also functions in cell defense against environmental osmotic pressure, can also be stored in the form of lipids when under stress conditions (Pareek et al., 2017).

Microalgae will modify lipid biosynthetic pathways to produce and accumulate neutral lipids, especially in the form of triacylglycerol (TAG) when under stress conditions. The increased content of neutral lipids, especially TAG, is caused by a shift in lipid metabolism from membrane lipid synthesis to neutral lipids or storage lipids (Hu et al., 2008), in other words, the carbon skeleton needed for protein and amino acid synthesis is diverted into carbon and energy sources for TAG biosynthesis (He et al., 2017). In addition, the increased lipid production under stress conditions is also due to the role of lipids as cell protectors because they are compounds that make up the cell membrane (Wardana et al., 2023).

The composition of fatty acids in microalgae is highly dependent on environmental conditions, including the availability of nutrients in the growth medium. According to Chiu et al. (2009), the factors that affect the fatty acid composition in microalgae consist of temperature, CO_2 gas, the addition of stressors such as heavy metals and the composition of the growth medium. Under nitrogen-limited conditions, the proportion of total lipids consisting of TAG increases. Stephenson et al. (2010) mentioned that *C. vulgaris* cultured after 12 days with nitrogen limitation accumulated more than 50% of total lipids. The fatty acid composition of lipids was also reported to change with nitrogen restriction. Previous research by Griffiths et al. (2014) explained that the metabolism of *C. vulgaris* shifted from the production of polyunsaturated fatty acids (C18:2 and C18:3) to the production of saturated or monounsaturated fatty acids (C18:0 and C18:1) under nitrogen deprivation conditions.

Table 2 shows that the composition of fatty acids in *Euglena* sp. contains saturated fatty acids (SFA) which are higher than monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). This is in accordance with the research of Zarrinmehr et al. (2020) that the amount of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) increases when under low nitrogen conditions, while the range of polyunsaturated fatty acids (PUFA) increases under high nitrogen concentration conditions. The fatty acid components in *Euglena* sp. were dominated by arachidic acid (C20:0), heneicosanoic acid (C21:0), and palmitoleic acid (C16:1). The dominating fatty acid components are classified into saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). Based on the research of Kottuparambil et al. (2019), lipids in *Euglena* sp. have a composition dominated by C16 methyl esters (42%). In addition, *Chlorella* sp. showed high amounts of saturated fatty acids and monounsaturated fatty acids, consisting of 36.45% stearic acid; 16.78% arachidic acid, 7.02% heneicosanoic acid, and 19.91% linoleic acid (Praveenkumar et al., 2012). Zienkiewicz et al. (2020) using different microalgae also showed that fatty acids belonging to the C16:1 group produced >40% of fatty acids under a concentration of 0 g L^{-1} nitrogen. The differences in fatty acid components in microalgae indicate that differences in treatment of the composition and concentration of nutrients in the culture greatly affect the composition of fatty acids.

Based on Figure 6, methyl arachid, methyl cis-10-heptadecenoate, and methyl cis-5,8,11,14,17-eicosapentaenoate are known to have VIP values greater than (VIP>1.5). Based on the results of significance features, methyl arachid is the most common and significant compound of *Euglena* sp. in nitrogen variation treatment. These saturated fatty acids have very important biochemical roles, including being direct precursors of bioactive lipid mediators such as prostaglandins and leukotrienes. In addition, arachidonic acid also keeps fatty acid liquids pure, even at sub-zero temperatures, and helps provide proper fluidity cell membranes at physiological temperatures (Martin et al., 2016).

The fatty acid composition of *Euglena* sp. will be very influential in the potential of *Euglena* sp. as a source of lipids that can be utilized for biofuel production. According to Demirbas (2010), high-quality biodiesel generally contains saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). Saturated fatty acids (SFAs) with chain lengths from C10 to C18 have properties such as low viscosity, higher cetane number, and lower pollutant emissions that make them superior for biodiesel

production. Monounsaturated fatty acids (MUFAs) in the range of e.g. C18:1, also exhibit characteristics suitable for biodiesel production (Suyono et al., 2015).

PUFAs exhibit different characteristics than SFAs and MUFAs, namely high viscosity and low cetane number, which can reduce the quality of biodiesel. These characteristics cause saturated fatty acids and monounsaturated fatty acids to be preferred for use in biodiesel (Knothe, 2013). Through the results obtained, it can also be recognized that cultivation of *Euglena* sp. under nitrogen deficit resulted in lower PUFA and higher SFA. Previous research by Ho et al. (2012) stated that under nitrogen deficiency conditions, the content of C16 or C18 fatty acid groups will increase and dominate the total fatty acids which are indicators of biodiesel quality. Based on Table 2, it is also known that *Euglena* sp. has less EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) content where the highest content of the two fatty acids is 4.75% and 6.11%. Through fatty acid characterization tests in this study, it was found that low ammonium sulfate concentrations produced highly saturated fatty acids suitable for biodiesel production.

In addition to lipid and FAME levels, nitrogen variation treatment can also impact protein content. In Figure 7, the F2 treatment group with ammonium sulfate levels of 2 g L^{-1} has the highest protein content and productivity, which is significant to the control group ($p<0.05$). However, the protein content in F2 is not significant to the F1 group (0.5 g L^{-1}) ($p>0.05$). According to recent research by Tossavainen et al. (2019), a medium with high nitrogen levels can boost the content and productivity of *Euglena* sp. protein, making it an ideal alternative for the production of protein-rich biomass. A common reaction to reduced cellular N content and increased C:N ratio in biomass is a decrease in both protein and non-protein N molecules (Xie et al., 2023).

The study's analysis of the amino acids that comprise the protein in *Euglena* sp. revealed that each treatment had 18 amino acid compounds, with L-Arginine having the greatest amino acid content. Arginine is a semi-essential amino acid obtained from food intake for protein biosynthesis; however, arginine can also be synthesized by the body via the urea cycle mechanism using L-citrulline as a substrate; the synthesis of arginine compounds can aid in wound healing, cell division, and ammonia removal. (Al-Koussa et al., 2020). The PLS-DA analysis of *Euglena* sp. amino acids revealed that eight compounds had a VIP score value more than 1, indicating that these amino acids are the most influential or typical amino acids of *Euglena* sp. (IDN33). The amino acids are L-alanine, L-histidine, L-tyrosine, L-tryptophan, L-aspartic acid, and L-serine. Alanine was the most influential in the F1 treatment group, whereas histidine, tyrosine, and tryptophan were the most influential in the F2 treatment group.

L-alanine, L-histidine, L-tyrosine, and L-tryptophan are amino acids that are needed for a variety of biological functions, including food metabolism and nutrition. L-alanine is a non-essential amino acid that acts as a precursor to the synthesis of other amino acids, including glutamate and aspartate. L-histidine is an important amino acid that aids in the production of histamine. L-tryptophan is an important amino acid that acts as a precursor for the synthesis of serotonin, which is involved in the production of melatonin, a hormone that regulates sleep-wake cycles. L-tyrosine is an important amino acid that acts as a precursor to the synthesis of neurotransmitters such as dopamine, norepinephrine, and epinephrine (Pokorný et al., 2021). L-Alanine, identified as a notable amino acid in this study, is a non-essential amino acid that can be produced from pyruvate, a transient result of glycolysis (Li et al., 2024). Alanine synthesis is facilitated by the enzyme Alanine Aminotransferase (ALT), which transfers the amino group from glutamate to pyruvate, resulting in the formation of alanine (Ingrisano et al., 2023). Alanine production transpires during the vigorous development phase when microalgae must equilibrate energy and nitrogen metabolism.

Conclusion

The variation of ammonium sulfate [$(NH_4)_2SO_4$] to *Euglena* sp. in large scale cultivation (500 L) with variation of 0.5 g L^{-1}, 1 g L^{-1}, and 2 g L^{-1} cultivated over 16 days. The modifications tested had a significant impact on growth, biomass, lipid content, and protein content. *Euglena* sp. grew the best in the F1 treatment group, which was also the group with the most significant biomass. The F1 treatment group exhibited a notable reduction in lipid levels due to fluctuations in ammonium sulfate concentration, whereas the F2 treatment demonstrated a considerable augmentation in protein content. The Fatty Acid Methyl Ester (FAME) content detected was 17 fatty acids, of which methyl heptadecanoate (C17:0) and methyl palmitoleate (C16:1) were the highest concentrations of fatty acids

characterized and belonged to saturated fatty acid (SFA) and monounsaturated fatty acids (MUFA). Nitrogen levels substantially influence the protein content of *Euglena* sp. during large cultivation, with treatment group F2 yielding 11.87% protein, which is 1.16 times greater than that of the control group. The study identified 18 amino acids from *Euglena* sp., with L-Arginine having the highest concentration in the control group and identifying distinctive amino acids like L-Alanine, L-Tyrosine, L-Histidine, L-Tryptophan, L-Aspartic acid, and L-Serine.

Ethical Statement

This study does not require ethical approval as it does not include human or animal subjects.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Author Contribution

E.A.S conceptualization , A.N.D. and I.W. collected the data, performed data processing and analysis, and composed the preliminary draft of the manuscript., S.N.Z. and T.A.P. preparation and collected data, D.K., T.E., R.A., and K.Q.M. assisted in formulating research strategies and overseeing the execution of research, R.A.E.P assisted in developing and amending the text in accordance with reviewer suggestions, and contributed to the data analysis. All authors examined, revised, and validated the final paper.

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Research Article

Synergistic Effect of Silica and NPK Fertilizer on Nutrient Status, Chlorophyll Content, and Rice Yield (*Oryza sativa* L.)

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Abstract: Rice productivity must be increased to meet the high food needs. One of them is by fulfilling nutrients through fertilization. This study aims to evaluate the use of Silica fertilizer on the effectiveness of NPK fertilizer on nutrient status, chlorophyll content, and rice yield. The experimental method with a Complete Randomized Block Design consisting of 9 treatments, namely A = control, B = recommended NPK, C = ¼ NPK + 2.6 kg ha^{-1} SiO_2, D = ½ NPK + 2.6 kg ha^{-1} SiO_2, E = ¾ NPK + 2.6 kg ha^{-1} SiO_2, F = 1 NPK + 2.6 kg ha^{-1} SiO_2, G = ¾ NPK + 0.65 kg ha^{-1} SiO_2, H = ¾ NPK + 1.3 kg ha^{-1} SiO_2, I = ¾ NPK + 1.95 kg ha^{-1} SiO_2. The fertilizers used were Urea, SP-36, KCl, and liquid K_2SiO_3. The study results showed that adding silica fertilizer could improve the effectiveness of NPK fertilizer in rice cultivation. The use of various doses of Si (¼ to 1 dose) and ¾ dose of NPK can increase the effectiveness of NPK fertilizer use, thereby reducing its use. The dose has also been proven to support producing better nutrient status values, chlorophyll levels content and rice yields than recommended NPK.

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1. Introduction

Rice is a staple food for most of the world's population including Indonesia, which ranks fourth after China, India, and Bangladesh (Shahbandeh, 2024). However, Indonesia's rice production in 2023 decreased by 767.98 thousand tons or 1.40% compared to 2022 (Statistic Indonesia, 2024), so several efforts are needed to increase rice productivity. Rice productivity in Indonesia in 2022 was 5.19 tons/ha (PBS, 22) lower than Vietnam at 5.89 tons ha^{-1} (FAO, 2023). However, Indonesia still imported rice reaching 3.48 million tons in 2024 (BPS 2024). Therefore, rice production must continue to be increased through several methods such as the use of superior varieties, pest, disease and weed control, application of cultivation methods and increased use of fertilizers (Jiraporncharoen, 1993).

One of the factors that supports the achievement of high productivity is the availability of nutrients; as stated by Bertham et al. (2022), the need for rice for the N element is 139.17 kg ha^{-1}. The need for phosphorus nutrients is 27 kg ha^{-1} (Jiang et al., 2021), and potassium nutrients are 29.1 – 386.7 kg ha^{-1} (Amin et al., 2019). Since the introduction of synthetic fertilizers, farmers have started using them as a source of NPK and there has been a tendency for the dosage to continue to increase, causing land degradation (Syamsiyah et al., 2023). This condition ultimately causes nutrient imbalances, one of which is in the Si element (Husnain et al., 2016).

The Si element is very abundant in the soil, but most of it is in the form of soil minerals such as feldspar, mica, quartz, and SiO_2, which are not available to plants (Hayati and Astuti, 2015; Amin et al., 2021). This makes using Si fertilizer very important, so it needs to be added. Based on the statement by Jayawardana et al. (2014), potassium silicate fertilizer can source highly soluble Si and K nutrients. The research results by Mini et al. (2023) stated that using of Si fertilizer is still limited and the results are varied. The use of 0.5% Si and NPK gave higher grain yields compared to the results of recommended NPK fertilization. In comparison, according to Shah (2022), the combination of NPK fertilizer and 1% silica increased plant height by 126.4 cm and the number of productive tillers was 185.5 tillers per m^2.

Providing Si to plants also increases chlorophyll content support photosynthesis efficiency thus resulting in the formation of energy as ATP (Silva et al., 2014). Rice is a high silicon accumulating plant, reaching 10% of the dry weight of the plant (Ma and Yamaji, 2006). The research results show that rice plants produce 5.0 tons ha^{-1} of grain, where the rice plants absorb 230 - 470 kg Si ha^{-1} from the soil (Rodrigues and Datnoff, 2005). According to Amrullah et al. (2014) Rice plants absorb Si approximately 10 times N, 20 times P, 6 times K and 30 times Ca. The critical level of Si in soil is 40 mg kg^{-1} and in rice (leaves and straw) 5% (Nagula, et al, 2015).

Silica although not an essential nutrient, is vital for rice plants (Patil et al., 2018), which are required in high amounts of 230–470 kg ha^{-1} (Sabatini et al., 2017). In plants, Si increases rice resistance to biotic and abiotic stresses (Hastuti et al., 2016), increasing N uptake, improving efficiency, and increasing crop yields (Mabagala et al., 2020). The provision of Si accompanied by P fertilization can increase the efficiency of P fertilizer (Singh et al., 2005; Wang et al., 2020) and increase K uptake by rice by 87.32% compared to the control (Nagula et al., 2018). This study aims to evaluate the use of Si fertilizer on the effectiveness of NPK fertilizer on nutrient status, chlorophyll content, and rice plant yields.

2. Material and Methods

2.1. Research site

Field research was conducted in April 2023 - July 2023 in Plupuh, Sragen Regency, Central Java, Indonesia, which is geographically located at 7°28'41.40" S and 110°53'15.08" E with an altitude of 100 m asl, average rainfall in one year 220 mm $month^{-1}$ (Statistic Indonesia, 2023). Laboratory analysis was conducted at the Soil Chemistry and Fertility Laboratory, Universitas Sebelas Maret. The type of soil includes Vertisols with characteristics pH H_2O 7.34, C-organic 1.71%, cation exchange capacity (CEC) 68.27 me 100 g^{-1}, total N 0.25%, available P 3.08 ppm, and available K 0.23 me 100 g^{-1}. Overall, the soil at the research location has a relatively low level of soil fertility (Soil Research Center Indonesia, 1995).

2.2. Sampling techniques

Experimental research with a completely randomized block design consisted of 9 treatments with three replications. Each plot was 3 m x 4 m in size, with a distance between blocks of 60 cm and a distance between plots in each block of 40 cm. The rice variety used was Inpari 32 with characteristics including having a plant age of approximately 120 days, plant height of 97 cm, having moderate resistance to brown stem leafhoppers biotypes 1, 2, and 3, resistant to leaf blight and blast, suitable for planting in lowland rice field up to an altitude of 600 masl (IAARD, 2019). This crop was planted 20 days after sowing with 25 cm x 25 cm distance, and 2 seedlings in each planting hole. Treatments include A = no fertilizer (control), B = recommended NPK (same doses as 1 NPK), C = ¼ NPK + 2.6 kg ha^{-1} SiO_2, D = ½ NPK + 2.6 kg ha^{-1} SiO_2, E = ¾ NPK + 2.6 kg ha^{-1} SiO_2, F = 1 NPK + 2.6 kg ha^{-1} SiO_2, G = ¾ NPK + 0.65 kg ha^{-1} SiO_2, H = ¾ NPK + 1.3 kg ha^{-1} SiO_2, I = ¾ NPK + 1.95 kg ha^{-1} SiO_2, this treatment

is in accordance with Minister of Agriculture Regulation no. 13 of 2022. The NPK fertilizer used is Urea 350 kg ha^{-1}, SP-36 50 kg ha^{-1}, KCl 50 kg ha^{-1}, and liquid K_2SiO_3 (K_2O 20.13% and SiO_2 13.80%). The NPK sources were Urea, SP-36, and KCl because these fertilizers are most commonly used by local farmers. Urea fertilizer was applied thrice at 0, 14, and 28 Days After Planting (DAP), while SP-36 and KCl fertilizers were applied once at 0 DAP. Silica fertilizer used is liquid and applied twice at 14 and 28 DAP by spraying it onto the soil. Plant care was carried out according to local farmers' habits.

2.3. Measurement of variables

Soil plant samples were taken at 50 DAP. Soil samples were taken in the area around the roots at a depth of 0-20 cm from the surface, while plant samples used for analysis used leaf tissue. harvesting was done at 93 DAP by cutting the base of the stem and threshing the grain using a thresher machine. The soil parameters observed included total N using the Kjeldahl method (Syamsiyah et al., 2023), available P using the $NaHCO_3$ (Olsen) extraction method (Tang et al., 2014), available K using the ammonium acetate ($NH_4CH_3CO_2$) extraction method (Zhang and Kong, 2014), and available Si using the $CaCl_2$ extraction method (Liang et al., 2015). Plant parameters included tissue N content using the Kjeldahl method (Oksana et al., 2012), tissue P and tissue K using the HNO_3 and $HClO_4$ wet ashing methods (Hazra et al., 2019), tissue Si using the colorimetric method (Pati et al., 2016), and chlorophyll content using the Arnon method (Rajalakshmi and Banu, 2015). Yield components included the number of productive tillers (Saragih and Wirnas, 2019), the weight of 1000 seeds with a water content of 14% (Herve et al., 2017), and the weight of grain at harvest.

2.4. Statistical analysis

Data were analyzed using Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) with a 95% confidence level to see the difference in influence between treatments, Pearson correlation to see the closeness of the relationship between treatments and the parameters tested, and linear regression to measure the influence of treatments on the parameters tested.

3. Results and Discussion

3.1 NPK and Si content in soil

Table 1. shows that the fertilizer addition treatment significantly affects total N. The addition of Si fertilizer with NPK showed a higher total N than the recommended NPK, although not statistically significant. This result aligns with Samaddar et al. (2019) that the NPK + Si treatment showed a total N that was not significantly different from without Si, but the total N value increased by around 28%. The provision of silica fertilizer with as much as one dose with ¼ to ½ NPK showed an increase in total soil N compared to the recommended NPK, but the results would be higher when given ¾ to 1 dose of NPK. The application of fertilizers such as Urea containing N will cause an increase in nutrients in the soil (Swify et al., 2023) thus creating optimal cultivation practices (Maulana et al., 2024). Silica plays a role in maintaining nutrient availability by reducing the leaching rate (Bocharnikova and Matichenkov, 2010) and increasing the soil exchange capacity so that N is weakly adsorbed on the Si surface and remains in a form available to plants (Matichenkov and Bocharnikova, 2001).

Table 1. Nitrogen, phosphorus, potassium, and silica content in soil

Code	Total N (%)	Available P (ppm)	Available K (me 100 g^{-1})	Available Si (ppm)
A	0.21±0.04 a	3.24±0.01 a	0.25±0.03 a	211.97±7.40 a
B	0.29±0.01 b	3.30±0.03 ab	0.26±0.03 ab	234.08±6.70 bc
C	0.30±0.04 bc	3.44±0.03 b	0.30±0.01 abc	252.62±6.85 e
D	0.30±0.04 bc	3.54±0.07 c	0.27±0.02 ab	228.18±4.05 b
E	0.35±0.02 cde	3.69±0.01 d	0.37±0.00 de	247.92±6.46 de
F	0.39±0.03 e	3.70±0.04 d	0.39±0.04 e	254.02±2.41 e
G	0.36±0.01 de	3.69±0.11 d	0.36±0.06 cde	251.87±2.18 e
H	0.33±0.03 bcd	3.57±0.01 c	0.32±0.03 bcd	224.94±3.43 b
1	0.30±0.03 bc	3.59±0.05 c	0.34±0.04 cde	240.10±5.82 cd

Note: Numbers followed by the same letter in the column show no significant difference at the 5% level.

Available P in the soil is significantly affected by the provision of Si with NPK fertilizer. In general, the availability of P increases along with the increasing dose of NPK fertilizer and the application of several doses of silica fertilizer (0.65 to 2.6 kg ha^{-1}). The increase in available P is due to the provision of SP-36 fertilizer containing P and Si which can mobilize P from the unavailable phase (Schaller et al., 2019). Monosilicic acid ($H_3SiO_4^-$) formed from the provision of silicate fertilizer will compete with $H_2PO_4^-$ in the absorption site, where $H_3SiO_4^-$ can replace $H_2PO_4^-$ so that it can be available to plants (Agostinho et al., 2017). The provision of Si and NPK fertilizers can increase available P by up to 12% compared to fertilization without Si. These results align with Greger et al. (2018), which concluded that Si fertilizer can increase P availability by up to 50%.

Table 1. shows that NPK + Si fertilization significantly increases soil available K. The provision of ¾ doses of NPK fertilizer with various doses of silica fertilizer showed significant and higher available K results compared to the control and recommended NPK. The increase in available K occurs due to the addition of KCl fertilizer and the effect of Si fertilizer on available K. According to Rao et al. (2019) and Al-Shahmani and Al-Juthery (2021), silica will push potassium from the exchange site to the soil solution. The added Si element synergizes with K to replace K from the exchange site to the soil solution (Savant et al., 1996). The highest potassium availability was obtained from the treatment of 1 NPK + 2.6 kg ha^{-1} SiO_2, which resulted in a 50% increase in available K compared to fertilization without silica.

The availability of Si in this study was significantly influenced by NPK and silica fertilization. Table 1. shows that the highest available Si was obtained from 1 NPK + 2.6 kg ha^{-1} SiO_2 treatment. This treatment showed an increase of 19.84% compared to the control and 8.52% compared to the recommended NPK. Increasing the NPK dose from ¼ to 1 total dose with one dose of Si fertilizer (2.6 kg ha^{-1}) showed an increase in the level of available Si, while increasing the dose of silica fertilizer from 0.65 to 2.6 kg ha^{-1} at the same NPK level, namely ¾ dose, resulted in varying levels of available Si. Silica fertilization causes an increase in the concentration of H_4SiO_4 in the soil solution (Tubaña and Heckman, 2015). A similar thing was also stated by Schaller et al. (2021) and Rupashinge et al. (2022) that adding Si fertilizer could increase the levels of available Si in the soil.

3.2 Uptake of NPK and Si

Fertilization of Si with NPK resulted in a significant increase in uptake of NPK and Si compared to the control and recommended NPK (P<0.05). This is related to the presence of Si, which improves the root system, thus stimulating higher nutrient uptake (Pati et al., 2016). Increased N uptake in rice is due to increased N availability by Si and NPK fertilizers (r=0.712**). Makka et al. (2015) stated that uptake of nitrogen is related to the total N content in the soil. Plants fertilized with Si and N fertilizers cause plant stems to be straighter and receive better sunlight, thus increasing photosynthesis activity (Siam et al., 2018).

Figure 1. shows an increase in P uptake due to adding silica fertilizer, although some Si and NPK + Si fertilization treatments are not significantly different from NPK treatment alone. This increase is related to the increase in available P (r=0.643**) caused by Si, which can help increase soil P availability (Greger et al., 2018). The results also align with research by Schaller et al. (2022) that the addition of Si causes an increase in the concentration of available P to exceed the critical limit of P availability for rice cultivation, which is around 3.5 mg kg^{-1} P.

Potassium uptake is determined by the concentration of K in the soil (r = 0.619**); the higher the concentration, the higher the uptake (Chatterjee et al., 2014). According to Hafez et al. (2021) using additional fertilizer in the form of potassium silicate will increase the source of highly soluble K nutrients; in addition, available K also comes from the KCl fertilizer given. This is in line with the results of research from Nagula et al. (2018) showing that the combination treatment of NPK and spraying potassium silicate can increase K absorption by 87.32% compared to the control.

The data shows that Si uptake tends to increase along with the increase in the dose of Si fertilizer (0.65 to 2.6 kg ha^{-1}) applied and increase in available Si content in the soil. This is reflected in the positive correlation between Si uptake of plants and available Si (r = 0.493**). These results align with Huang et al. (2024) that there is a significant linear correlation between available Si and Si content in plants. According to Pati et al. (2016), the addition of silica fertilizer in a readily available condition causes the nutrients to be ready for plant use.

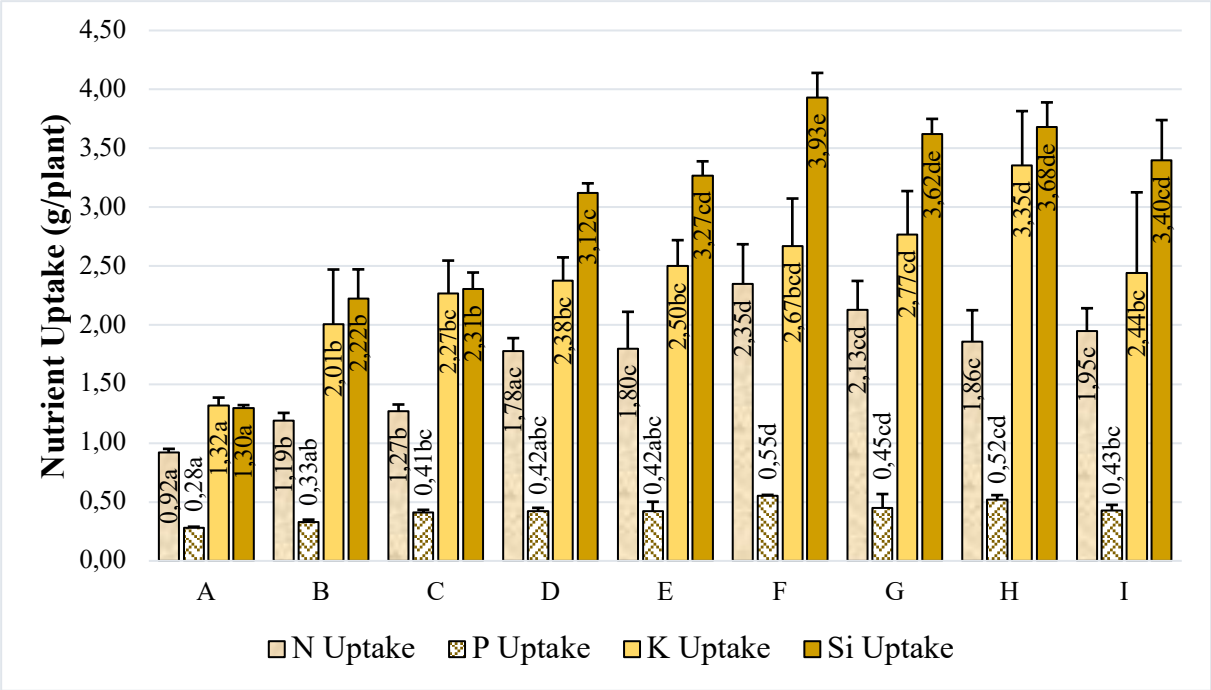

Figure 1. Effect of silica and NPK fertilization on plant nutrient uptake.

	Total N	Available P	Available K	Available Si	N Uptake	P Uptake	K Uptake	Si Uptake
Total N	-							
Available P	0.787**	-						
Available K	0.698**	0.780**	-					
Available Si	0.635**	0.628**	0.644**	-				
N Uptake	0,712**	0.773**	0.795**	0.696**	-			
P Uptake	0.700**	0.682**	0.458*	0.416*	0.703**	-		
K Uptake	0.569**	0.600**	0.619**	0.299	0.620**	0.532**	-	
Si Uptake	0.796**	0.888**	0.666**	0.493**	0.716**	0.814**	0.691**	-

Note: *Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level

Figure 2. Relationship between nutrient content in soil and plant nutrient uptake.

3.3 Chlorophyll content and plant yield

Figure 3. shows increased chlorophyll A, B, and total chlorophyll levels (P<0.05). This is due to increased availability and uptake of NPK as an energy source for N transport to leaves (Isnaini and Novitasari, 2020), as well as increasing the quality of chloroplasts by the element K resulting in good grana stacks and expanding the stroma in the lamella with a small amount of starch granules (Zhao et al., 2001). At the same time, Si plays a role in osmoregulation, which fulfills the essential nutrient needs that are important in chlorophyll formation (Zainul et al., 2022). The results showed a linear relationship between NPK and Si uptake with chlorophyll (Figure 4a). Chlorophyll A levels ranged from 1.09 to 1.28 µg ml^{-1}, chlorophyll B ranged from 0.57 to 0.73 µg ml^{-1}, and total chlorophyll ranged from 1.66 to 1.98 µg ml^{-1} (Table 2). These results align with Ramirez-Olvera et al. (2021) that applying Si fertilizer will increase chlorophyll levels, both chlorophyll A, B, and total chlorophyll.

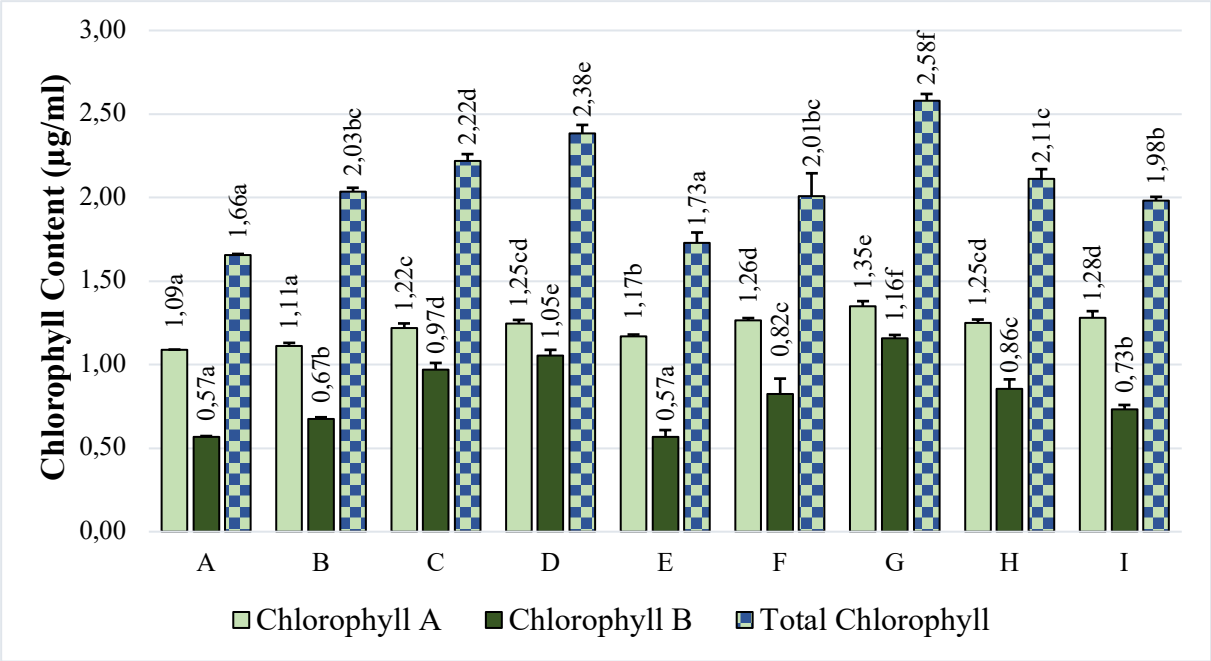

Figure 3. Chlorophyll A, B, and total chlorophyll levels in various treatments.

Table 2. Number of productive tillers (tillers per clump), weight of 1000 seeds (gram), and weight of grain (ton ha^{-1}) from Si and NPK fertilizer treatments

Code	Number of Productive Tillers	Weight of 1000 Seeds	Weight of Grain
A	14.87±2.01 a	21.52±0.48 a	3.42±0.45 a
B	21.80±1.83 b	23.31±1.42 b	5.28±0.91 b
C	26.60±3.42 bcd	23.83±0.72 b	5.27±0.42 b
D	23.93±3.20 bc	24.66±1.05 b	5.63±0.44 bc
E	29.53±3.52 cd	24.44±0.14 b	5.73±0.75 bc
F	31.00±3.67 d	25.03±0.65 b	6.66±0.68 c
G	30.27±2.08 cd	24.58±0.90 b	5.79±0.59 bc
H	29.60±3.14 cd	23.93±1.69 b	5.35±0.93 b
I	29.93±5.90 cd	23.71±0.46 b	5.32±0.11 b

Note: Numbers followed by the same letter in the column show no significant difference at the 5% level.

The application of Si fertilizer together with NPK fertilizer significantly increased the number of productive tillers, the weight of 1000 seeds, and the weight of grain (Table 2) compared to control. The formation of optimal productive tillers will support the seed filling process effectively, and can contribute to increasing the weight of 1000 seeds as an indicator of the quality and weight of grain. Results revealed that increasing the availability and uptake of NPK and Si elements from Si and NPK fertilizers significantly supports rice yields. Uliyah et al. (2017) states that the amount of nutrient absorption is closely related to the weight of the plants produced. This is align with Basha et al. (2013), which states that the application of silica can affect in NPK uptake, thus affecting the accumulation of protein in seeds and straw. These elements play an essential role in plant growth and production. The N element can stimulate tiller growth through the formation of hormones and enzymes (Widodo and Damanhuri, 2021), P plays a role in root formation and increasing the number of tillers (Rosalina and Nirwanto, 2021). Potassium increases productive tillers because it stimulates ATPase, an enzyme that plays a crucial role in plant growth and development (El-Mageed et al., 2023). At the same time, Si is needed in the cell division process (Hasmeda et al., 2023), thus encouraging the formation of productive tillers. The relationship between nutrients uptake and production components is presented in Figures 4b, 4c, and 4d. which show a linear relationship between uptake of nutrients and grain weight. Based on Figure 4, it is known that Si plays an important role in influencing the number of productive tillers,

weight of 1000 seeds, and weight of grain, which are respectively 82.64%, 75.04%, and 71.88%. Silica application can increase productive tillers and grain yield by 28.23% compared to without Si addition (Dehaghi et al., 2018). The increased grain yield in this study is in line with research by Wissa et al. (2017) that silica plays a role in meeting rice nutrient needs and supporting productive growth, thereby maximizing grain yield.

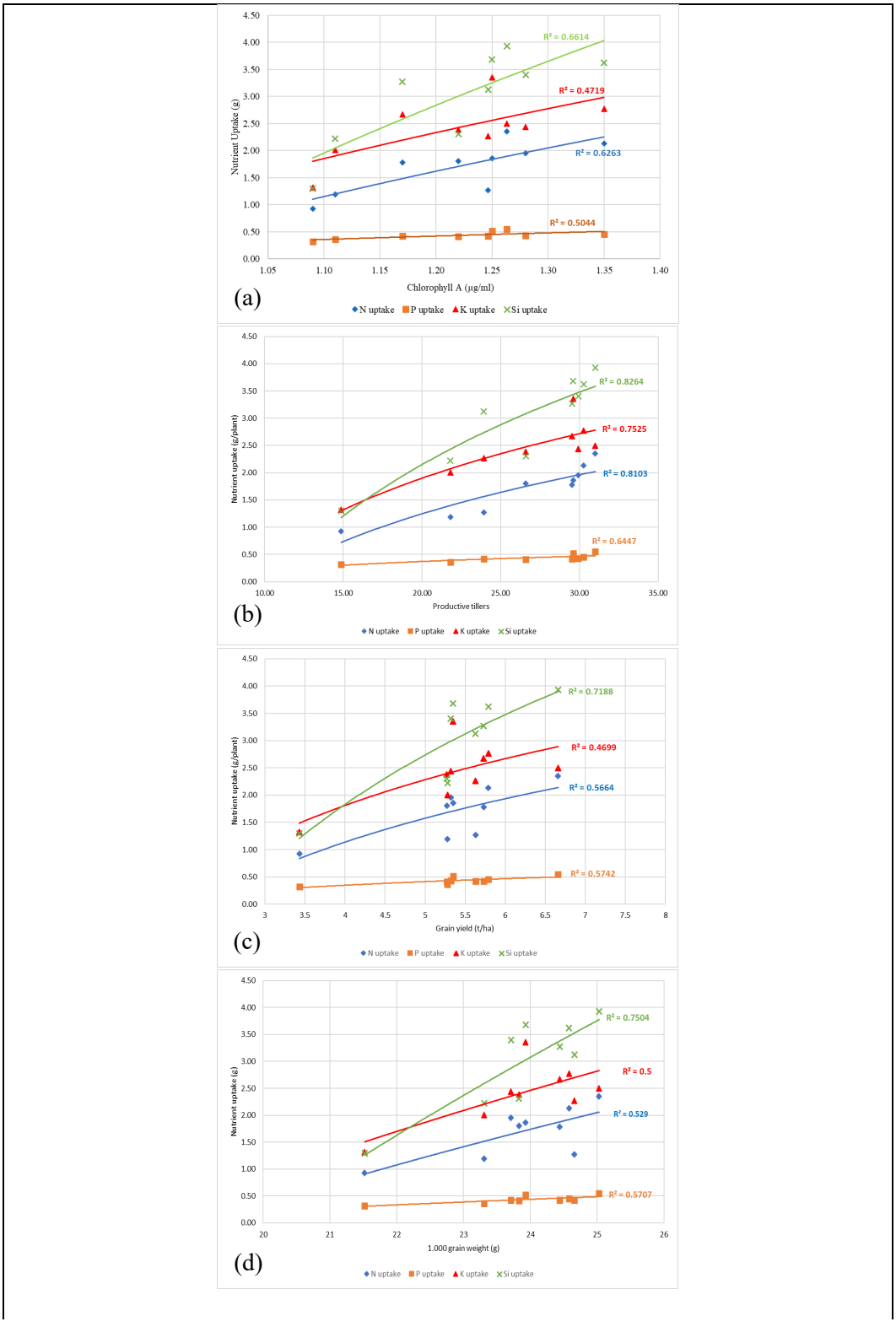

Figure 4. Regression analysis between nutrients uptake and chlorophyll content (a), productive tillers (b), grain yield (c), and 1 000 grain weight (d).

Conclusion

Adding Si fertilizer can improve the availability and uptake of nutrients, as well as crop yields. The use of various doses of Si (¼ to 1 dose) and ¾ dose of NPK can increase the effectiveness of NPK fertilizer use, thereby reducing its use. The dose has also been proven to support producing better nutrient status values, chlorophyll levels content, and rice yields than recommended NPK. To maintain the sustainability of rice productivity, Si fertilizer needs to be given continuously so that farmers can benefit from it.

Ethical Statement

Ethical approval was not required for this study as there was no specific type of research involving humans or animals.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Author Contributions

The first and second authors as research supervisors, outlined the framework of the research task. The third and fourth authors provided input to present the findings. The fifth and sixth authors conducted the research and analyzed the data and drafted the manuscript. The seventh and eighth authors provided additional feedback and refined the manuscript.

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Research Article

Physicochemical, Microbiological, and Organoleptic Properties of Quail (*Coturnix-coturnix Japonica*) Meat Fed with Black Soldier Fly Maggot Meal Added Diet

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Abstract: Feed is one of the important factors that support quail productivity. One of the sources of feed protein that is usually used in quail feed is Meat Bone Meal (MBM). Meat Bone Meal (MBM) is a feed ingredient that serves as a source of animal protein as well as minerals, particularly calcium (Ca) and phosphorus (P), which play a crucial role in the growth and health of livestock. MBM is an imported product, causing it to be expensive. One of the alternatives to replace MBM is by utilizing local feed ingredients, namely black soldier fly (BSF) maggots. This study aims to evaluate the physicochemical, microbiological, and organoleptic characteristics of quail meat given BSF maggot flour in feed. The quail used are female birds quail with a laying period of 44-50 weeks. Research procedures include maintaining and making BSF maggot flour, producing feed, preparing and maintaining cages, slaughtering, and testing meat's physicochemical, microbiological, and organoleptic characteristics. The treatment given is R0 (feed containing MBM) and R1 (feed containing black soldier fly maggot). The data obtained was analyzed using a two-sample t-test. The results showed that quail fed diets containing the main protein source of black soldier fly maggot produced physical and microbiological characteristics of meat that were not different from quail fed diets containing MBM. Quail fed with maggot produced meat with a significantly lower cholesterol content and a more savory taste and preferred color.

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1. Introduction

Quail is one of the potential poultry livestock to be developed. Quail are cultivated in Indonesia mainly as egg producers. In addition to eggs, quail meat is also rich in protein, like other poultry meat. Quail productivity for both egg and meat production is influenced by genetic and environmental factors

(Sembor and Tinangon, 2022). Ecological factors consist of rearing management, microclimate, and feed.

Feed is a crucial factor for achieving quail productivity, accounting for 60%-80% of operational costs associated with feed (Kurniati and Vaulina, 2021). In general, quality feed is feed that contains nutrients such as energy, protein, fat, vitamins, minerals, crude fiber, and amino acids. The feed provided must be according to the needs of the livestock. One of the nutrients that has an important role in feed is protein. The protein required for the quail laying period is at least 17% (BSN, 2006). Protein functions for the growth of new tissues, repairing damaged tissues, and productivity (Wang et al., 2022).

The main protein source commonly used in poultry feed is soybean meal, while Meat Bone Meal (MBM) serves more as a supplementary protein source and a source of minerals. MBM has a high crude protein content of 47.35% (Citra et al., 2019). MBM is rich in essential amino acids such as lysine (3.87 g 100 g^{-1} protein), methionine (1.18 g 100 g^{-1} protein), isoleucine (2.18 g 100 g^{-1} protein), leucine (4.41 g 100 g^{-1} protein), and tryptophan (0.63 g 100 g^{-1} protein) (Barua et al., 2020). The disadvantages of MBM include its risks, such as the potential spread of infectious diseases, contamination with pathogenic bacteria or toxins due to improper processing, and its impact on animal health and food safety. Additionally, MBM is an imported and expensive ingredient, leading to high feed prices. Therefore, efforts are needed to reduce feed costs. One alternative is to replace MBM with local raw materials that have a similar protein content, such as black soldier fly maggots.

Black soldier fly maggot is one type of insect larvae that is widely found. Maggots can also be produced easily and at low cost. According to Jayanegara et al. (2017), black soldier fly maggot contains nutrients including crude protein 44.9%, crude fat 29.1%, crude fiber 16.4%, and ash content 8.1%. Essential amino acids contained in black soldier fly maggot protein include lysine (4.23 g 100 g-1 protein), methionine (1.82 g 100 g^{-1} protein), isoleucine (3.05 g 100 g^{-1} protein), leucine (6.35 g 100 g^{-1} protein) and tryptophan (3.17 g 100 g^{-1} protein) (Djissou et al., 2018). Harianja et al. (2024) examined the quail laying period by adding black soldier fly maggot flour to feed. The parameters observed were quail egg quality. The levels of maggot flour were 0%, 15%, 20% and 25%. The optimal results were achieved with an egg weight of 10.93 g per grain and a yolk index of 0.62, which was significant at the 25% level. Meanwhile, Siregar and Warisman (2023) examined the quail laying period by adding black soldier fly maggot flour in feed. The parameter observed was quail performance. The levels given were 0%, 3%, 6%, 9% and 12%. The best results were obtained at the 6% level with a feed conversion value of 2.04. Until now, it is still difficult to find information about studies that evaluate the physicochemical, microbiological, and organoleptic characteristics of quail meat fed with black soldier fly maggot flour in feed. Therefore, this study aimed to evaluate the feeding of black soldier fly maggot meal in feed on the physicochemical, microbiological, and organoleptic characteristics of quail meat.

2. Material and Methods

This study was conducted for 5 months, from April to August 2024. The barn preparation took 1 month, feed preparation took 1 month, rearing until maggot meal production took 1 month, rearing with treatments lasted for 6 weeks, and parameter observation took 2 weeks. Maintenance was carried out at Arkan Quail Farm, Ciampea, Bogor Regency, West Java. Physical tests, microbiological tests, organoleptic tests, pH values, and MDA levels of meat were carried out at the Animal Product Technology Laboratory of the Department of Animal Production Science and Technology, Faculty of Animal Husbandry, IPB University. The meat cholesterol test was conducted at the Integrated Laboratory of the Bogor Agro Industry Center.

The tools used in the study consisted of tools for maintenance and tools for laboratory analysis. The materials used in this study were 100 female quails of the egg-laying period aged 44-50 weeks, drinking water, black soldier fly maggot, feed ingredients, and laboratory analysis materials tailored to the parameters observed. This research was approved by the Animal Ethics Committee School of Veterinary Medicine and Biomedicine, IPB University, with number 198/KEH/SKE/IV/2024.

2.1. Raising and producing black soldier fly maggot meal

Maggot rearing begins with egg hatching, enlargement, and harvesting (Citra et al., 2019). Preparation of maggot meal involves sorting, cleaning, and roasting (120 ˚C for 10-20 minutes) (Dortmans et al., 2021). Maggots were ground and sieved to a particle size of 100 mesh.

2.2. Feed production

The feed was prepared from ingredients including corn, rice bran, soybean meal, MBM, BSF maggot meal, CPO (Crude Palm Oil), $CaCO_3$, DCP, salt, L-Lysine, DL-Methionine, and premix. Feed is self-prepared on an iso-protein and iso-energy basis. Feed is prepared according to the nutrient requirements of quail for the egg-laying period (BSN, 2016). Maggot meal completely replaces MBM as the protein source in the feed.

2.3. Cage preparation, rearing, and slaughter

The feed used consisted of two types: feed containing 6% MBM and feed containing 12% BSF maggot. This study consisted of two treatments, R0 and R1. Each treatment was repeated 5 times. The rearing period lasted for 6 weeks, starting from 44 weeks of quail age until they reached 50 weeks of age. The cages used for rearing were 10 cage plots. Each plot was filled with 10 quails and the placement was randomized. Before use cages were cleaned and disinfected. The average initial body weight of quail was 190 ± 9.66 g $bird^{-1}$. Feed and drinking water were provided *ad libitum*. Temperature recording is carried out every day, namely morning (06.00-07.00 WIB), afternoon (12.00-13.00 WIB), and evening (16.00-17.00 WIB).

The quails were reared for 6 weeks, starting at 44 weeks of age, with the first 2 weeks for feed adaptation, followed by 4 weeks of treatment feed administration until they reached 50 weeks of age. On 50 weeks of age, the birds were weighed and then slaughtered. Quail samples were randomly taken from each plot at 30% (3 birds), where 1 bird was used for physical and chemical variables, and 2 birds were used for microbiological and organoleptic variables. Before slaughter, quails were fasted for 12 hours (Citra et al., 2019). Slaughter was by CAC/GL 24-1997 (BSN, 2009).

2.4. Testing of physical characteristics of meat

The meat samples tested were taken from the breast. One sample was taken for each replicate. The meat samples tested were taken from the breast. One sample was taken for each replicate. The physical characteristics of the meat tested included a_w (water activity), cooking shrinkage, and tenderness. The aw value was measured using an aw meter (Saledja et al., 2014). Cooking shrinkage (%) was measured by subtracting the weight before and after cooking divided by the initial weight (Bouton et al., 1971). Meat tenderness was measured using a texture analyzer (Soeparno, 2005).

2.5. Testing of meat chemical characteristics

The chemical characteristics of the meat tested included pH, cholesterol content, meat MDA (malondialdehyde) content, and protein content. The pH value of meat was measured with a meat pH meter (Petracci and Baeha, 2011). Determination of meat cholesterol levels was carried out by Liebermen-Burchard method (Sahriawati et al., 2019) using a spectrophotometer at the highest wavelength. Meat MDA levels were measured using a spectrophotometer at a wavelength of 532 nm and calculated the concentration with the TEP standard curve (Singh et al., 2008). Protein content testing was conducted using the Kjeldahl method.

2.6. Testing of meat microbiological characteristics

The microbiological characteristics of meat analyzed were TPC (Total Plate Count) and *Salmonella sp.* content. TPC analysis was conducted using the spread plate method (Yusuf et al., 2016). Determination of the number of colonies was based on the Bacteriological Analytical Manual (BAM) method. *Salmonella sp*. content was tested by following the Badan Standarisasi Nasional (2008) method.

2.7. Testing of meat organoleptic characteristics

Organoleptic tests conducted include hedonic and hedonic quality assessments of color, texture, aroma, and taste. The test was conducted on meat in raw condition (color, aroma, texture) and cooked (color, aroma, texture, taste) by 30 semi-trained panelists using a questionnaire (Smith et al., 2012). Panelists were asked to assess with a score of 1-5.

2.8. Data analysis

The treatments in this study were R0 (feed containing MBM) and R1 (feed containing black soldier fly maggot). Each treatment was repeated 5 times and each replicate consisted of 10 quails. Each treatment was repeated 5 times, and each replicate consisted of 10 quails. The total number of quails used was 100. Organoleptic test data were analyzed using the Kruskal-Wallis test, while physicochemical characteristics test data and microbiological test data were analyzed using t-tests according to Mattjik and Sumertajaya (2013).

$$t_{hit} = \frac{(\bar{x}_1 - \bar{x}_2)}{\sqrt{\frac{(n_1-1)S_1^2 + (n_2-1)S_2^2}{n_1+n_2-2}\left(\frac{1}{n_1}+\frac{1}{n_2}\right)}} \quad (1)$$

t : t-value;
$\bar{x}1$: average of quail treated with feed containing MBM;
$\bar{x}2$: average of quail treated with feed containing maggot;
S1 : standard deviation of quail-fed diets containing MBM;
S2 : standard deviation of quail-fed diets containing maggot;
n1 : number of quail samples fed with feed containing MBM
n2 : number of quail samples fed with feed containing maggot.

3. Results and Discussion

The temperature of the quail rearing environment during the study was recorded in the morning 23.7-27.0 °C, afternoon 25.3-37.0 °C, and evening 24.1-32.8 °C. According to Ulupi et al. (2016), a comfortable ambient temperature for quail is in the range of 20-24 °C. During rearing, part of the time from morning to evening showed an ambient temperature that was above the comfort zone for quail. This causes heat stress, which is characterized by panting, indicating that the quail is experiencing oxidative stress. Oxidative stress is a condition that occurs when free radicals exceed the ability of the body's antioxidant system leading to cell and tissue damage. During oxidative stress, the production of ROS increases beyond the ability of the body's antioxidant system (SOD) to neutralize them. As a result, ROS damages lipids in the cell membrane and produces MDA as a by product (Zaboli et al., 2019).

Physical characteristics testing included cooking shrinkage, a_w (water activity), and tenderness. The results of the analysis of the physical characteristics of quail breast meat are presented in Table 1.

Table 1. Physical characteristics of quail breast meat fed with different main protein sources

Variable	R0	R1
Cooking shrinkage (%)	37.16 ± 1.62	37.24 ± 2.01
a_w (water activity)	0.86 ± 0.01	0.85 ± 0.01
Tenderness (kg cm^{-2})	1.55 ± 0.05	1.61 ± 0.04

Notes: R0 = feed containing MBM as the main protein source, R1 = feed containing black soldier fly maggot as the main protein source.

The different main protein sources of feed between treatments R0 and R1 did not affect the percentage of quail meat cooking shrinkage produced. This was due to the same age and breed of the animals in both treatments. According to Nurwanto & Mulyani (2003), the age of livestock is one of the factors that affect cooking shrinkage. The cooking shrinkage value in this study was still in the normal range. According to Soeparno (2005), the cooking shrinkage value of meat in general for all types of meat varies between 1.5%-54.5% with a normal range of 15%-40%.

The a_w value is the amount of free water that can be used to grow microorganisms. The a_w value of quail meat showed no significant difference between the R0 and R1 treatments. Age correlates with water holding capacity and the composition of connective tissue in muscles. Both are closely related to the amount of free water. The a_w value in this study was below the minimum limit for bacterial growth. Bacteria have a minimum a_w value to grow, which is 0.91 (Beuchat, 2002). This condition means that the a_w value in the research results is not a good value for bacterial growth.

The tenderness value of quail breast meat fed with different main protein sources (MBM for R0 and maggot meal for R1) showed no significant difference. Warner et al. (2021) opine that various factors, such as species, age, management practices, and gender, affect the tenderness of meat. The meat tenderness value of both treatments is in the range of very tender meat tenderness values. Nurcahaya et al. (2022) mentioned that tenderness is classified according to its value, namely very soft (<3.3 kg cm^{-2}), soft (3.3- 5.0 kg cm^{-2}), somewhat soft (5.0-6.71 kg cm^{-2}), somewhat tough (6.71-8.42 kg cm^{-2}), tough (8.42-10.12 kg cm^{-2}), and very tough (>10.12 kg cm^{-2}).

The chemical characteristics observed in this study include pH, cholesterol, meat MDA, and protein content. The chemical characteristics of the meat are presented in Table 2.

Table 2. Chemical characteristics of quail breast meat fed with different main protein sources

Variable	R0	R1
pH	5.78 ± 0.13	5.77 ± 0.18
Cholesterol (mg 100 g^{-1})	68.77 ± 2.33[a]	50.27 ± 3.92[b]
MDA (µg g^{-1})	1.15 ± 0.04	0.92 ± 0.25
Protein content (%)	20.70 ± 0.75	21.71 ± 0.53

Notes: Different superscripts on the same line indicate significant differences (P<0.05); R0 = feed containing MBM as the main protein source, R1 = feed containing black soldier fly maggot as the main protein source.

The results of the analysis of the pH value of quail meat fed with different main protein sources showed no significant difference. This may be because both rations consist of isoprotein and isoenergy. These conditions have an impact on body weight which is relatively the same so that the muscle glycogen produced is the same. According to Kim et al., (2014) one of the factors that affect the pH value is muscle glycogen. Nkukwana et al. (2015) reported the range of pH values as <5.7 (PSE meat), 5.7-6.1 (standard meat), and >6.1 (DFD meat). The pH value of meat in this study is still in the normal/standard category reported by Nkukwana et al. (2015). DFD (Dark, Firm, and Dry) meat is dark-colored, firm-textured, and appears dry due to a high pH (>6.1) after slaughter. Meanwhile, PSE meat (Pale, Soft, and Exudative) is pale in color, has a soft texture, and releases a lot of liquid due to a drastic drop in pH (<5.7) after slaughter.

Quail fed diets containing black soldier fly maggot meal produced meat with a cholesterol level 26.9% lower than quail fed diets containing MBM (P<0.05). The results of the analysis showed that the two treatments were significantly different. This may be because black soldier fly maggot contains chitin, which is the main component of maggot skin (exoskeleton). Chitin plays a role in lowering cholesterol because it has hypocholesterolemic properties that function to inhibit fat absorption (Silva et al., 2021).

MDA (malondialdehyde) is an indicator to assess the severity of oxidative stress (Cordiano et al., 2023). The analysis of meat MDA levels showed no significant difference in both treatments. The MDA value of meat in both treatments was lower than the research conducted by Bulbul et al. (2021) on the meat of quail breast meat, which was 1.21 µg g^{-1}. This means that the meat in both treatments has a lower level of oxidative damage than the results of the study by Bulbul et al. (2021), so the quality is better and safer for consumption.

Different main feed protein sources did not affect the meat protein content (P>0.05). This is due to the same total feed protein content in both treatments. Based on the calculation, both treatments produce protein intake is almost the same. Protein levels in quail meat range from 18 to 23% (Genchev et al., 2008). This shows that the protein content of the tested quail meat is still within the reported range.

The microbiological characteristics of meat analyzed in this study were TPC and *Salmonella sp*. content. The results of the analysis of microbiological characteristics are presented in Table 3.

Table 3. Microbiological of quail breast meat fed with different main protein sources

Variable	R0	R1
Salmonella sp.	negative	negative
Total Plate Count (TPC) log CFU g^{-1}	6.22 ± 0.64	6.20 ± 0.23

Notes: R0 = feed containing MBM as the main protein source, R1 = feed containing black soldier fly maggot as the main protein source.

Based on the test results of *Salmonella sp.* content of quail meat given different feed protein sources, the results showed no *Salmonella sp.* contamination in both treatments. This means that the meat has met the requirements set by the Indonesian National Standard (SNI) 7388:2009. The maximum limit of *Salmonella sp.* contamination in fresh poultry meat is negative 25 g^{-1} (BSN, 2009). The negative *Salmonella sp.* content in both treatments is due to the feed used of the same quality not contaminated with pathogenic contaminants (*Salmonella sp.*) and the process of raising and cutting is carried out hygienically by the applicable SOP. Diyana et al. (2021) believe negative *Salmonella sp.* content can be influenced by factors including good hygiene and sanitation and the quality of feed free from *Salmonella sp.* contamination.

Different feed protein sources did not significantly affect the total bacterial colonies. This may be explained by the a_w values of the two groups. We know that there is a correlation between the aw value and the total bacteria. A_w value is the free water used to grow microorganisms, including bacteria (Eskin and Robinson, 2010). The average TPC content of quail meat is within the BSNI (2009) standard limit of 1 x 10^6 CFU g^{-1} (6 Log CFU g^{-1}). This means that the quail meat meets microbiological safety standards with TPC within safe limits, making it suitable and safe for consumption.

Organoleptic testing includes hedonic test and hedonic quality on raw and cooked meat which is presented in Table 4. The results of the hedonic quality assessment of cooked and raw meat by panelists showed that meat from quail fed with feed containing black soldier fly maggot meal was more reddish in color than quail meat fed with feed containing MBM. The difference in meat color can be seen in Figure 1. It is possible that the reddish color in the R1 treatment is due to the higher Fe content (iron) of the maggots of the black soldier fly compared to MBM. Fe is contained in myoglobin. The pigment that gives red color to meat is myoglobin (Suman and Joseph, 2013). Higher Fe will result in a redder meat pigment.

Table 4. Organoleptic characteristics of raw and cooked quail meat fed diets with different main protein sources

Variable	Raw Meat		Cooked Meat	
	R0	R1	R0	R1
Hedonic Quality				
Color	3.27 ± 0.78	4.17 ± 0.75	2.77 ± 0.57	3.43 ± 0.50
Texture	3.37 ± 0.67	3.27 ± 0.69	3.67 ± 0.55	3.63 ± 0.81
Aroma	2.77 ± 1.10	3.67 ± 0.96	2.90 ± 1.06	3.50 ± 0.86
Hedonic				
Color	2.90 ± 0.66	3.73 ± 0.74	3.07 ± 0.74	4.07 ± 0.78
Texture	3.17 ± 0.59	3.33 ± 0.71	3.30 ± 0.60	3.37 ± 0.72
Aroma	4.13 ± 0.51	4.17 ± 0.53	4.23 ± 0.50	4.13 ± 0.57
Flavor	-	-	3.00 ± 0.95	4.20 ± 0.55

Notes: R0 = feed containing MBM as the main protein source, R1 = feed containing black soldier fly maggot as the main protein source. Hedonic quality (product quality based on panelist assessment); color brightness: 1:very pale, 2:pale, 3:slightly pale, 4:slightly reddish bright, 5:reddish bright; texture hardness level 1:very hard, 2:hard, 3:slightly soft, 4:soft, 5:very soft; aroma level 1:very fishy, 2:fishy, 3:slightly fishy, 4:not fishy/no aroma, 5:typical meat aroma; taste level 1:very bland, 2:bland, 3:slightly tasty, 4:tasty, 5:very tasty. Hedonic (degree of panelist liking): 1:dislike, 2:somewhat like, 3:like, 4:very like, 5:very much like.

Figure 1. Differences in quail meat color in R0 and R1 fed different protein sources in the diet.

The organoleptic data analysis using the Kruskal-Wallis method showed no significant differences ($p > 0.05$) between the treatment groups in the assessment of taste, aroma, texture, and color of quail meat fed with black soldier fly maggot meal compared to the control feed. The observation data in Table 4 shows that the panelists' assessment of meat texture has the same hedonic quality value of slightly tender in both raw and cooked conditions. The hedonic test on the meat texture of both treatments was equally favored by panelists in both raw and cooked conditions. Panelists liked both raw and cooked conditions.

Hedonic quality assessment of meat aroma in raw and cooked conditions showed that panelists stated that quail meat fed with black soldier fly maggot flour was not more fishy than quail meat fed with MBM as the main protein source. This is due to differences in MBM and maggot flour processing methods. Based on the hedonic test results, panelists gave the same assessment of the meat aroma of both treatments, which was very like (both in raw and cooked conditions).

Organoleptic testing of flavor is only done on cooked meat. In the assessment of the level of savor (hedonic quality), panelists considered that the taste of quail meat fed with feed containing black soldier fly maggot flour was more savory than quail meat fed with feed containing MBM. This may be due to the difference in the glutamic acid content of the two types of meat. Glutamic acid is the determinant of savory taste in meat. Based on the glutamic acid analysis in the laboratory Saraswanti Indo Genetech laboratory, it is known that the glutamic acid content in black soldier fly maggot is 3.42% and in MBM is 0.92%. Hedonic test results showed that panelists preferred quail meat fed with maggot meal because of its more savory taste. This was indicated by the higher hedonic test score of hedonic test score which was higher than quail meat fed with MBM in the feed.

Conclusion

Quail fed diets containing the main protein source of black soldier fly maggot produced physical and microbiological characteristics of meat that were not different from quail fed diets containing the main protein source of MBM. Quail fed with maggot as the main protein source produced meat with a significantly lower cholesterol content and a more savory taste and preferred color. Research suggests that black soldier fly maggots can be a primary protein source in quail feed to replace MBM.

Ethical Statement

Ethical approval for this study was obtained from Animal Ethics Committee School of Veterinary Medicine and Biomedicine, IPB University, with number 198/KEH/SKE/IV/2024.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Author Contributions

The authors contributed equally.

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Research Article

The Evaluation for Recent Growth Performance of Bali Cattle using Non-linear Models

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Inflection points,
Modified Von Bertalanffy model

Abstract: Growth curve modeling is essential for understanding livestock development, productivity, and efficiency. This study evaluated the growth patterns of Bali cattle, a resilient and economically significant breed in Indonesia, using five non-linear growth models: Brody, Gompertz, Logistic, Von Bertalanffy, and Modified Von Bertalanffy. Body weight data were collected from 256 males and 279 females at key growth stages from birth to 730 days. Goodness-of-fit criteria including Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), coefficient of determination (R^2), and correlation coefficient (r) were applied to identify the most suitable model for describing growth curves. The Gompertz model exhibited the best fit for males, with the lowest AIC (29.76) and BIC (28.58) and highest R^2 (0.9913) and r (0.9956). For females, the Modified Von Bertalanffy model performed best, with superior goodness-of-fit metrics. Growth parameter analysis revealed that males achieved higher mature weights (A) and slower growth rates (K), whereas females exhibited faster growth rates but matured at smaller sizes. These findings indicate distinct growth dynamics between sexes, influenced by genetic and physiological factors. This research emphasizes the importance of selecting appropriate models to understand critical growth stages, optimize nutrition, and enhance management and breeding strategies. The results offering valuable insights for breeders, farmers, and policymakers aiming to bolster beef production.

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1. Introduction

One of the most important indicators to predict livestock development, production and efficiency is by using growth curve as the parameter. Mathematical models that illustrate the relationship between age and body weight are commonly used to describe growth curve (Bahreini et al., 2014; Regadas Filho, 2014; Filipe et al., 2018). Non-linear models are particularly suitable for analyzing

growth curve patterns of livestock. They effectively demonstrate how sigmoidal pathways appear. Through accurately growth curve modeling, these methods offer important insights into crucial growth stages and performance (Zimmerman et al., 2001; Cantalapiedra-Hijar et al., 2018). This allows livestock producers to make decisions regarding nutrition, management, and breeding programs. Ultimately, optimizing these factors contribute to better production outcomes, increased productivity and efficiency.

Bali cattle is one of Indonesian indigenous cattles originated from Bali Island. This particular type of cattle was domesticated breed of wild Banteng (Kikkawa et al., 2003; Mohamad et al., 2012; Wijaya et al., 2023). Bali cattle is well-known for its adaptability, which thrived under tropical environmental conditions and can utilize low-quality feed efficiently. Their adaptability and economic significance make them a cornerstone of Indonesia's beef production, particularly in traditional and semi-intensive farming systems (Martojo et al., 2012; Firman and Nono, 2021). Additionally, Bali cattle hold considerable cultural value, further solidifying their importance in local communities (Lisson et al., 2010). Understanding their growth characteristics is crucial for improving productivity, guiding genetic selection, and enhancing resource management strategies to support sustainable livestock development.

Non-linear growth models were widely used to analyze and predict growth patterns of cattle due to their ability to capture the sigmoidal characteristic of animal growth. These models, such as Gompertz, Logistic, and Von Bertalanffy, provide a better representation of biological growth processes compared to linear models, as they account for acceleration and deceleration phases and inflection points of livestock growth (Nogales et al., 2017; Selvaggi et al., 2017; Júnior et al., 2022). By accurately estimating growth parameters, non-linear models help identify critical growth stages, assess feeding efficiency, estimate breeding program outcomes, and determine the optimal slaughtering time. Furthermore, non-linear models were favorable tools for genetic selection, allowing breeders to assess individual growth trajectories and select animals with superior growth potential (Weber et al., 2021; Benvenga et al., 2022; Araujo et al., 2023). Overall, non-linear models enhance decision in nutrition, breeding, and management, leading to improved productivity and profitability in cattle production system. The best-fit model can be implemented to attain more reliable estimated growth parameters which are essential for effective cattle management.

This study aims to compare the performance of five non-linear models Brody, Gompertz, Logistic, and Von Bertalanffy, by assessing the growth performance of Bali cattle. The findings will determine the model which would be appropriate to describe the growth pattern and provide meaningful biological interpretations of the growth parameters of Bali cattle. The results of this research are expected to contribute to improved management practices, sustainable productivity, and better decision-making for government-owned breeding center, farmers and stakeholders.

2. Material and Methods

2.1. Data collection

The body weight records of Bali Cattle were obtained from Bali cattle breeding center of Republic Indonesia, Denpasar. Data comprised of recorded body weight at birth (0 day), body weight at weaning (205 day), then body weight at 365, 550, and 730 days. A data set included 256 males and 279 females Bali cattle born between 2017 and 2021. Bali cattle growth stages at each age are presented in Figure 1 and Figure 2, respectively for male and female.

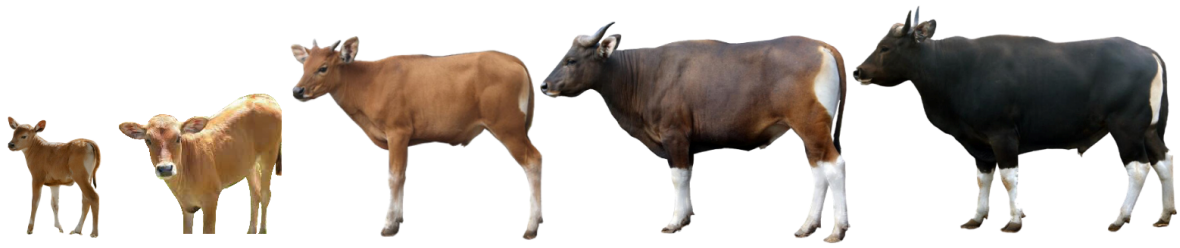

Figure 1. The illustration of male Bali cattle in each age stage.

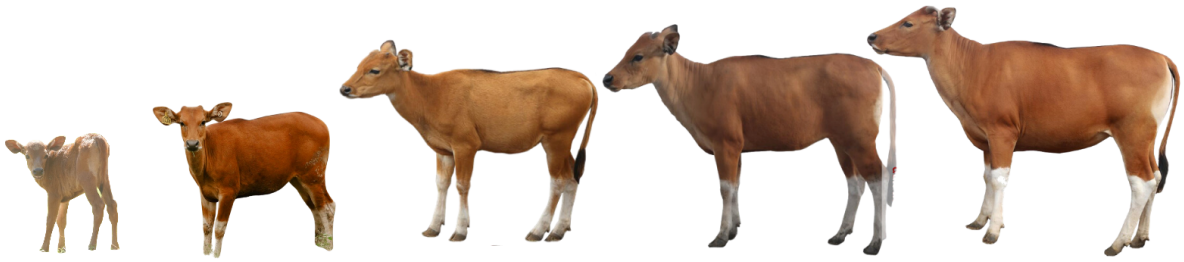

Figure 2. The illustration of female Bali cattle in each age stage.

2.2. Non-linear growth model

Individual body weight of Bali cattle was analyzed using five nonlinear growth curves: Gompertz, Logistic, Brody, Von Bertalanffy, and The modiffied Von Bertalanffy models. The NLIN procedure of Statistical Analysis System (SAS) OnDemand for Academics (SAS, 2021) was utilized to fit the observed body weights to these nonlinear models. The models used for predicting body weight is shown on Table 1.

Table 1. Mathematical equations of five growth models

Model	Equations	Inflection Point	
		Wi	***Ai***
Brody	$y = A(1 - Be- Kt)$	n/a	n/a
Gompertz	$y = Ae- Be- Kt$	$e^{-1}A$	*(ln B)/K*
Logistic	$y = A/(1 + Be- Kt)$	*A/2*	*(ln B)/K*
Von Bertalanfy	$y = A(1 - Be- Kt)^3$	*A(8/27)*	*(ln3B)/K*
Von Bertalanfy Modified	$y=A*(1- B*exp(-K*t))^2$	*8A/27*	*(ln3B)/K*

y, Body weight at t time; A, Asymptote body weights; B, Integral constant; e, Basic logarithm (2,71828); K, Average growth rate until adult age; Wi, Weight at inflection; Ai, Age of inflection; t, Time unit (week); n/a, not available.

2.3. Goodness of fit criteria

The appropriate model to describe the growth curve of Bali cattle was chosen using the goodness of fit criteria listed below. The Akaike Information Criterion (AIC) equation computed according to Narinc et al. (2014) as follows:

$$AIC = n\ ln\left(\frac{SSE}{n}\right) + 2p \quad (1)$$

The Bayesian Information Criterion (BIC) was computed using the equation Lewis et al. (2010) below:

$$BIC = n\ ln\left(\frac{SSE}{n}\right) + p\ ln\ (n) \quad (2)$$

Coefficient of determination (R^2) is used to evaluate the reliability of a model in linear regression analysis Equation provides the definition of this coefficient.

$$R^2 = 1 - \left(\frac{SSE}{SST}\right) \quad (3)$$

Where SSE is Error Sum of Square, SST is Total Sum of Square, n is the number of observations, ln indicates the natural logarithm, and p is the number of model parameters. The Pearson's correlation between prediction and observed body weight of Bali cattle chicken were estimated by using the CORR Procedure of SAS.

3. Results

The descriptive statistic of body weight for each age stage is presented in Table 2. Body weights of 205, 365, 550 and 730 days in female Bali cattle showed lower coefficients of variance than male Bali cattle. The estimated growth parameters for Bali cattle showed variation between males and females in all models. For males, the parameter A ranged from 410.17 kg (Logistic) to 542.64 kg (Modified Von Bertalanffy), with the Modified Von Bertalanffy model predicting the highest weight but slower growth rates (K = 0.002).

Table 2. The descriptive statistic of the body weight measured

Body weight	**Male**				**Female**			
(kg)	**Minimum**	**Maximum**	**Means**	**CV**	**Minimum**	**Maximum**	**Means**	**CV**
0 day	14	25	18.25	0.11	13	24	18.17	0.11
205 days	57	167	91.56	0.21	51	142	90.30	0.20
365 days	81	226	138.71	0.21	80	200	127.48	0.18
550 days	113	330	193.61	0.22	103	270	165.09	0.18
730 days	133	427	273.95	0.22	147	306	205.00	0.14

CV, coefficient of variation.

In females, the estimated growth parameters were ranging from 239.04 kg (Logistic) to 366.12 kg (Brody), with the Logistic model showed the fastest growth rate (K = 0.076). The Modified Von Bertalanffy model generally predicted higher A and slower K for both sexes. The detailed growth parameter for Bali cattle estimated by five models are presented in Table 3.

Table 3. Estimated growth parameters for Bali cattle using five non-linear models

Parameters	Brody	Gompertz	Logistic	Von Bertalanfy	Modified Von Bertalanfy
Male					
***A* [Coeff. ± SE]**	522.98±35.95	485.49±19.36	410.17±15.80	516.86±21.25	542.64±24.56
***B* [Coeff. ± SE]**	0.95±0.003	3.74±0.886	17.79±6.331	0.63±0.006	0.79±0.006
K	0.001	0.014	0.047	0.002	0.002
Wi	n/a	441.36	495.60	383.40	628.90
Ai	n/a	178.60	205.08	153.14	160.78
Female					
***A*[Coeff. ± SE]**	366.12±14.49	299.13±26.47	239.04±10.27	291.85±11.27	296.41±9.85
***B*[Coeff. ± SE]**	0.94±0.002	2.41±0.027	8.27±0.804	1.63±1.060	0.72±0.003
K	0.006	0.032	0.076	0.005	0.009
Wi	n/a	273.31	324.66	234.86	365.12
Ai	n/a	110.04	119.52	86.47	87.82

SE, Standard error; A, Asymptote body weights; *B*, Integral constant; *K*, Average growth rate until adult age; *Wi*, Weight at inflection; *Ai*, Age of inflection.

The goodness of fit for growth curve models in Bali cattle was evaluated using AIC, BIC, R^2, and correlation coefficient (r) values (Table 4). For males, the Gompertz model showed the best fit with the lowest AIC (29.76) and BIC (28.58), and the highest R^2 (0.9913) and r (0.9956), indicating excellent predictive accuracy. Similarly, Gompertz model also performed the best with the lowest AIC (26.59) and BIC (25.42), along with the highest R^2 (0.9937) and r (0.9969) for females. Other models, such as Logistic and Von Bertalanffy also showed strong fits, while the Gompertz model consistently provided the most accurate representation of growth for both male and female Bali cattle.

Table 4. Goodness of fit of growth curve models for body weights of Bali cattle

Model	Male				Female			
	AIC	BIC	R^2	r	AIC	BIC	R^2	r
Brody	246.68	35.06	0.8862	0.8862	26.71	25.64	0.9649	0.9820
Gompertz	29.76	28.58	0.9913	0.9956	26.59	25.42	0.9937	0.9969
Logistic	30.80	29.63	0.9923	0.9961	28.46	27.23	0.9920	0.9957
Von Bertalanffy	29.99	28.82	0.9904	0.9952	26.31	25.14	0.9935	0.9967
Modified Von Bertalanffy	30.92	29.72	0.9857	0.9927	26.25	25.03	0.9933	0.9966

AIC, Akaike Information Criterion; BIC, Bayesian Information Criterion, R^2, Coefficient of determination; r, Coefficient of correlation.

4. Discussion

The average body weights of male and female Bali cattle at birth (0 days) were similar, with males at 18.25 kg and females at 18.17 kg. By 730 days, males Bali cattle reached 273.95 kg, significantly heavier than females at 205.00 kg, indicating that males grow faster and reach maturity at earlier age. Compared to previous studies on Bali cattle, the body weight at 205 days was similar with Azis et al. (2023). The body weight of 730 days 273.95 kg for males and 205.00 kg for females were higher than the findings of Jakaria et al. (2019) at extensive rearing system (208.7 kg and 168.0 kg, respectively). Garantjang et al. (2020) reported body weight of Bali bull at the Village Breeding Center Bone South Sulawesi were 210.25 kg. Environmental factors such as feeding, housing conditions, and climatic stressors can significantly influence growth rates in cattle. These conditions may affect males and females differently, particularly in terms of metabolic demands, stress tolerance, and nutrient utilization. While this study did not specifically differentiate management strategies by sex, potential differences in how males and females respond to environmental factors could partially explain the observed growth variations. In comparison to other Indonesian breeds, the result of this study was lower than Madura cattle as reported by Prihandini et al. (2020) that body weight for male and female at 2 years were 306.66 and 282.76 kg respectively. The body weight of male Bali cattle at 2 year was higher

than male Pasundan cattle (257.0 kg), whereas female Bali cattle were similar with female Pasundan cattle (204.5 kg) (Sumaryadi et al., 2021).

The estimated *A* parameter of Bali cattle showed that males consistently have higher mature weights than females across all models. The Brody and Modified Von Bertalanffy models predicted the highest *A* for males (522.98 kg and 542.64 kg, respectively), while the Logistic model provided the lowest estimate (410.17 kg). For females, the Brody model estimated the highest *A* (366.12 kg), whereas the Logistic model produced the lowest (239.04 kg). Compared to other breeds Bali cattle show lower *A* value. Adinata et al. (2022) reported higher *A* value of Ongole grade cattle ranged from 596.87 to 857.64 for male and ranged from 504.24 to 692.35 for female. The parameter *A* represented the maximum body weight cattle can achieve, reflecting its growth potential and genetic capacity (Grossi et al., 2008; Walmsley et al., 2016). production in tropical regions. Sex-based differences in mature body weight and growth potential are influenced by genetics and hormones. Males generally express more growth-related genes and produce higher levels of testosterone, which enhances muscle development and overall size. These factors, along with the influence of sex chromosomes, help explain why males have higher mature weights and slower, sustained growth compared to females (Kassahun et al., 2022).

The estimated of parameter *B* for Bali cattle varied across the five models. The Logistic model produced the highest value of *B* (17.79) and (8.27), respectively for male and female. The Von Bertalanffy model provided the lowest estimates (0.63) and (1.63) respectively. This result were similar to previous studies in Bahashwan et al. (2015) which reported the highest *B* values estimated by Logistic model and lowest *B* (0.52) estimated by Von Bertalanffy model in Dhofari cattle. Marinho et al. (2013) also reported lower *B* values in Nellore cattle (0.52) and (0.92) respectively, estimated by Von Bertalanffy and Brody models. These results suggested that Bali cattle exhibit a relatively faster early growth phase indicated by higher *B* values, which may contribute to their efficiency in reaching moderate mature weights. Differences between studies may be attributed to variations in genetic background, environmental conditions, and management practices. For example, Bali cattle raised under improved feeding and housing conditions tend to show enhanced growth compared to those in extensive systems, as seen in the studies by Jakaria et al. (2019) and Kurlyana et al. (2023). These comparisons highlight how the current results reflect a unique interaction of breed genetics with optimized management strategies, contributing to improved performance.

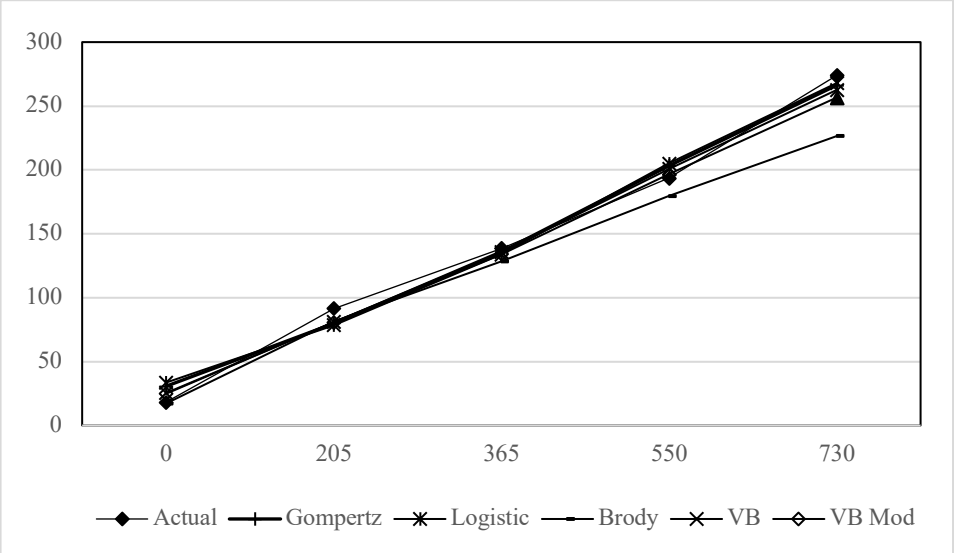

Figure 3. Actual and prediction of growth curve for male Bali cattle.

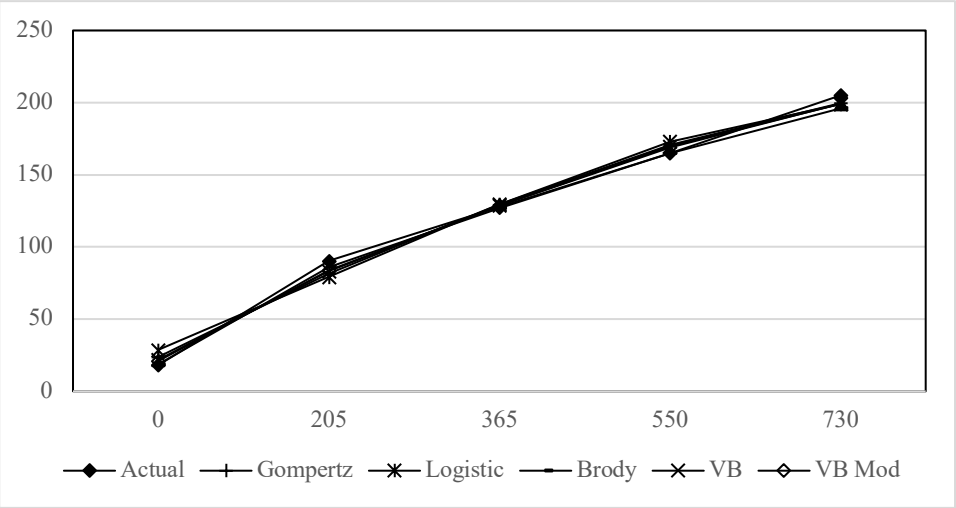

Figure 4. Actual and prediction of growth curve for female Bali cattle.

The growth rates (*K*) estimated for Bali cattle demonstrated significant variability across models. Logistic model showed the highest values and the Brody model showed the lowest, suggesting the differences in how these models captured early compared with sustained growth. Female cattle exhibited consistently higher *K* values than males across all models (Figure 3 and 4), aligning with findings in other breeds such as Nelore cattle (Forni et al*.,* 2009; Marinho et al*.,* 2013; Júnior et al*.,* 2022), and Angus cattle (Goldberg and Ravagnolo, 2015), where females often showed acceleration in early growth due to physiological maturity differences. Gompertz and Logistic models frequently provided higher *K* values than the Brody and Von Bertalanffy models. This result is similar with the studies on Zebu cattle (Domínguez-Viveros et al*.,* 2020). The higher *K* values estimated for the Logistic model emphasize rapid early growth which was crucial for efficient meat production, while the lower *K* estimated by Brody model reflected a focus on long-term growth sustainability. These results suggested that sex and growth models significantly influenced growth rate predictions, highlighting the need for tailored approaches in beef cattle management.

The estimated *Wi* and *Ai* for Bali cattle exhibited notable variations across models, reflecting differences in how these models capture dynamics of growth. The Modified Von Bertalanffy model predicted the highest *Wi* for both males (628.90 kg) and females (365.12 kg), while the Von Bertalanffy model estimates the lowest values, indicating a more conservative projection of Wi. The Logistic model estimates slightly higher *Wi* and *Ai* than the Gompertz model, suggesting a delayed, however rapid growth phase. The *Ai* values were consistently lower for females across all models, indicating earlier attainment of peak growth compared to males, similiar with Cano et al. (2016) findings. Gompertz and Logistic models showed higher *Wi* and *Ai* than Von Bertalanffy. The result was similar with previous studies by Domínguez-Viveros et al. (2020) on Zebu cattle, and Domínguez-Viveros et al (2023) on pure breed and crossbreed Limousin cattle. Bali cattle exhibited comparatively lower *Wi* due to their smaller frame size. These variations underscored the influence of breed characteristics and growth model selection on predicting growth patterns, essential for optimizing cattle production strategies.

The lowest values of AIC and BIC in Gompertz model indicated that this model was the best fit for males Bali cattle, whereas Modified Von Bertalanffy was the best fit model for females. The result was slightly different with the study growth model for Madura cattle by Hartati and Putra (2021) which reported the lowest AIC and BIC was in Von Bertalanffy model both for male and female. The correlation between prediction and observed body weight and R^2 were superior in all model except for Brody model, in both male and female. The result was in contrast to the estimated growth cruve for Nellore cattle which showed lower R^2 (89.16%) and (88.70%) respectively for Brody and Bertalanffy (Marinho et al*.,* 2013). Gompertz model was frequently reported to provide a superior fit for body weight growth for male Bali cattle due to its flexibility in capturing the sigmoidal growth pattern. On the other hand, Modified Von Bertalanffy model showed reasonable fit for female Bali cattle.

Conclusion

Bali cattle exhibited distinct growth characteristics between males and females, with males achieving higher mature weights (A) and slower growth rates (K), while females grew faster but matured at smaller sizes. The Gompertz model provided the best fit for males, while the Modified Von Bertalanffy model was most suitable for females, demonstrating that model performance varies with sex. These findings emphasize the importance of selecting sex-specific growth models to improve prediction accuracy and management strategies. Understanding these differences can help farmers and breeders optimize feeding regimes providing higher energy diets for males over a longer growth period to support their extended growth phase, while focusing on nutrient-rich early-life feeding for females to match their faster maturation. Strategically adjusting nutrition and management based on sex-specific growth patterns can improve feed efficiency, reduce production costs, and enhance the overall productivity and sustainability of Bali cattle farming in tropical environments.

Ethical Statement

The ethical approval from the committee of Animal Care and Welfare for this research was not necessary due to the used of secondary data only and there was no field experiment conducted.

Conflict of Interest

The authors declare that there were no conflicts of interest in any other financial institution nor organization regarding the material in this research.

Author Contributions

AS and PNG designed the study; PNG, DAL, NSP, MAM, FTK, and SVP performed the experiment; DAL, STP, AS, PNG and MAM analyzed and interpreted the data and prepared and wrote the manuscript; and AS and STP took part in critically checking the manuscript.

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Research Article

Determination of Juglone Content of Some Walnut cultivars and the Light Fastness of Dyestuffs Obtained from These Cultivars in the Dyeing of Cotton

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Abstract: This study was carried out in 2020 to determine the light fastness in dyeing cotton fabrics to test the juglone values in the content of domestic and foreign walnut cultivars as well as the use of these cultivars in natural dyeing. According to the results of the study, the light fastness test results obtained after dyeing the dyestuffs obtained from domestic and foreign cultivars of *Juglans regia* L. walnut species on cotton fabrics were calculated with the lowest 2 values in Serr and Şen 2 walnut cultivars, while the highest 3 were Maraş 18, Şebin, Fernette, and Fernor cultivars. *J. regia* juglone analysis results obtained from domestic and foreign cultivars of walnut species, from lowest to highest, were determined in Şen 2, Maraş 18, Fernette, Şebin, Maraş 12, Pedro, Serr, Sundland, Bilecik, Chandler, Franquette and Fernor cultivars, respectively. In addition, according to the results of the percentage similarity analysis, in which the similarities between the juglone contents of domestic and foreign walnut cultivars were calculated, it was determined that domestic and foreign cultivars were generally grouped among themselves and these groups had higher similarities among themselves. When the tested juglone amounts of domestic and foreign walnut cultivars included in the experiment were compared statistically, it was found that Fernor (0.2595) and Franquette (0.2581) cultivars were not statistically different, but together with Chandler (0.2538), the juglone amounts of these cultivars were statistically significantly different between almost all cultivars.

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1. Introduction

Türkiye is one of the rare countries that has 3 hotspots (Hotspot: priority protection areas) (Aydın, 2005 and 2006; Aydın and Kazak, 2010; Aydın, 2011 and 2018; Aydın and Karaca, 2009 and 2011). For this reason, plant diversity and endemism in Türkiye, which has many endemic species, is quite high compared to European countries (Şekeroğlu and Aydın, 2002; Yaşar et al., 2003; Aydın et al., 2005; Lillig and Aydın, 2006; Dinç et al., 2015; Aydın and Şen, 2020). Walnuts are among the fruit species grown in our country; It shows a wide natural distribution area from the Carpathian Mountains to Türkiye, Iran, Iraq, Afghanistan, Southern Russia, India, Manchuria and Korea. There are 25 walnut

species that are widely accepted in the world, and the most common species grown in Türkiye is *Juglans regia* L. (Akça, 2009; Şen, 2011).

Walnut production in Türkiye is provided from trees grown from seeds and orchards established with grafted saplings in recent years. The presence of walnut trees in Türkiye creates a valuable population in terms of genetic material (Akça, 2009).

Unlike other fruit species, walnuts contain juglone (5-hydroxy-1,4-naphthalenedione). This chemical is found in different amounts in different organs of the plant and shows different effects. There are several studies on the amount of juglone as well as changes in its quantity with time and other factors (Tekintaş et al., 1988; Karadağ, 2007; Turan, 2008; Cosmulescu et al., 2011; Yurtçu, 2014).

Moreover, the fruit, leaves and shells of walnut plant are a type of fruit that has a wide area of use in the paint and cosmetics industry. Considering the richness of species in our country, the number of scientific studies on the possibilities of using especially domestic and foreign walnut cultivars in juglone and natural dyeing is still rare. Present study was aimed to determine the light fastness of cotton fabrics in order to test the juglone values in the content of domestic and foreign walnut cultivars and the use of these cultivars in natural dyeing.

2. Material and Methods

2.1. Material

The study was carried out in 2020. Some domestic cultivars belonging to *Juglans regia* walnut species such as Bilecik, Maraş 12, Maraş 18, Şebin, Şen 2 (Sütyemez, 2016; Sütyemez et al., 2019), as well as some foreign cultivars including Chandler, Fernette, Fernor, Franquette, Pedro, Serr and Sundland were selected for trial. The green dry shells of the domestic and foreign walnut cultivars selected were used as material. These materials were obtained from ten-year-old walnut trees grown under the same conditions in Eğirdir district of Isparta province. The green peels separated from the fruit after harvest were dried in the natural environment with shade and ventilation. The peeled walnut shells were analyzed after they were ground in the mill. Organic knitted cotton fabric was used to determine the light fastness values of domestic and foreign walnut cultivars mentioned above. The light fastness values of the samples obtained after dyeing the natural dyestuffs of domestic and foreign walnut cultivars on cotton fabric were carried out in Türkiye Instrumental Analysis Laboratory (CHT).

2.2. Method

2.2.1. Painting process

The dyeing processes of natural dyestuffs obtained from domestic and foreign walnut cultivars on cotton fabrics were carried out in the Paint Workshop of the Faculty of Fine Arts, Süleyman Demirel University, Türkiye. Because walnut is a direct dye, it has not been subjected to a pre-dyeing process (mordant). The dyestuffs were obtained by grinding the shell part of the walnut fruit into powder. The dyestuffs obtained from walnut cultivars were added separately after the solution, in which 2.5 liters of water was added into a 3-liter beaker, started to boil slightly, and was boiled at 85 °C for one hour with continuous stirring. After the dyeing process was completed, the fabrics were left to cool in the solution overnight. The fabrics were then rinsed with cold water and dried in a shaded and airy environment. These processes were applied to all domestic and foreign walnut cultivars on cotton fabrics and a total of 12 dyeing were carried out (see Table 1 for details).

Table 1. Dyeing recipe of dyestuff obtained from domestic and foreign cultivars of *Juglans regia* L. Walnut on cotton fabrics

Paint Equipment	*Juglans regia* L. (1753)
Amount Of Paint	20 gr
Mordant Type	-
Mordant Amount	-
Solution Ratio	2.5 lt
pH	7.6
Boiling Temperature	85 °C
Boiling Time	60 min
Painting Type	direct painting
Fabric Used	organic cotton
Painting Process	Add 2.5 liters of water into a 3 liter beaker. 20 gr walnut fruit peel is weighed and put into this solution. After the solution starts to boil slightly, 20 g of cotton fabric is wetted and thrown into the solution and boiled at 85°C for one hour with constant stirring. After the dyeing process is finished, the fabrics are kept in the solution for 1 night. The next day, the fabrics are removed, rinsed and left to dry.

2.2.2. Determination of light fastness

Light fastness measurements were made using the Procedure 3 method on the Atlas Xenotest machine and 8 replications on the D 65 light source and evaluated.

2.2.3. Juglone analyses

2.2.3.1. Sampling and extraction

Juglone was analysed using the method reported by Tekintaş et al. [20]. In order to determine the Juglone content of the seedlings, two-gram samples were weighted from the fruit peels, with 3 replications on the basis of variety. The samples were extracted in 50 ml petroleum ether (40-60%) for a total of 24 hours, including 6 hours in a shaker. For the analysis, the standard curve was prepared as follows.

2.2.3.2. Standard curve preparation

First, crystal juglone stock solution was prepared and this solution was than diluted to different concentrations (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, and 0.60 mg). Briefly, 500 ml of 50 ppm stock solution was prepared by dissolving 0.025 g of crystal juglone in 500 ml of petroleum ether. Samples taken from the stock solution were made up to 25 ml with petroleum ether (Table 2).

The absorbance values of the samples taken from the prepared extracts were measured at 410 nm. A linear relationship emerged between absorbance values and the amount of crystal juglone. The regression equation of the relationship between the determined absorbance values and the amount of crystal juglone in the solutions was created.

$$(y= 0.0455*X + 0.0429), (R\ 2=0.9945) \quad (1)$$

Crystal juglone values were calculated according to the formula below:

$$\text{Crystal juglone (mg/25ml)} = (0.0455*\text{absorbance value}) + 0.0429 \quad (2)$$

After preparing the standard curve, 5 ml samples taken from the extracts were completed to 25 ml with petroleum ether and absorbance values were determined at 410 nm wavelength in a spectrophotometer. By adapting these values to the standard curve prepared with crystal juglone, the juglone content of seedling shells was determined with the equation (iii).

$$\text{Juglone (mg/g)} = ((0.0455*\text{absorbance value}) + 0.0429)*5 \quad (3)$$

Juglone analysis studies were carried out in Karamanoğlu Mehmetbey University, Central Research Laboratory.

Table 2. Standard curvature values for Juglone analysis

Standard Curb Sequence No:	Amount of juglone required for standard curd (mg)	Quantity taken from stock solution (ml)	Amount completed with Petroleum Ether (ml)
1	0.05	1	25
2	0.10	2	25
3	0.15	3	25
4	0.20	4	25
5	0.25	5	25
6	0.30	6	25
7	0.35	7	25
8	0.40	8	25
9	0.45	9	25
10	0.50	10	25
11	0.55	11	25
12	0.60	12	25

2.2.4. Evaluation of data

2.2.4.1. Light fastness

As a result of the application of dyestuffs of domestic and foreign cultivars of *Juglans regia* walnut species on cotton fabrics, the "Light Fastness" values were entered into the Microsoft Excel program and X and Y graphic distributions were created.

2.2.4.2. Juglone analysis

The differences between the juglone content of domestic and foreign cultivars of *J. regia* walnut species were evaluated by ANOVA-LSD test. The data obtained were calculated in the JMP Statistics program. In addition, the similarities and differences between the amount of Juglone were subjected to classification analysis. Classification analysis was calculated using the Multi-Variate Statistical Package (MVSP) 3.11c program (Kovach, 1999). The distances between the amounts of juglone were measured with percent similarity.

3. Results and Discussions

3.1. Determination of light fastness

The light fastness test results obtained after dyeing the dyestuffs obtained from domestic and foreign cultivars of *J. regia* walnut species on cotton fabrics were calculated with the lowest 2 values in Serr and Şen 2 walnut cultivars, while the highest 3 values were in Maraş 18, Şebin, Fernette and Fernor cultivars. The light fastness results stained with Bilecik, Maraş 12, Chandler, Franquette, Pedro and Sundland cultivars were found in the range of 2.5 to these two values (Figure 1 and Table 3).

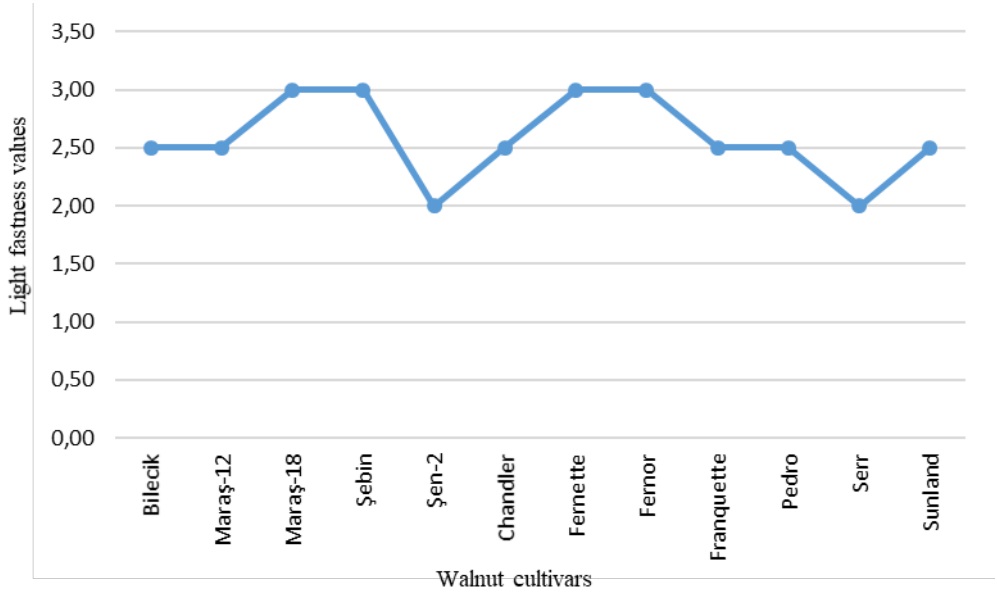

Figure 1. Light fastness values obtained after dyeing materials obtained from domestic and foreign walnut cultivars on cotton fabrics.

Gözütok (2017) determined the light fastness value obtained from walnut shell in the range of 5.5 in the application without mordant. In the study, it was also mentioned that the walnut species included in the experiment was *J. regia*, but walnut cultivars were not mentioned. It can be said that the difference between the light fastness values is also caused by the dyestuff applied on the woolen fabric samples (Gözütok, 2017). In the study where light fastness values were measured, it was determined that the light fastness results painted on the skin by applying a single dose were between 2 and 4, and the light fastness results painted on the skin by applying multiple doses were between 2 and 4 (Arabacıoğlu, 2017). Ghaheh et al. (2012) emphasized that the light fastness value varies between 3 and 4 as a result of the dyeing process without pre-dyeing (without mordant). Ghaheh et al. (2012) and our results were found to be similar, however, walnut cultivars were not specified in this study, and only *J. regia*, which is a walnut type, was specified. Eser et al. (2015) stated in their study that the light fastness values of the dyestuffs obtained from walnut leaves by using different mordant substances changed between 1 and 6, and the light fastness values of the dyestuffs applied without using mordant changed between 4 and 5. The difference between the results obtained in our study and the results of Eser et al. (2015) obtained the natural dyestuff from walnut leaves.

Mirjalili et al. (2010) calculated the light fastness values of fabrics treated with walnut natural dyestuff on silk fabrics boiled in ethanol and water as 6 and 5, respectively. The high light fastness values obtained in the mentioned study may be due to the silk fabric. Sharma and Grover (2011) stated in their study that the light fastness values of walnut natural dyestuff treated with different mordant substances on cotton fabrics vary between 6 and 8. The light fastness values obtained in our study were obtained as a result of application without mordant. Sadeghi-Kiakhani et al. (2019) measured the light fastness values in the range of 4 to 8 on fabrics dyed with walnut natural dyestuff using different mordant substances. Khan et al. (2016) found all the light fastness values measured as a result of the application of walnut natural dyestuff on wool yarn with and without mordant method as 5. Zakaria et al. (2017) reported in their study that the light fastness values measured as a result of the application of walnut natural dyestuff on knitted cotton fabrics before, during and after mordanting generally vary in the range of 2-3. Doğan-Sağlamtimur et al. (2017) determined that the light fastness values obtained as a result of the application of the walnut natural dye obtained from the dry and wet shells of Kaman variety on the leather were 2 in the control group and in the wet shell test, and 3.5 in the dry shell test. As it can be understood from previous studies, it can be said that light fastness is affected by many factors such as the difference in the material to which the natural dyestuff is applied, the properties of the plant part used in dyeing, applications with and without mordant, among the reasons for the different results of light fastness values.

3.2. Juglone analysis

Juglans regia juglone analysis results obtained from domestic and foreign cultivars of walnut species, from lowest to highest, were determined in Şen 2, Maraş 18, Fernette, Şebin, Maraş 12, Pedro, Serr, Sundland, Bilecik, Chandler, Franquette and Fernor cultivars, respectively (Figure 2). Analysis results showed that the juglone content values of foreign origin cultivars were higher than domestic cultivars. However, the domestic Bilecik walnut variety was among the juglone values obtained from foreign cultivars with a value of 0.2536. The measured juglone values of all other domestic cultivars were generally lower than those obtained from foreign cultivars. It is thought that the difference between the amount of juglone between domestic and foreign cultivars may be due to the genetic structure between cultivars and the different foliation periods and harvest times of many foreign cultivars. Chandler, Franquette and Fernor foreign walnut cultivars are the last leafing cultivars compared to other walnut cultivars tested. The highest amount of juglone was calculated in these cultivars. This may be due to late leafing. Among the foreign cultivars, since Pedro is a mid-leafing variety, the amount of juglone is between late leafing foreign cultivars and early leafing domestic cultivars. In addition, among the reasons for measuring the amount of juglone between cultivars with different values, it is a fact that although values such as location, tree age, soil characteristics were taken as homogeneous in the experiment, the juglone values may be affected by some ecological factors.

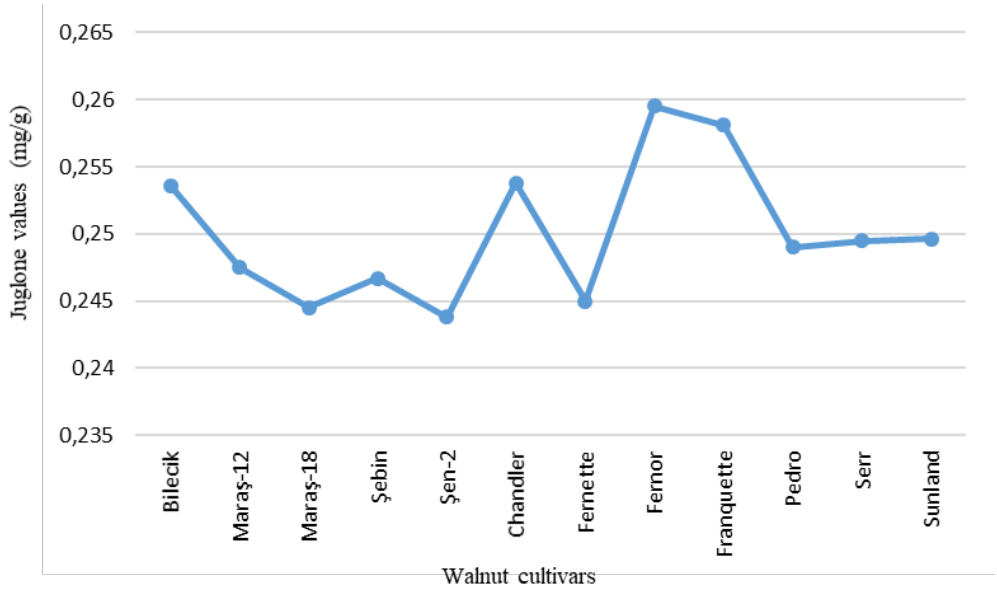

Figure 2. Juglone values calculated from domestic and foreign walnut cultivars.

Uğurlu et al. (2019) examined the juglone amounts of walnuts harvested in different periods and grown in different locations in their study and determined it in the range of 0.132 to 0.278 mg g^{-1}, which are different than the results of our study. These results are reasonable due to the fact that regional and variety differences. Cosmulescu et al. (2011) evaluated the juglone content between cultivars in their study. In the study, the average of the juglone values measured from the leaf was found to be 0.4879±0.028 mg g^{-1}, and the average of the juglone values measured from the green bark was 0.4877±0.012 mg g^{-1}. The measured juglone values of the Franquette variety, which is the common variety with our study, were found to be 0.1521±0.043 and 0.4034±0.123 mg g^{-1} in the leaf and green fruit peel, respectively. Although the juglone values were measured from the dry bark in our study, it is among the values measured from the leaf and green bark in the study of Cosmulescu et al. (2011). In the study of Sesli (2016), the juglone values calculated from the fruits of different walnut cultivars vary between 0.22 and 0.29 mg g^{-1}. When the walnut cultivars used in Sesli (2016) were compared with our study results, Bilecik (0.292 mg g^{-1}), Chandler (0.258 mg g^{-1}), Fernor (0.268 mg g^{-1}), Pedro (0.277 mg g^{-1}) and Serr (0.244 mg g^{-1}) were found to be very close to the juglone values obtained from cultivars. There are not many studies with juglone values measured from dried fruit shells of different walnut cultivars.

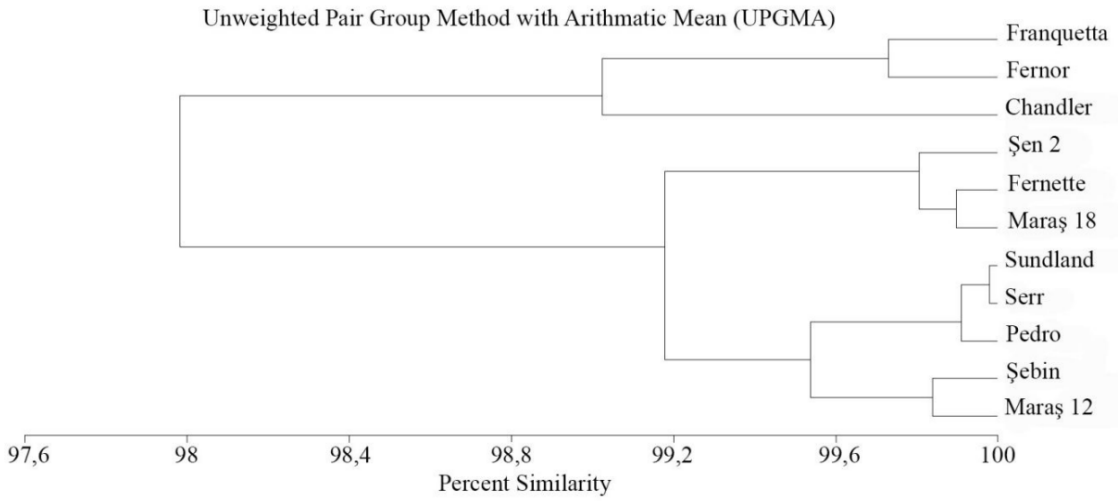

Figure 3. Percent similarity analysis of juglone values calculated from domestic and foreign walnut cultivars.

According to the results of the percentage similarity analysis, in which the juglone content of domestic and foreign walnut cultivars is calculated, it is seen that domestic and foreign cultivars are generally grouped among themselves. It was determined that the most similar cultivars were between Serr and Sundland foreign cultivars with 99.98%. 99.91% similarity of Pedro cultivar to the group consisting of Serr and Sundland cultivars was calculated. Figure 3 shows that there is 99.90% similarity between Maraş 18 and Fernette, another similar group, and that Şen 2 is 99.81% similar to this group. It has been calculated that two local cultivars, Maraş 12 and Şebin, are 99.84% similar to each other in terms of juglone content. Fernor and Franquette cultivars were 99.73% similar to each other, and Chandler's was 99.03% similar to this group they formed.

Table 3. Light fastness values on cotton fabrics and juglone values measured from fruit peels of natural dyestuff obtained from domestic and foreign walnut cultivars

Cultivars	Light Fastness Values	Amount of Juglone (mg/g) ±standart deviation
Fernor	3	0.2595 a*
Franquette	2.5	0.2581 ab
Chandler	2.5	0.2538bc
Bilecik	2.5	0.2536 cd
Serr	2	0.2495 de
Sunland	2.5	0.2496 e
Pedro	2.5	0.2491ef
Maraş-12	2.5	0.2475efg
Şebin	3	0.2467efg
Fernette	3	0.2450fg
Maraş-18	3	0.2445 g
Şen-2	2	0.2438 g
P		0.0001
F		11.7621

* Means within a column followed by the same letters are not significantly different by the LSD test at P < 0.01.

While the group consisting of Sundland, Serr and Pedro cultivars and the groups formed by Şebin and Maraş 12 cultivars were found to be 99.54% similar to each other, the similarity of all these cultivars was calculated to be 99.18% to the group consisting of Şen 2, Fernette and Maraş 18. Finally, the group consisting of Franquette, Fernor and Chandler cultivars was determined to be 97.98% similar

to the group consisting of all other cultivars. The reason for this dissimilarity is thought to be due to the different genetic structure as well as the different foliation and harvest periods.

When the classification analysis is examined in general terms, it is seen that mostly domestic and foreign cultivars have higher similarities among themselves (Figure 3).

When the tested juglone amounts of domestic and foreign walnut cultivars included in the experiment were compared, there was no statistical difference between Fernor (0.2595 mg g^{-1}) and Franquette (0.2581 mg g^{-1}) cultivars, but the juglone amounts of these cultivars were statistically significantly different between Chandler (0.2538) and almost all cultivars. Tested juglone values ranged from 0.2595 to 0.2438 mg g^{-1}, standard errors are reflected in Figure 4.

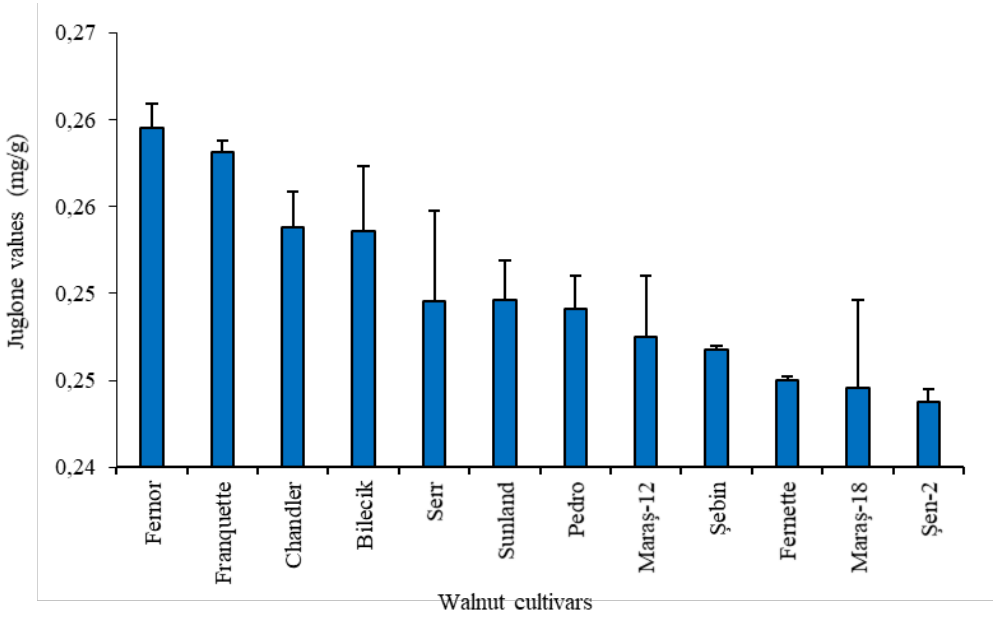

Figure 4. Juglans values and standard deviations measured from walnut cultivars.

Conclusion

In this study, the juglone amounts of different walnut cultivars including domestic and foreign cultivars were calculated, and differences between the juglone amounts and different walnut cultivars selected were determined. The origin of the cultivars, the cultivar characteristics, the material used and the ecology have an effect on the formation of the differences. Among the cultivars included in the study, the juglone content of the native cultivars is generally at a lower level. On the other hand, according to the early or late leafing characteristics of the cultivars in terms of vegetative period; it is seen that the juglone content amounts of late leafing cultivars are higher, it is thought that new studies can be done on this subject. In addition, it was concluded that it would be beneficial to work with the same cultivars in different ecologies in order to determine the effect of ecology.

Considering the relationship between juglone amounts and light fastness, although there is no significant difference between the juglone amounts of the cultivars, the light fastness values are different. In this case, the use of different dyeing materials with the same cultivars or the use of different dyeing materials with a single variety in new studies to be conducted may be beneficial for studies

Ethical Statement

The authors state that ethical approval is not required for the present study, as the study consists of walnut materials that do not require ethical approval.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

Each author made an equal contribution to the article (33.3%).

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Research Article

Cationic and Anionic Dye Removal of Modified *Ulva lactuca* L. and Antioxidant Activity

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Dye removal,
Modified biomass,
Pollution,
Ulva lactuca

Abstract: This study aimed to investigate the removal of fuchsin and nigrosin synthetic dyes using modified *Ulva lactuca* L. The study used the adsorption process under laboratory conditions to determine the removal effect of initial dye concentration (25, 50, 100 mg L^{-1}) at different exposure times (60, 90, 120 min), constant temperature (26±1 ºC), biomass dosage (1 g dw) and pH (7–8) values on the dried biosorbent chemically treated with magnesium chloride ($MgCI_2$) and potassium hydroxide (KOH). The research also provides information on changes in some biochemical properties of the biosorbent by exposure to $MgCI_2$ and KOH. The adsorption of the dyes on *U. lactuca* was modeled with the Langmuir and Freundlich isotherms. The study results determined maximum dye removal for fuchsin (70.81%) and nigrosin (61.29%) dyes at 120 min exposure time and 50 mg L^{-1} dye concentration onto non-modified *U. lactuca* biomass. The mean dye removal for fuchsin (60.08%, 99.12%) and nigrosin (56.23%, 54.27%) was obtained on *U. lactuca* biomass treated with $MgCI_2$ and KOH, respectively. The sample prepared at 60 min contact time and 50 mg L^{-1} dye concentration had the highest adsorption efficiency for fuchsin on *U. lactuca* biomass treated with KOH (99.40%). These results demonstrated that the KOH exposure onto *Ulva* is an efficient, non-polluting, and economical process for eliminating fuchsin from aqueous solutions.

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1. Introduction

As a result of the direct discharge, with or without pre-treatment, of wastewater generated in different industrial departments like paper, leather, textile, plastic, and medicine to the environment, it has become inevitable for dye-laden wastewater to affect the environment and organisms negatively (Li et al., 2022). Today, water pollution, which occurs primarily as a result of the inability of textile industries to fully treat wastewater containing dyes, negatively affects the global economy and environmental pollution in many countries (Almroth et al., 2021; Olisah et al., 2021; Ali et al., 2022). Approximately 7×10^7 tons of synthetic dyes are produced worldwide each year. Textile industries use ten thousand tons of dyes such as azo, direct, reactive, mordant, acid, basic, disperse, and sulfur dyes (Chandanshive et al., 2020). Textile dyes, however, do not adhere well to fabric and are released into

lakes, rivers, streams, and ponds without first being treated. They are also released with wastewater, constituting a significant ecotoxicological hazard that can harm living things (Parmar et al., 2022).

Contaminated colored wastewater is formed due to the combination of aromatic, water-soluble, and dispersible dyes used extensively in the textile industry. This dirty paint discharge into the environment causes an aesthetic problem and serious environmental problems due to its durability in nature and non-biodegradable properties (Stefanakis et al., 2014). In addition to threatening human health due to their carcinogenic and mutagenic properties, dyes significantly affect the distribution of plants, plankton, and neuston organisms in the aquatic ecosystem by blocking the penetration of sunlight into the water (Abbas et al., 2021; Alprol et al., 2021; Ashour et al., 2021; Sardar et al., 2021). These artificial synthetic dyes, which have very high pollutant levels even at low concentrations and negatively affect the aquatic food chain, are generally light-stable, oxidizing agents and are resistant to aerobic digestion, making it challenging to treat colored wastewater and difficult to degrade (Tahir et al., 2008; Oladipo et al., 2013; Stefanakis et al., 2014).

Dyes used in the textile industry are generally classified as cationic, anionic, and nonionic dyes (Demirbas, 2009). cationic dyes (Crystal violet, Methylene blue, Fuchsin, etc.) carrying a positive charge in their molecules are toxic basic dyes widely used in wool, nylon, and silk dyeing. They are water-soluble (Eren and Afsin, 2008). Anionic dyes (Indigo carmine, Methyl orange, reactive Brilliant red, Nigrosin, etc.) bound to a negative ion are acidic and reactive toxic dyes used in dyeing cotton, silk, and wool, and are well soluble in water (Qin et al., 2009; Eren et al., 2010). Some methods have been developed to purify wastewater from the harmful effects of these toxic dyes. These physical and chemical methods include flotation, flocculation, coagulation, ion exchange, irradiation, ozonation, precipitation, oxidation, and reduction. The low removal rate, large secondary sludge, and high cost of these methods make them unfavorable since the dyes' aromatic structures are resistant to heat, light, and oxidizing agents (Akceylan et al., 2009; Gupta et al., 2009; Chequer et al., 2013; Brahmbhatt and Jasrai, 2016). Conversely, in the bioremediation technique, where microorganisms such as fungi, bacteria, or micro-macro algae are used *in-situ* or *ex-situ*, pollutants can be converted into non-toxic products while being cleaned from the environment or an aqueous medium. In addition, this technique is environmentally friendly, economical, has a high absorption capacity, and is widely available in physico-chemical treatment technology (Abatenh et al., 2017; Bhuyar et al., 2020).

The biomaterial pores must be opened to be used as an efficient bioadsorbent in removing toxic materials. Studies have determined that cone-shaped cavities are formed with physical activation and bottle-shaped cavities with chemical activation. Alkaline agents that chemically activate the pores of the biomaterial are some acids and salts such as KOH, K_2CO_3, Na_2CO_3, and $MgCl_2$, and alkaline earth agents are H_3PO_4, H_2SO_4, $AlCl_3$, and $ZnCl_2$ (van Oss, 1990; Nahil and Williams, 2012; Nazem et al., 2020).

According to studies, the biomass of many microorganisms such as macroalgae, seaweeds, microalgae, fungi, and yeasts have been used as biological, sustainable, and low-cost adsorption materials for the removal of toxic dyes in the aqueous environment (El-Sheekh et al., 2009; Blaga et al., 2021; Kapoor et al., 2021; Mishra et al., 2021). Among all microorganisms, non-living or living algal cells are the most promising, sustainable, and low-cost biomaterials for adsorption due to the cell wall containing various functional groups such as phosphate, carboxyl, amino, carbonyl, and hydroxyl groups (Alprol et al., 2021). Algae can take up pollutants in the aquatic environment bioaccumulate and biotransform organic matter and immobilize inorganic elements, making them less toxic (Saleh, 2015). It has been stated that dye removal using especially non-living algae biomass as a result of physicochemical interactions such as electrostatic interaction, ion exchange, complexation, and microprecipitation is more advantageous due to reasons such as not needing maintenance and nutrition, being independent of metabolism and being able to be stored and reused (Aksu, 2005).

Ulva Linnaeus is a cosmopolitan genus that can grow very quickly, has a short life cycle and high pollution tolerance, and is tolerant to high pollutant concentrations, eutrophication, and wide changes in other abiotic factors (Koeman, 1985; Steneck and Dethier, 1994; Amaral et al., 2018; Eismann et al., 2020). *Ulva lactuca* (Sea lettuce), a type of green macroalgae that can be obtained in large quantities in shallow water near low water bodies, is a biomaterial that is suitable for the extraction of color and metal ions from water, is highly preferred in the removal of toxic heavy metals, and is used in biological treatment methods (Salima et al., 2013; Ibrahim et al., 2016; Heidarpour et al., 2019; Mourad et al., 2019; Soliman et al., 2019). However, most previous studies used *U. lactuca* without activation (El Sikaily et al., 2006; Tahir et al., 2008; Pratiwi et al., 2019; El Nemr et al., 2021). The

study aimed to improve the biosorption surface by activating the surface of the seaweed *U. lactuca* biomass as a low-cost adsorbent. The removal of fuchsin (cationic) and nigrosin (anionic), widely used in different industrial fields such as textile, pharmaceutical, and chemical, was analyzed on modified (with $MgCl_2$ and KOH chemicals, 2:1 ratio) and non-modified dried *U. lactuca* L. biomass collected from the Aegean Sea. The adsorption capacity of *U. lactuca* towards fuchsin and nigrosin was studied using different concentrations of dye solution and different contact times for the adsorption performance. The experiment under laboratory conditions kept pH, algal biomass dose, and temperature constant. Additionally, the research also provides information on changes in some biochemical properties of the biosorbent, including total phenolic, flavonoid ingredient, and antioxidant activity in the modified and non-modified green macroalgae *U. lactuca*. The adsorption of the dyes on *U. lactuca* was supported by Langmuir and Freundlich isotherm models. The results obtained from this study, which was conducted on water contaminated by the used synthetic dyes, would be significant in addressing the increasing demand for bioabsorbents for cleaning toxic dyes from aqueous solutions.

2. Material and Methods

2.1. The pretreatment of the organism and modified biomass

The seawater and organism employed in this study were gathered between March 9−10, 2024, from the coastal zone of İzmir Bay, İnciraltı (38°24'39"N, 27°02'10"E). After sampling, the *U. lactuca* sample was transferred to the laboratory (4 °C). The thallus was washed with double distilled water to eliminate salts and other particles on the surface. The cleaned thallus was dried at room temperature and then in an oven (Memmert UNB 100) at 60 ºC for 24 h until the mass stabilized and measured the dry weight.

The dried biomass, which was ground into a fine powder with the grinder, was sieved to obtain particle sizes below 350 µm. The sieved powdered biomass was stored in glass containers for future use.

2.2. Dyes and batch adsorption experiment

The adsorbates used in this study were basic fuchsin (Synthetic cationic dye, Merck 42510, with the molecular formula $C_{19}H_{17}N_3 \cdot HCl$, 323.82 g mol^{-1}) and nigrosin disodium (Synthetic anionic dye, Merck 115924, with the molecular formula $C_{22}H_{14}N_6Na_2O_9S_2$, 202.21 g mol^{-1}).

The chemical activation is carried out by impregnating small particle-sized biomass into an aqueous solution containing an activating agent such as $MgCI_2$ or KOH at a ratio of 2:1 (biomass: chemical). This chemical method can also be modified to related literature according to the purpose of the study (Tseng, 2006; Cazetta et al., 2011; Sultan et al., 2020; Wahlström et al., 2020).

An adsorption experiment was conducted in batch conditions with 250 mL glass beakers containing 100 mL of dyes in an aqueous solution. Using the prepared 1000 mg L^{-1} stock solution, dilutions were prepared in three different concentrations. Makeswari et al. (2016) determined in their dye removal study that the absorption rate decreased with the increase in the initial dye concentration. The concentration ranges were adjusted taking this study into account. 1 g of dry algal powder was added to each beaker containing 100 mL of dye solution and agitated continuously using an orbital shaker. Beakers were shaken on an incubator at 100 rpm and 26 ºC. The effect of suitable time interval was tested at 60, 90, and 120 min, then centrifuged for 10 min at 2000 rpm. pH was kept constant between 7 and 8. All dye experiments were carried out at room temperature. Fuchsin and nigrosin concentrations were assayed colorimetrically using a spectrophotometer (Varian Cary 50). Spectrophotometric wavelengths of 544 nm and 570 nm were used for fuchsin and nigrosin, respectively. The initial pH was calibrated with concentrated 0.1 M HCl or 0.1 M NaOH.

The absorption capacity and its effect are given in the Equations 1 and 2, respectively.

$$qe\ (\mathrm{mg/g}) = \frac{(\mathrm{CO} - \mathrm{Ce}) * \mathrm{V}}{\mathrm{m}} \quad (1)$$

$$\text{Dye removal efficiency (\%)} = \frac{(\text{CO} - \text{Ce}) * 100}{\text{CO}} \quad (2)$$

Co and Ce (mg L^{-1}): Initial and final concentrations of dyes in the solution,
V (L): The volume of the solution,
m (g): The mass of the adsorbent. The percentage of dye removal can also be displayed by the dye removal efficiency (Denniz and Saygideger, 2011).

All experiments in the study were performed with 3 repetitions, and the results were expressed as an average value.

2.3. Total phenolic compounds (TPC), total flavonoids content (TFC), and antioxidant activity

TPC was determined with Folin-Ciocalteu and gallic acid (1 mg mL^{-1}) as a reagent and standard, respectively, according to the described method by Saeed et al. (2012). Briefly, 50 µL of the crude extract (5 mg) in 150 µL of the methanol at different concentrations was taken and followed by the addition of 250 µL of Folin-Ciocalteu diluted in a 1:1 ratio was added and left for approximately 1 min. Then, 1 mL of Na_2CO_3 (10%) was added, and the total reaction volume was completed to 5 mL with ddH_2O, with the remaining 95 min in darkness for the reaction. After the reaction period was completed, the color change in the reaction solution was determined at 760 nm absorbance and the results were expressed as mg equivalents per gram of gallic acid equivalents (mg GAE g^{-1}) by comparing it with the standard absorbance subjected to the reaction under the same conditions.

The TFC was determined by the colorimetric method with minor amendments, using ddH_2O solutions of 10% $AlCl_3$ and 1 M sodium acetate as a reagent. Briefly, 100 µL of extract was taken and followed by the addition of 1700 µL of methanol, 100 µL of $AlCl_3$, and 100 µL of sodium acetate. After, it was incubated at room temperature for 30 min for the reaction, then, the absorbance measurements were completed at 415 nm with quercetin (1 mg mL^{-1}) as a standard prepared in different concentrations under the same reaction conditions. TFC of the extract was expressed as quercetin equivalents per gram of sample (mg QE g^{-1}) (Zhishen et al., 1999).

Antioxidant activity was assayed using the standard method with a different concentration of 1,1-diphenyl-2-picrylhydrazyl (DPPH) (0.002%, 2 mg 100 mL^{-1} in methanol). Briefly, different methanol extracts (0.1-1 mg mL^{-1}) of the *U. lactuca* (5 mg stock solution) were added to 150 µL methanol; then, 2 mL of DPPH was added to each tube and kept for 40 min at a room temperature in the darkness and the absorbance was measured at 517 nm. The various ascorbic acid concentrations from the stock solution (1 mg mL^{-1}) were tested as a standard at the same experiment conditions for comparing the results. Thereafter, DPPH radical scavenging activity was determined at 517 nm, and the antioxidant activity was calculated using the following equation:

DPPH free Radical scavenging activity:

$$RSA\% = \frac{\text{ControlA517} - (\text{SampleA517} - \text{BlankA517})}{\text{ControlA517}} X100 \quad (3)$$

ControlA517: The absorbance of the control (DPHH-without sample),
SampleA517: The absorbance of the test sample (the sample and DPPH),
BlankA517: The absorbance of the sample blank (Sample without the DPPH) (Huang et al., 2005; Prakash et al., 2007).

2.4. Adsorption isotherms

The adsorption isotherm and the Langmuir and Freundlich isotherm models supported the results obtained. These adsorption isotherms are two popular isotherm models frequently preferred in studies that provide information about the adsorption capacity among the adsorbate and the adsorbent. The Langmuir isotherm is a model that predicts the monolayer absorption (homogeneous points) of a solute from a liquid solution without the adsorbate movement on the surface plane (Langmuir, 1916). In other words, this isotherm was used to estimate the highest layered adsorption capacity. The linear form of the Langmuir isotherm is represented in the Equation 1 (Langmuir, 1917).

$$qe = \frac{qmkLCe}{1 + kLCe} \quad (4)$$

qe (mg g^{-1}): The equilibrium amount of biosorbent,
qm (mg g^{-1}): The maximum capacity for sorption at equilibrium,
kL (L mg^{-1}): Langmuir constant represents a coefficient related to the affinity.
Ce (mg L^{-1}): Dye concentration at balance.

R^2, the regression correlation coefficient value, is used to determine whether the isotherm model is suitable. R^2 value close to 1 indicates that the isotherm model is suitable. The kL constant factor is the separation factor of the Langmuir isotherm, which indicates the suitability of the absorption process. If the kL value is between 0 and 1, it is suitable; if it is greater than 1, it is not appropriate (kL = 1 linear; kL = 0 irreversible). Ce/qe and Ce graphs were created using experimental data to determine the regression coefficient and Langmuir isotherm model parameters.

A model known as the Freundlich adsorption isotherm assumes that the dye molecule is adsorbed on the solid surface in several layers or heterogeneous spots. When all of the surface's points are filled with dye molecules, the system reaches equilibrium, forming an isotherm (Freundlich, 1906). The following equation provides the Freundlich adsorption isotherm in its linear form.

$$qe = kFCe^{n} \quad (5)$$

kF (L mg^{-1}): Freundlich constant related to the adsorption capacity of adsorbent,
n: The intensity of biosorption (the adsorbate's affinity for the adsorbent),
Favorable sorption is indicated by a value of 1/n that falls between 0 and 1 (El Qada et al., 2006).

3. Results and Discussion

3.1. Effect of initial dye concentrations on *U. lactuca* dye uptake

To determine the effect of dye concentration on *U. lactuca*, pH, temperature, and time were kept constant at 7−8, 26 °C, and 120 min., respectively. The removal of fuchsin by both modified and non-modified *U. lactuca* increased in correlation with the increase in dye concentration after exposure to three different dye concentrations. However, high nigrosine removal was found at 50 mg L^{-1} dye concentration (Figure 1).

For non-modified *U. lactuca* biomass, maximum fuchsin removal was determined as 420.5±1.072 mg g^{-1} dw at 100 mg L^{-1} dye exposure. In comparison, it was defined as 104.14±0.193 mg g^{-1} dw at 50 mg L^{-1} dye concentration for nigrosin. A decrease in dye removal was observed in *U. lactuca* biomass modified with $MgCI_2$. Dye removal decreased from 270.12 ±2.570 mg g^{-1} dw to 234.01±1.855 mg g^{-1} dw for fuchsin and from 85.69±0.977 mg g^{-1} dw to 75.12±0.814 mg g^{-1} dw for nigrosin on average compared to non-modified biomass. On the other hand, an increase (46.29%) in fuchsin removal was determined in KOH-modified *U. lactuca* biomass (average value 395.06±3.455 mg g^{-1} dw). In contrast, a decrease in nigrosin removal (average value 66.41±0.947 mg g^{-1} dw) was observed compared to unmodified biomass. Similar to the results obtained in the study, Salima et al. (2013) obtained maximum uptake of 400 mg g^{-1} and 526 mg g^{-1} for the removal of malachite green and safranin by phosphoric acid-modified *Ulva lactuca* and *Systoceira stricta*, respectively. To obtain an effective biosorbent for dye removal, the pores of the biomass must be formed into a porous structure on the biomass surface by activating agents such as $MgCI_2$, KOH, H_2SO_4, and NaOH. These agents' primary purposes are to eliminate water from the material structures and lower the temperature needed for carbonization. In this way, a bottle-shaped porous structure is obtained on the biomass surface (Bansal et al., 1988; Nahil and Williams, 2012). As a result of the porous structure formed on the biomass surface due to the KOH treatment, the removal of fuchsin, in particular, is higher than that of the nonmodified biomass. This situation has been confirmed by the information given in other studies (Bansal et al., 1988; Nahil and Williams, 2012).

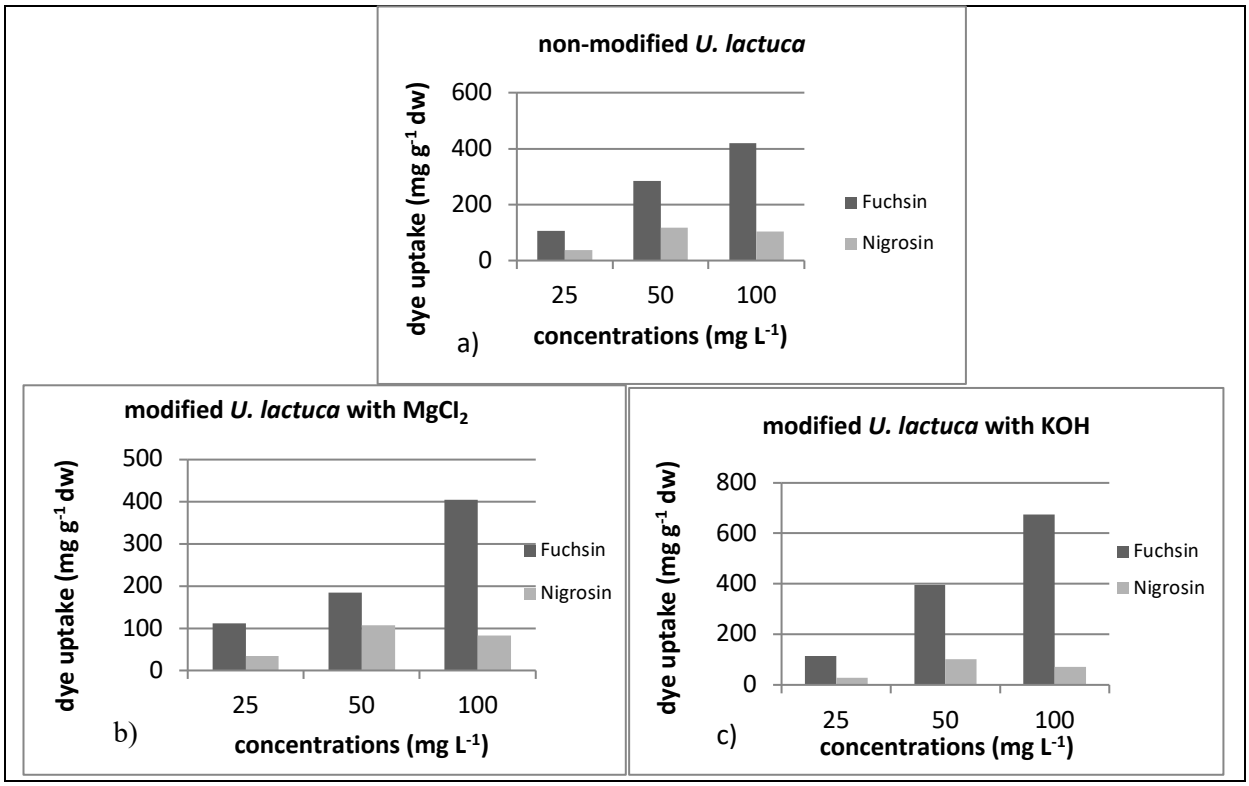

Figure 1. The effect of different concentrations of dye treatments on non-modified (a) and modified (b-c) *U. lactuca* biomass dye removal (mg g $^{-1}$).

3.2. Effect of different contact times on dye removal efficiency

To determine the effect of contact times on *U. lactuca*, pH and temperature were kept constant at 7−8 and 26 °C, respectively. In non-modified *U. lactuca* biomass, fuchsin and nigrosin uptake was exceptionally high at 50 mg L^{-1} dye concentration and 90-120 min dye exposure. Average fuchsin removal was determined as 33.68%, 68.20%, and 60.92% at three different dye concentrations (25, 50, and 100 mg L^{-1}), respectively. Similarly, the average nigrosin removal was determined to be 43.91, 61.17, and 41.43% in non-modified biomass, respectively (Figure 2).

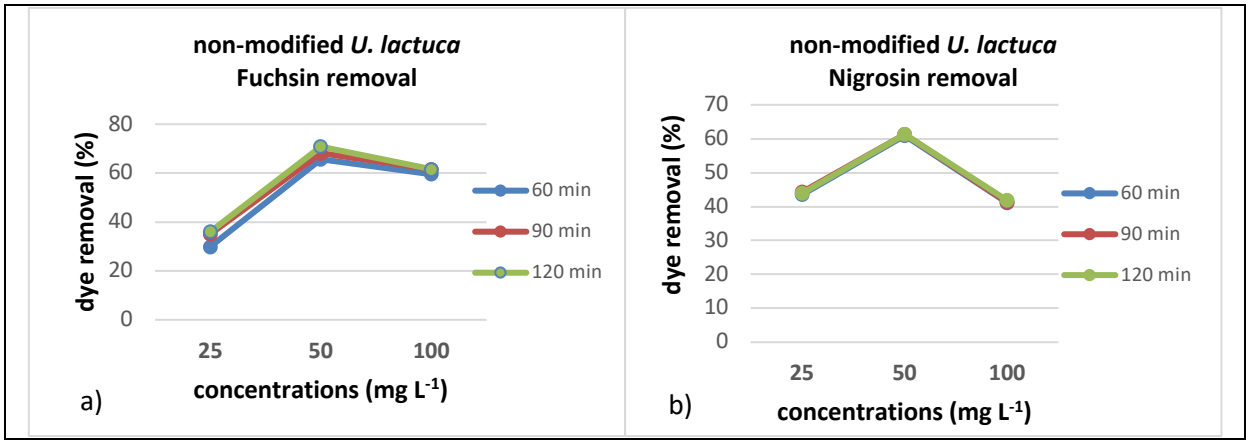

Figure 2. The effect of different contact times on fuchsin (a) and nigrosin (b) removal efficiency (%) of non-modified *U. lactuca*.

The fuchsin removal of *U. lactuca* modified with $MgCl_2$ increased parallel to the increasing time and dye concentration. The average fuchsin removal percentage was determined as 33.78%, 43.22%, and 60.08%, respectively. In nigrosin removal, the highest removal percentage was determined to be 56.23% at a 50 mg L^{-1} dye concentration ratio. Dye removal of *U. lactuca* modified with KOH

was determined to be 99.12% and 98.46%, especially in fuchsin removal at 50 and 100 mg L^{-1} dye concentration exposure, respectively. In nigrosin removal, these values were determined as 35.12%, 54.27%, and 26.13%, respectively (Figure 3). The findings indicate that, for most treatments at low and high concentrations, but not all of them, the ideal contact duration was 120 min, which equates to a sufficient elimination of the dyes. Additionally, it was found that increasing the contact period from 60 to 120 min had no discernible effect on the rates of dye removal for either low or high concentrations of the modified or unmodified *U. lactuca* adsorbent. However, El-Skaily et al. (2006) studied the removal of methylene blue at different concentrations by *U. lactuca* biomass at various contact times, and they found that dye removal stabilized and reached saturation after a contact time of 45 min, after which it remained more or less constant up to 120 min. In another study (Safarik et al., 2024), it was determined that *Ulva rigida* modified with iron oxide had maximum capacity for malachite green (202 mg g^{-1}) and safranin (227 mg g^{-1}) removal among fifteen macroalgae species collected from Saros Bay, Türkiye.

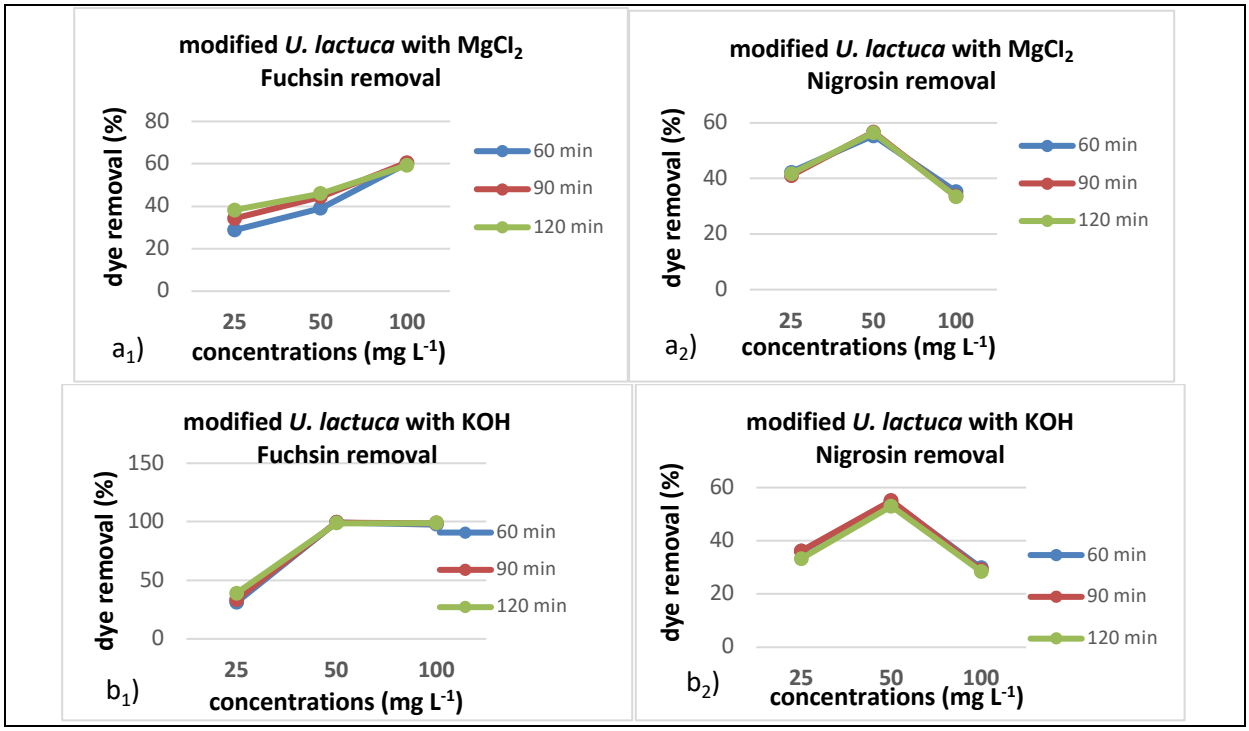

Figure 3. The effect of different contact times on dye removal efficiency (%) of modified *U. lactuca* with $MgCl_2$ (a_1: fuchsin, a_2: nigrosin removal) and with KOH (b_1: fuchsin, b_2: nigrosin removal).

It can be seen that the removal percentage of fuchsin increased from 68.20% to 99.12% on average for *Ulva* biomass modified with KOH at low dye concentration (50 mg L^{-1}) compared to unmodified biomass. This could be because the adsorbent surface has unoccupied functional groups. Active sites may become occupied if the dye concentration rises further. As a result, the algal cell surface becomes saturated, preventing further adsorption. This is confirmed by previous studies (M'sakni and Alsufyani, 2021). In addition, the fact that the absorption capacity of fuchsin, a cationic dye, increased more than that of unmodified biomass as a result of KOH application can be explained by the increase in the binding sites formed in the surface active regions of the adsorbent as a result of modification.

3.3. Effects of $MgCl_2$ and KOH on total phenolic, flavonoid content, and antioxidant activity of modified *U. lactuca*

The present study determined the TPC, TFC, and DPPH radical scavenging activity (RSA%) of the methanol extract from *U. lactuca* (Table 1.). The TPC values were 7.8, 4.6, and 30.40 mg GAE g^{-1} for the non-modified, modified with $MgCl_2$, and modified with KOH of the *U. lactuca,* respectively. Interestingly, the TPC value of *U. lactuca* modified with KOH was the highest and may have shown a synergistic effect in the reactions. On the other hand, the TPC amount of *U. lactuca* that was not treated

at all was determined to be consistent with previous studies. Tong et al. (2020) determined the TPC value of the distilled water extract of *U. lactuca* as 7.72. mg GAE g^{-1}. In the same study, the RSA value of *U. lactuca* was approximately 85%, which is considerably lower than our values (98.13%). However, although the TPC value of *U. lactuca* treated with KOH was high (30.40 mg GAE g^{-1}), the RSA value decreased considerably (38.70%). The reason for this is not understood; perhaps DPPH, which was used as a reagent to determine antioxidant activity, may have reacted negatively with KOH. As a result, there was no positive correlation between the TPC value and RSA value of *U. lactuca* extract treated with KOH. In a recent study, the amount of TPC (2.4 mg g^{-1}) was lower than the value obtained in this study. The same survey clearly emphasizes that the amount of TPC can be affected by the extraction process, the organic solvent used, and the extraction time (Pappou et al., 2022). It was also observed that the highest RSA value was obtained in 0.11 mg mL^{-1} of *U. lactuca* methanol extract, which is much lower than in earlier research (Farasat et al., 2014; Tong et al., 2020; Ouahabi et al., 2024). The organic solvent used in extraction significantly affects the determination of primary and secondary metabolites and pigments of macroalgae. In a study, the ratio of Chlorophyll *a*, carotenoids, and total phenolic compounds was higher in ethyl acetate extracts obtained from *U. lactuca* (Hidayati et al., 2020).

Table 1. The effect of $MgCI_2$ and KOH treatments on TPC, TFC, and antioxidant activities of *U. lactuca*.

	TPC* (mg GAE g^{-1})	**TFC (mg QE g^{-1})**	**RSA (%)**
Non-modified *U. lactuca*	7.8±0.015	0.74±0.00025	98.13±2.95
Modified *U. lactuca* with $MgCI_2$	4.6±0.050	1.47±0.00050	93.90±0.42
Modified *U. lactuca* with KOH	30.40±2.14	0.82±0.00062	38.70±0.05

*TPC, TFC, and RSA refer to Total Phenolic Contents, Total Flavonoid Contents, and DPPH Free Radical Scavenging Activity, respectively.

Besides, total flavonoid amounts were determined between 0.74 and 1.47 (mg QE g^{-1}), and the highest TFC value was determined in *U. lactuca* treated with $MgCI_2$. Interestingly, these amounts are much lower than the results obtained in previous studies from the literature (Prasedya et al., 2019; Benítez García et al., 2020; Ouahabi et al., 2024). Many studies have been conducted on the health benefits of *U. lactuca*; these benefits are generally based on the amounts of proteins, fatty acids, phenolics, flavonoids, polysaccharides, minerals, and other bioactive components. In addition, the antimicrobial, antioxidant, and anti-inflammatory properties of *U. lactuca* increase due to these compounds, especially flavonoids (Shobier and El Ashry, 2021; Madhusudan and Baskaran, 2023; Putra et al., 2024). As a result, it is thought that the differences in TPC, TFC values, and antioxidant activities in our research may be due to differences in extraction method, organic solvent, time, habitat, and period in which *U. lactuca* grows.

3.4. Adsorption isotherms

Langmuir and Freundlich's isotherms were used to indicate the efficiency of the dye activation process on *Ulva lactuca*. The Langmuir Model is a model that predicts the monolayer absorption (homogeneous points) of a solute from a liquid solution without the adsorbate movement on the surface plane (Langmuir, 1916). The Freundlich isotherm model describes the binding occurring in a heterogeneous adsorption site (multiple layers). Ce/qe and Ce graphs were created to determine the regression coefficient and Langmuir isotherm model parameters using experimental data. In qe and Ce graphs were created to determine the Freundlich isotherm model parameters. Figure 4 shows the Langmuir and Freundlich isotherm models for dye adsorption on unmodified *U. lactuca* biomass.

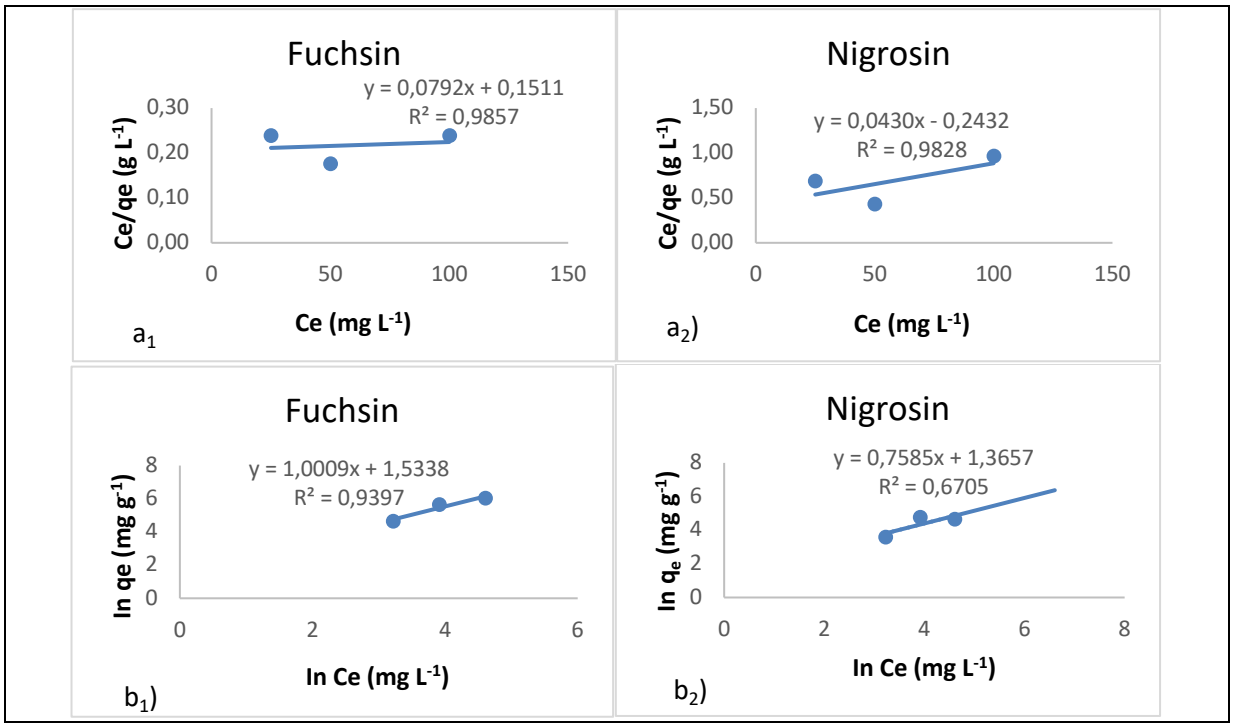

Figure 4. Langmuir (a_1/a_2) and Freundlich (b_1/b_2) Isotherms for the fuchsin and nigrosin (100 mg L^{-1}) dye onto non-modified *U. lactuca*.

Because the Langmuir isotherm model had the highest regression coefficient (R^2 = 0.9857 and 0.9828, respectively), the results demonstrated that it was the best model for the adsorption of fuchsin and nigrosins onto non-modified *U. lactuca*. According to the Langmuir model, which was used to fit the adsorption data, the monolayer adsorption capacity for the removal of fuchsin and nigrosin was 420.50 and 104.14 mg g^{-1}, respectively. However, as it has the highest regression coefficient (R^2 = 0.9832 and 0.9035 for modified biomass with $MgCl_2$; 0.9462 and 0.9492 for modified biomass with KOH), it has been demonstrated that the Freundlich isotherm model is the most suitable model for the removal of dyes onto modified *U. lactuca* biomass (Figure 5 and 6; Table 2).

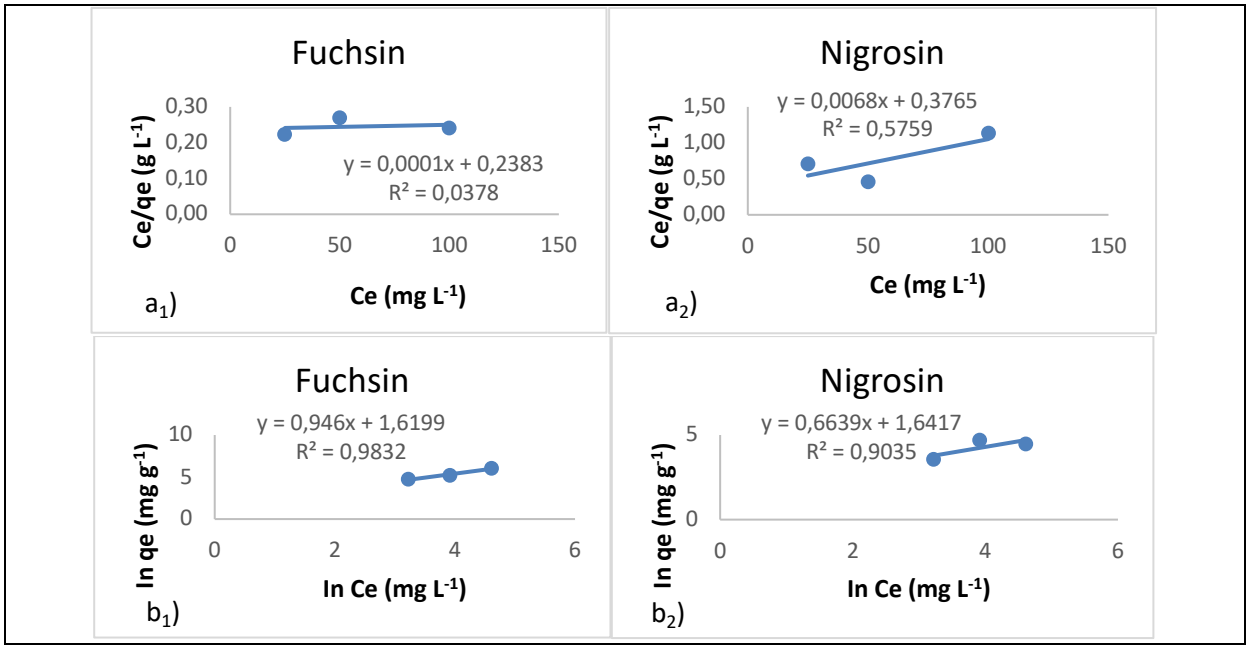

Figure 5. Langmuir (a_1/a_2) and Freundlich (b_1/b_2) Isotherms for the fuchsin and nigrosin (100 mg L^{-1}) dye onto modified *U. lactuca* with $MgCl_2$.

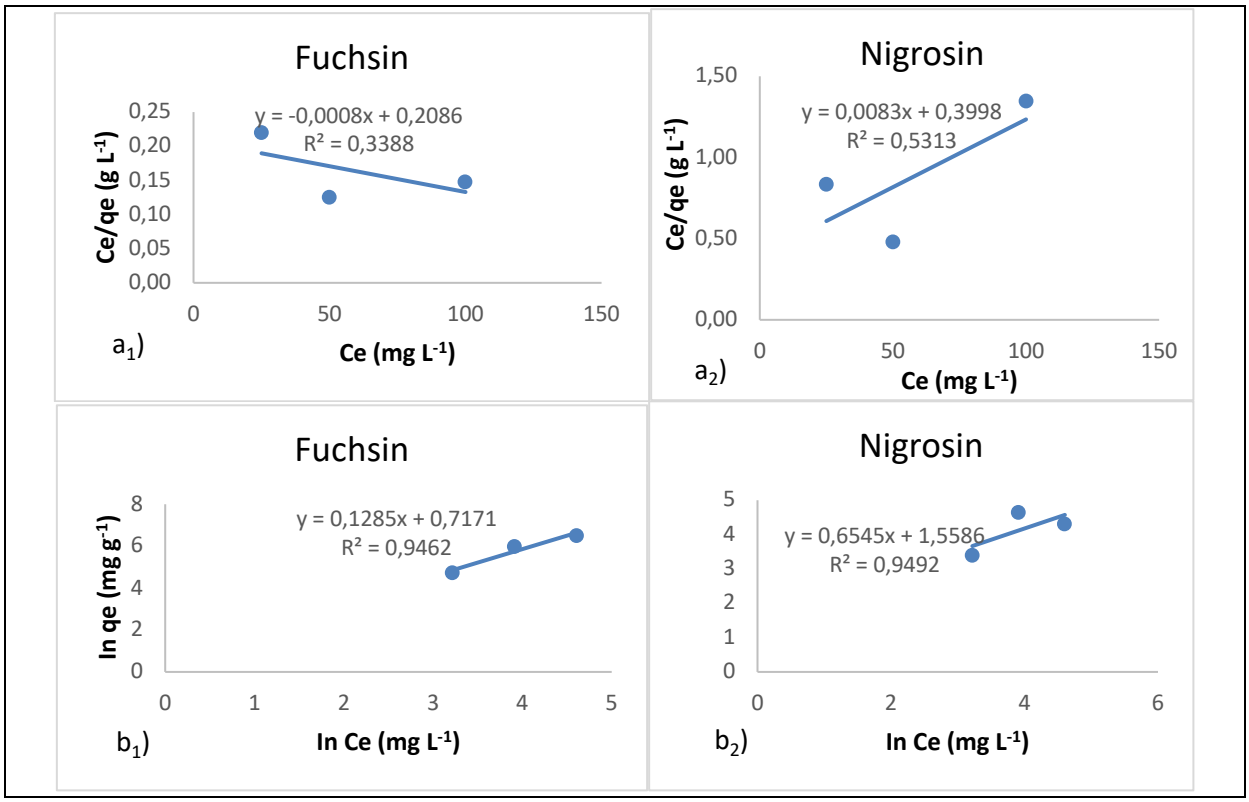

Figure 6. Langmuir (a_1/a_2) and Freundlich (b_1/b_2) Isotherms for the fuchsin and nigrosin (100 mg L^{-1} cons.) dye onto non-modified *U. lactuca* with KOH.

Table 2. Isotherms for the adsorption of fuchsin and nigrosin (100 mg L^{-1} cons.) dye onto modified and non-modified *U. lactuca*

	Adsorption Isotherm Constants	Fuchsin	Nigrosin
Non-modified *U. lactuca*	**Freundlich**		
	1/n (L g^{-1})	0.759	1.001
	k_F (L g^{-1})	0.65	0.55
	R^2	0.9397	0.6705
	Langmuir		
	q_m (mg g^{-1})	420.50	104.14
	k_L (L mg^{-1})	0.52	0.16
	R^2	0.9857	0.9828
Modified *U. lactuca* with $MgCl_2$	**Freundlich**		
	1/n (L g^{-1})	0.9460	0.6639
	k_F (L g^{-1})	0.5839	0.4043
	R^2	0.9832	0.9035
	Langmuir		
	q_m (mg g^{-1})	414.12	107.93
	k_L (L mg^{-1})	4.1963	0.018
	R^2	0.0378	0.5759
Modified *U. lactuca* with KOH	**Freundlich**		
	1/n (L g^{-1})	0.1285	0.6545
	k_F (L g^{-1})	0.1791	0.4199
	R^2	0.9462	0.9492
	Langmuir		
	q_m (mg g^{-1})	674.71	104.69
	k_L (L mg^{-1})	0.003	0.020
	R^2	0.3388	0.5313

As shown in Table 2, the maximum adsorption capacity of fuchsin was determined to be higher than the adsorption of nigrosin on unmodified *U. lactuca*. Similarly, when previous studies were examined, it was stated that cationic dyes were absorbed faster than anionic dyes (Gong et al., 2005; Namane et al., 2005; M'sakni and Alsufyani, 2021). The negatively charged carboxyl group in the cell wall molecular structure of *U. lactuca* is one of the main functional groups in cationic dye adsorption (Gong et al., 2005). For this reason, it was predicted that the cationic dye would be absorbed more than the anionic dye by the adsorbent. In addition, dye removal studies conducted on *Ulva lactuca* determined that biosorption showed a monolayer, i.e., homogeneous absorption, and was by the Langmuir isotherm model (M'sakni and Alsufyani, 2021). Removal of cationic organic dye from aqueous solution by chemical and pyrolysis-activated *U. lactuca* biomass by the other studies conducted is consistent with the results of this study. However, it was determined that the surface biosorption of the adsorbent modified with $MgCl_2$ and KOH was heterogeneous, and the adsorption was multilayered for the two dyes determined, i.e., it was more suitable for the Freundlich model. As indicated in Table 2, the 1/n value between 0 and 1 and the R^2 value between 0.9 and 1 support the result.

Conclusion

This study investigated the removal of fuchsin and nigrosin synthetic dyes by using nonmodified and modified *U. lactuca* with $MgCl_2$ and KOH. According to the results, the sample prepared at 60 min contact time and 50 mg L^{-1} dye concentration had the highest adsorption efficiency for fuchsin on *U. lactuca* biomass treated with KOH (99.40%). In addition, the TPC value of *U. lactuca* modified with KOH was the highest and may have shown a synergistic effect in the reactions. The RSA value decreased significantly. No positive correlation was found between the TPC and the RSA values of the *U. lactuca* extract treated with KOH. As a result, it is thought that the differences in TPC, TFC values, and antioxidant activities in our study may be due to differences in extraction method, organic solvent, time, habitat, and the period in which *U. lactuca* was grown. The most suitable adsorption isotherm for *U. lactuca* biomass treated with $MgCl_2$ and KOH was determined as the Freundlich isotherm model. These results demonstrated that applying KOH on *U. lactuca* is an effective, non-polluting, and economical process, especially for removing fuchsin cationic dye from an aqueous solution.

Ethical Statement

Ethical approval is not required for this study because it does not include any studies on human or animal subjects.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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This study is performed by both authors. Both authors contributed 50%.

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Research Article

Antifungal Activity of Endophytic *Bacillus* spp. Bacteria and Its Effect on Root and Coleoptile Length during Germination Period

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Abstract: In this study, endophytic bacteria were isolated from roots and crowns of rosemary (*Rosmarinus officinalis* L.), olive (*Olea europaea* L.), and loquat (*Eriobotrya japonica* L.) plant samples. The morphological and physiological properties of nine isolated endophytic bacteria were determined. All isolates were identified as Gram-positive, oxidase-positive and catalase-positive. Amylase, cellulase, and carbohydrate tests gave positive results. Antagonistic activities of the isolates against fungal pathogens varied between 85.7% and 52.9% against *Fusarium culmorum* and between 86.0% and 65.1% against *Neoscytalidium dimidiatum*. BMBA2 isolate gave the best results both in Petri dish antagonistic activity and in wheat seed germination in terms of root length and coleoptile length. BMBA2 isolates gave the best results with a coleoptile length of 7.58 cm and root length of 8.33 cm. In wheat seeds treated with *F. culmorum* and bacteria, the BMBA2 isolate gave the best result with a coleoptile length of 6.98 and a root length of 7.30 cm. For the identification of bacteria, in vitro BiBA1 and ND3BA were determined as *Bacillus amyloliquefaciens* subsp. *plantarum*; BiBA2 and YDBA as *Bacillus subtilis*; NDBA, ND2BA, BMBA1, BMBA2, and BMBA3 as *Bacillus mojavensis*. Since this is the first study to use endophytic *Bacillus mojavensis* as a biological agent against *F. culmorum* and *N. dimidiatum* pathogens, the results obtained from this study are thought to be important and promising in terms of application.

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1. Introduction

Wheat production increased by 11.4% compared to the previous year and reached 22 million tons of wheat. Wheat production accounts for 28.3% of the production of cereals and other plant products (TURKSTAT, 2023). Wheat is an annual plant and a staple food widely used in many countries of the world. Unfortunately, wheat grown under field conditions is exposed to various factors such as biotic and abiotic factors such as high salt concentrations, water scarcity, and high temperatures that may affect it at different developmental stages (Ghazala et al., 2023).

In recent times, agriculture has been exposed to biotic factors originating from pathogens, stress, and abiotic factors such as salinity, drought, and extreme temperatures. These problems have been exacerbated by the continuous increase in population and the combined effects of extreme climate events

such as drought, floods, and high temperatures have also led to significant decreases in crop yields. It has also faced problems such as excessive use of chemical pesticides and fertilizers and the reduction of biodiversity in agriculture. In addition, the excessive use of chemical compounds has caused pathogen resistance and environmental pollution (Bódalo et al., 2023). The use of synthetic pesticides has increased since the 1940s, and pesticides damage non-target plants and the environment and can cause adverse health effects (Hernández et al., 2013; Bernardes et al., 2015; Carvalho, 2017; Tudi et al., 2021). Therefore, it is necessary to develop alternative methods to reduce the resistance of agricultural products to chemical compounds.

The *Neoscytalidium dimidiatum* pathogen has been widely distributed in different plants and has spread to 37 countries. The presence of this pathogen has been detected in various regions, including where *N. dimidiatum* pathogen was observed, 30 in Türkiye (Akgül et al., 2019; Güney et al., 2021; Alkan et al., 2022; Özer et al., 2022; Derviş and Özer, 2023), 47 in Iran (Ahmadpour et al., 2023; Esmaeil et al., 2023), 14 in Brazil (Monteles et al., 2020; Mello et al., 2021) 16 in South America (Arrieta-Guerra et al., 2021; Espinoza-Lozano et al., 2023), 18 in Malaysia and 6 in China (Ismail et al., 2021; Sha et al., 2022). The United States, particularly in California, displays a substantial presence, with 38 occurrences (French, 1989; Farr et al., 2005). Middle Eastern countries have also documented the detection of this pathogen in 6 different plants in Iraq (Al-Tememe et al., 2019; Abdulrahman and Haleem, 2023) and 9 in Oman (Al-Sadi et al., 2014). The distribution indicator of this pathogen shows that *N. dimidiatum* poses a global threat. This pathogen, seen in different examples in Türkiye, emphasizes that it is an important pathogen that needs to be addressed in the region (Derviş and Özer, 2023). Symptomatic expression is particularly pronounced in perennial plants such as dragon fruits (Chuang et al., 2012; Espinoza-Lozano et al., 2023; Khoo et al., 2023; Salunkhe et al., 2023), citrus fruits (Polizzi et al., 2009; Mayorquin et al., 2016), grapevines (Al-Saadoon et al., 2012; Rolshausen et al., 2013; Oksal et al., 2019), pines (Türkölmez et al., 2019a), stone fruits (Nouri et al., 2018; Oksal et al., 2020), *Ficus* species (Güney et al., 2022), pistachios (Derviş et al., 2019), and willows (Hashemi and Mohammadi, 2016), leading to reduced yields and shorter life spans (Türkölmez et al., 2019b). It has also been stated that *N. dimidiatum* has the potential to spread from seeds, soil, and air and to persist in the soil (Çiftçi et al., 2023; Güney et al., 2023).

Investigating the potential of environmentally friendly biological control agents and environmentally friendly biopesticides in controlling *N. dimidiatum* and related pathogens may provide sustainable alternatives for disease management. The use of biological control agents with broad-spectrum bioactive metabolites is recommended as an alternative to chemical pesticides against fungal phytopathogens. It has been reported that various bacterial genera such as *Bacillus* sp., *Pseudomonas* sp., and *Streptomyces* sp. are widely used in biocontrol studies (Albayrak, 2019; Singh et. al., 2024). It has been observed that the *F. culmorum* pathogen causes serious decreases in both yield and quality in wheat production regions, thereby further worsening the difficulties faced by the wheat production sector (Gökçe and Kotan, 2016; Bozoğlu et al., 2022). Güler Güney et al. (2024) reported that the endophytic *Stenotrophomonas rhizophila*, *Pseudomonas putida,* and *Pseudomonas orientalis* (EY1+EM9+MM21) combination gave the best results against *F. culmorum* in terms of disease severity, plant height, fresh weight, dry weight, root fresh weight and root dry weight in wheat plants. They reported that various endophytic bacteria can be used against *F. culmorum*.

Bacteria have different mechanisms to manage plant biocontrol, such as antagonistic activities, production of secondary metabolites or some lytic enzymes, competition, and induction of plant resistance. It is known that most endophytes are effective in plant growth and that without them, plants have difficulty coping with biotic and abiotic stresses (Santoyo et al., 2016). Endophytes play critical roles in plant ecology, evolution, and development, including nitrogen fixation (Carvalho et al., 2014), growth promotion, and production of antimicrobial substances (Singh et al., 2017), and are also considered important in increasing plant resistance to plant stress (Li et al., 2019) and remediating environmental pollution (Barik et al., 2021). Endophytes (bacteria and fungi) constitute a group of endosymbiotic microorganisms that are ubiquitous in nature. It is known that they live in the plant endosphere and do not show any significant harmful effects on any host plant (Compant et al., 2021; Hazarika et al., 2021). Studies have stated that endophytes facilitate nutrient uptake, promote growth, are effective against pathogenic microorganisms, and activate the defense systems of plants by increasing the production of secondary metabolites (Aeron et al., 2020; Patel et. Al. 2024).

Bacillus is a Gram-positive, endospore-forming, rod-shaped, aerobic, or facultative anaerobic bacterium (Logan and De Vos, 2009). *Bacillus* species are among the most widespread bacteria in nature and constitute one of the most important microbial sources in studies of plant-endophyte interactions (Izumi, 2011). Endophytic *Bacillus* species are generally preferred over other bacteria in biological control studies due to their ability to produce a significant number of secondary metabolites and hydrolytic enzymes and their biological and environmentally friendly properties (Sari et al., 2006).

Bacillus sp. appears to be more effective as a biological control agent due to its resistance to adverse environmental conditions, and its antagonistic effect against a wide range of pathogens (Shafi et al., 2017). According to the latest studies, *B. mojavensis, B. subtilis, B. amyloliquefaciens, B. thuringiensis,* and *B. polymyxa* species appear to be effective as biological agents (Bacon and Hill, 1996; Bacon and Hinton, 2002; Snook et al., 2009; Wang et al., 2019; Roy et al., 2021). *Bacillus mojavensis* is known to produce certain biosurfactants such as surfactin A, phenytoin, and iturin derivatives, as well as hydrolytic enzymes that play a role in antifungal activities. *Bacillus mojavensis* is preferred as a biological control agent due to its ability to inhibit many pathogens (Xiao et al., 2009; Jasim et al., 2016).

In this study, the antifungal activities of Endophytic *Bacillus* sp. isolates against *F. culmorum* and *N. dimidiatum* pathogens and the effects on some growth parameters of wheat seeds inoculated with *F. culmorum* and endophytic bacteria were investigated and effective results were obtained.

2. Material and Methods

2.1. Isolation of endophytic bacteria

Rosemary, olive, and loquat plant samples (1-2 cm long) were disinfected from the root and root collar parts and left to grow in Nutrient agar (NA)-containing media (Zvyagintsev, 1991). All representative isolates, bacteria taken from 24-48 hours fresh cultures for other studies, were cultured on NA-containing slant agar media. For long-term storage, all representative isolates were re-purified into NA medium and stored at –80 °C in 30% glycerol for *in vitro* tests.

2.2. Procurement and storage of pathogenic isolates

Fusarium culmorum and *N. dimidiatum* pathogens were obtained from the Mardin Artuklu University laboratory. Fungal pathogens were grown in Petri dishes on Potato dextrose agar (PDA) medium at 24 °C and stored in a tilted agar medium containing PDA at +4 ºC until used.

2.3. Disinfection of seeds

The seeds used in the experiment were surface disinfected before the experiment. The seeds were kept in sdH_2O for 5 minutes, 75% ethanol for 30 seconds, and 0.5% NaOCl for 1 minute to perform surface disinfection. Then, they were washed twice in sdH_2O and dried in a laminar cabinet. They were placed in sterile containers (Gargouri-Kammoun et al., 2009).

2.4. Examination of morphological characteristics of bacteria

Gram staining: Gram staining of isolates was performed (Demirbağ and Demir, 2005). Catalase: Isolates were grown in nutrient broth medium at 28 °C for 2 days. Foaming status will be checked by adding 3% H_2O_2 to the isolates. If there is foaming, it is evaluated as positive (+), if not, it is evaluated as negative (-) (Holt et al., 1994). Oxidase: A loopful of freshly developed samples from bacterial isolates were taken and spread on blotting paper, then oxidase was poured and color change was observed. Blue color formation was concluded as positive (Holt et al., 1994).

2.5. Carbohydrate tests

The growth of the isolates was evaluated at 28 °C for 3, 7, and 14 days after being separately filtered through a 0.45 µm filter into sterile mineral salt broth according to the method of Ji and Wilson (2002).

2.6. Enzymatic activity

In all enzyme experiments, cultures of the isolates prepared according to Mc Farland No. 5 were used. All enzyme experiments were performed with 3 replicates. Amylase (Hydrolysis of Starch): Isolates were streaked onto Petri dishes containing starch agar and incubated at 28 °C for 2 days. Lugol solution was dropped on the colonies that developed on the medium and the formation of a bright color was evaluated as positive for starch hydrolysis, and the formation of a blue-black color was evaluated as negative (Egamberdieva et al., 2008). Cellulase: 1 ml was taken from the sterilized B and D (Solution B: $MgSO_4.7H_2O$ 1M; Solution D: $CaCl_2$ 7.5% (V/W) solutions and added to the sterilized A+C (Solution A: NaCl 0.25 g, carboxymethylcellulose 2.5 g, K_2HPO_4 1.5 g, distilled water 400 ml; Solution C: Na_2HPO_4 3 g, yeast extract 0.5 g, glycerol 2.5 g, NH_4Cl 0.5 g, agar 6.5 g, distilled water 100 ml) solutions. Isolates were subjected to streaking onto this composite medium and were subsequently incubated at 28 °C for a duration of 96 hours. At the end of incubation, 0.1% Congo red solution was dropped on the colony and kept for 20 min. Petri dishes were washed with 1 N NaCl solution. Those showing a clear zone around the colony were evaluated as cellulase activity positive (Egamberdieva et al., 2005).

2.7. Antagonistic activity

The effects of endophytic bacterial isolates with detected antagonistic effects against *F. culmorum* and *N. dimidiatum* pathogens were performed in vitro in Petri dishes. The antagonistic activities of bacteria against pathogens were determined. The antagonistic activity of bacteria in Petri dishes against pathogens was calculated according to the formula below (Ahmad et al., 2008).

$$\%RI = R\text{-}r / R \times 100 \quad (1)$$

R: : Development of pathogenic fungus on the bacteria-free side
r : Development of pathogenic fungus towards bacteria
%RI : Inhibition rate

2.8. Effect of isolates on wheat seed germination period characteristics and examination of their effect against *Fusarium culmorum* pathogen

Two different applications were examined for their effect on seed germination and growth as well as their effect against the *F. culmorum* pathogen. In the first application, 144 wheat seeds were used in an endophytic bacterial suspension (10^7 CFU ml^{-1}) with 4 wheat varieties in each Petri dish and 36 Petri dishes (9 × 4) for each isolate and four replicates. For the control, 160 wheat seeds and 40 Petri dishes were used with 16 seeds (4 × 4) by applying distilled water to the seeds. Wheat seeds were sterilized and dried in a hood; dried sterilized seeds were placed on a sterile agar plate for 24 h at 21 °C to allow germination. Embryos were then inoculated with endophytic bacteria suspension (Simons et al., 1996).

In the second application, in order to examine its effect on the *F. culmorum* pathogen, only *F. culmorum* was planted in Petri dishes with 4 replicates as control, and wheat seeds in other Petri dishes were inoculated with nine bacteria, and *F. culmorum*, and in order to investigate their effectiveness against the disease, 144 wheat seeds (9*4*4) were contaminated with *F. culmorum* in 40 Petri dishes, and 16 wheat seeds (4*4) were left as control. Wheat seeds were sterilized and dried in a hood; dried sterilized seeds were placed on a sterile agar plate for 24 h at 21 °C to allow germination. Embryos were then inoculated with *F. culmorum* by adding 10 ml of a conidial suspension (3×10^5 spores ml^{-1} H_2O) to each embryo (Jaber, 2018).

2.9. Statistical analysis

Antagonistic activities of endophytic bacteria and statistical analyses of data obtained from seed germination experiments in Petri dishes were evaluated according to the JMP program Tukey's Test Method. The means and applications were considered significant when $P<0.01$. Differences analyses of groups were subjected to one-way ANOVA t-test analysis of variance according to Tukey's HSD test.

3. Results and Discussion

3.1. Examination of morphological characteristics and phenotypic tests of bacteria

Nine of the most effective isolates were selected in the endophytic bacteria isolation process. All endophytic bacteria gave positive results for oxidase and catalase. All isolates showed growth in carbohydrate tests. In enzyme tests, all were evaluated positive in amylase and cellulase tests (Table 1, Figure 1).

Table 1. Phenotypic tests of endophytic bacteria

Isolates	Oxidase	Catalase	Amylase	Cellulase	Carbohydrates (glucose, fructose, mannitol, maltose, m-inositol
BiBA1	+	+	+	+	+
BiBA2	+	+	+	+	+
NDBA	+	+	+	+	+
ND2BA	+	+	+	+	+
ND3BA	+	+	+	+	+
YDBA	+	+	+	+	+
BMBA1	+	+	+	+	+
BMBA2	+	+	+	+	+
BMBA3	+	+	Z	+	+

+: Positive; Z: Weak.

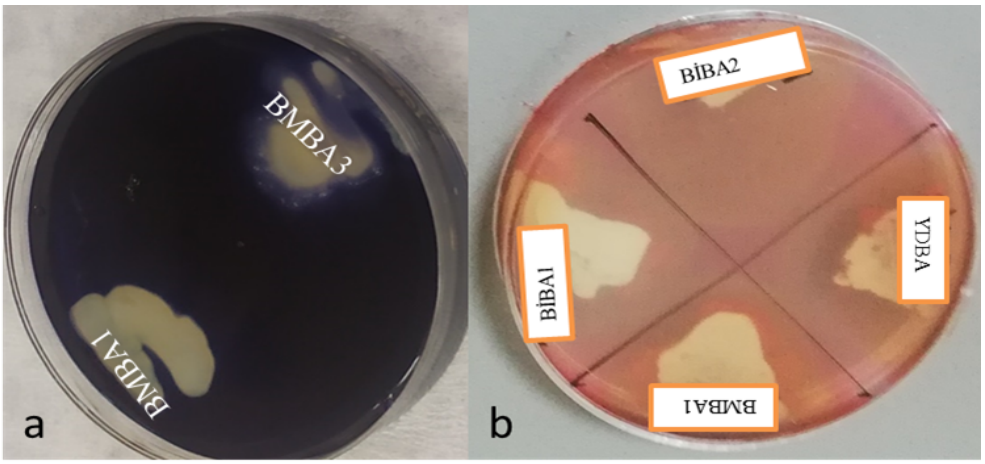

Figure 1. a) Amylase activity of endophytic bacteria b) Cellulase activity of endophytic bacteria.

The 9 isolates tested gave promising results. Camele et al. (2019) also found that it is an endophytic bacterium with fungicidal effects against some phytopathogens in their study with *B. mojavensis*. They stated that bioactive secondary metabolites produced by *B. mojavensis* may have promising applications in the agricultural, food industry, and clinical fields (Camele et al., 2019).

3.2. Antagonistic activity

The isolates' effectiveness against *F. culmorum* varied between 85.7 and 52.9%. Inhibition rates against *N. dimidiatum* varied between 85.7 and 65.1%. BMBA2 isolate was selected as the most effective isolate against *F. culmorum* with 85.7%. YDBA isolate was selected as the most effective isolate against *N. dimidiatum* with 86.0% of BMBA2 isolate (Table 2).

Table 2. Antagonistic activities of endophytic bacteria against *Fusarium culmorum* and *Neoscytalidium dimidiatum*

Isolates	**%RI *F.c***	**%RI *N.d***
BiBA1	52.90[h]	65.10[g]
BiBA2	57.10[g]	74.40[f]
NDBA	64.30[e]	81.40[c]
ND2BA	62.10[f]	74.40[f]
ND3BA	71.40[c]	76.70[e]
YDBA	78.60[b]	86.00[a]
BMBA1	65.70[d]	79.10[d]
BMBA2	85.70[a]	83.70[b]
BMBA3	64.30[e]	79.10[d]
Mean	**66.9**	**77.8**
SE	**0.05**	**0.12**

F.c: Fusarium culmorum; N.d: Neoscytalidium dimidiatum; %RI: Inhibition rate SE: Std Error.
There is no statistically significant difference between values.

Endophytic bacteria *F. culmorum* were found to be effective in their antagonistic activities against *N. dimidiatum* (Figure 2, Figure 3). According to the Tukey multiple comparison test, the differences between the means of the effects of the bacteria were found to be significant (Table 2).

Table 3. Variance analysis of the antagonistic activities of endophytic bacteria

Fusarium ***culmorum***					
Sources of Variation	**DF**	**SS**	**MS**	**F Value**	***P***
Isolates	8	3362.48	420.31	43647.58	<0.0001***
Rep.	3	0.0089	0.003	0.3077	0.8196
Error	24	0.2311	0.010		
General	35	3362.72			
Neoscytalidium ***dimidiatum***					
Sources of Variation	**DF**	**SS**	**MS**	**F Value**	***P***
Isolates	8	1216	152	2533.3	<.0001***
Rep.	3	0.72	0.24	4	0.0192
Error	24	1.44	0.06		
General	35	1218.16			

DF: Degrees of freedom SS: Sum of squares MS: Mean of squares.
*** Difference between bacteria according to Tukey LSD Significant according to $\alpha = 0.05$.

According to the one-way analysis of variance (ANOVA) obtained from the observation values, the differences between the antagonistic activities of the bacteria were found to be statistically significant (Table 3).

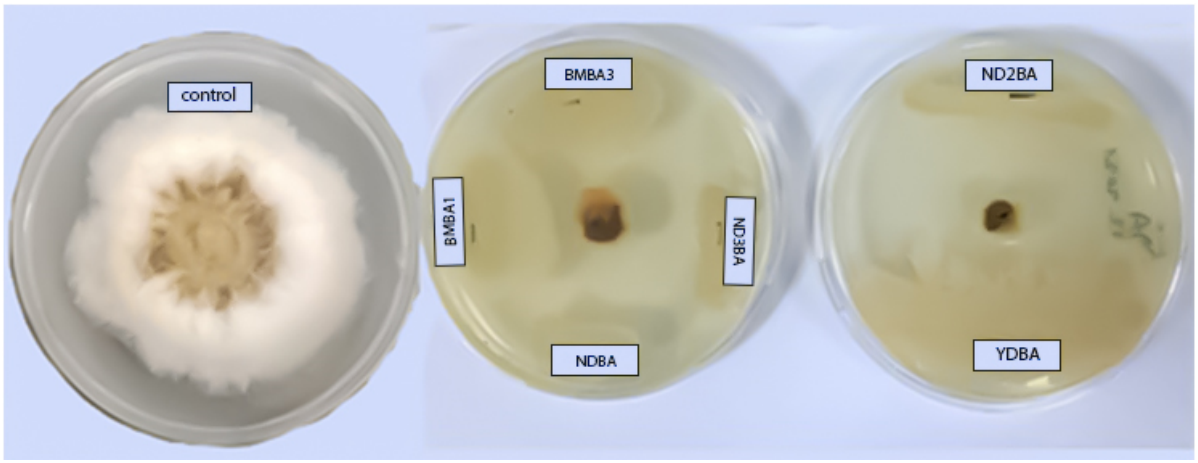

Figure 2. Antagonistic activities of endophytic bacteria against *Fusarium culmorum.*

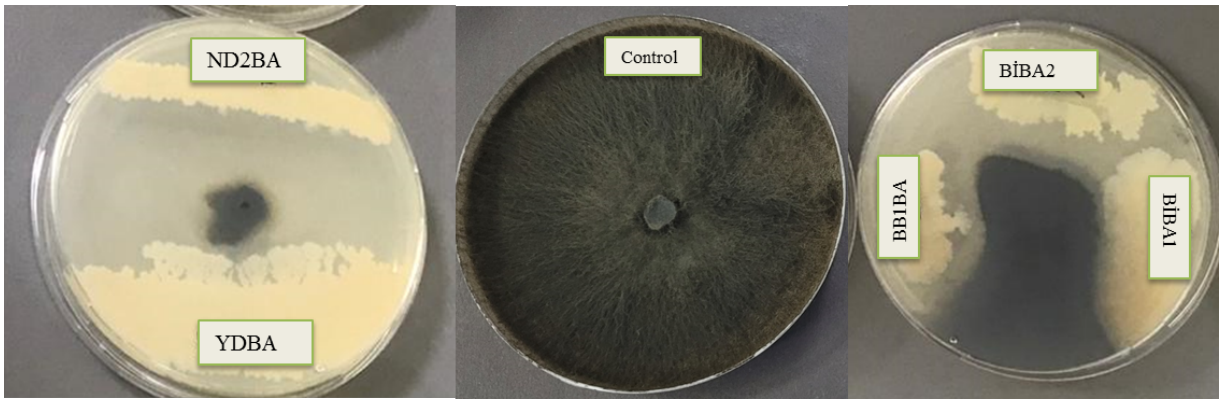

Figure 3. Antagonistic activities of endophytic bacteria against *Neoscytalidium dimidiatum.*

Al Hamad et al. (2021) studied the potential of actinobacterial isolates as biological control agents (BCAs) against the pathogen *N. dimidiatum*. Their research reported the inhibitory ability of *Streptomyces griseorubens* (UAE2) and *Streptomyces wuyuanensis* (UAE1). They reported that these strains exhibited strong antifungal activity by producing antifungal compounds and lytic enzymes. Since this is the first study to study endophytic *Bacillus* sp. against the pathogen *N. dimidiatum*, these studies are important for further studies.

3.3. Applications

According to the one-way analysis of variance (ANOVA) of the obtained observation values, the differences between the averages of the data shown by the root and coleoptile lengths of endophytic bacteria were found to be significant (Table 4).

Table 4. Variance analysis table of root and coleoptile lengths of endophytic bacteria

Coleoptile Length					
Sources of Variation	**DF**	**SS**	**MS**	**F Value**	***P***
Isolates	9	53.791	5.977	60.673	<.0001***
Rep.	3	0.203	0.068	0.686	0.568
Error	27	2.660	0.099		
General	39	56.654			
Root Length					
Sources of Variation	**DF**	**SS**	**MS**	**F Value**	***P***
Isolates	9	81.180	9.020	84.894	<.0001***
Rep.	3	0.159	0.053	0.498	0.687
Error	27	2.869	0.106		
General	39	84.208			

DF: Degrees of freedom SS: Sum of squares MS: Mean of squares.
*** Difference between bacteria according to Tukey LSD Significant according to $\alpha = 0.05$.

Table 5. Variance analysis table of root and coleoptile lengths of wheat inoculated with endophytic bacteria and *Fusarium culmorum* pathogen

Coleoptile Length					
Sources of Variation	**DF**	**SS**	**MS**	**F Value**	***P***
Isolates	9	87.336	9.704	154.236	<.0001***
Rep.	3	0.439	0.146	2.325	0.097
Error	27	1.699	0.063		
General	39	89.473			
Root Length					
Sources of Variation	**DF**	**SS**	**MS**	**F Value**	***P***
Isolates	9	78.007	8.667	178.251	<.0001***
Rep.	3	0.106	0.035	0.728	0.544
Error	27	1.313	0.049		
General	39	79.426			

DF: Degrees of freedom SS: Sum of squares MS: Mean of squares.
*** Difference between bacteria according to Tukey LSD Significant according to $\alpha = 0.05$.

As a result of the applications, the root and coleoptile lengths of wheat inoculated with endophytic bacteria were classified as short, medium, medium-long, and long. Coleoptile class values were given to classes with 30-40 mm coleoptile lengths as short, 41-51 mm as medium-short, 52-62 mm as medium, 63-73 mm as medium, and 74-84 mm as long (Table 6). In the application of only bacteria, the BMBA2 isolate with a coleoptile length of 76 mm was classified as long, while the control was classified as short, with a length of 35 mm (Table 6).

Root lengths of wheat inoculated with endophytic bacteria were evaluated as short for those with 30-40 mm root lengths, 41-51 mm as medium short, 52-62 mm as medium, 63-73 mm as medium long, and 74-84 mm as long (Table 6). Only in the bacterial application, the BMBA2 isolate, which had a root length of 83 mm from wheat, was classified as long, while the control was classified as short, with a length of 31 mm (Table 6). In the study conducted by Çelikten and Bozkurt (2018), 69 of the tested isolates caused an increase in root length by 7.1-70.6% compared to the control application. In terms of shoot development, all of the isolates positively increased shoot development and supported our study by increasing shoot length by 6.6-108.6% compared to the control application.

Table 6. Root and coleoptile lengths of plants treated with endophytic bacteria

Isolates	Coleoptile Length (cm)	Root Length (cm)
Control	3.50^{e}	3.05^{g}
BiBA1	4.58^{cd}	4.30^{f}
BiBA2	4.20^{de}	4.73^{ef}
NDBA	5.48^{b}	4.90^{ef}
ND2BA	5.58^{b}	5.25^{de}
ND3BA	5.78^{b}	6.38^{c}
YDBA	7.08^{a}	7.18^{b}
BMBA1	5.35^{b}	5.05^{def}
BMBA2	7.58^{a}	8.33^{a}
BMBA3	5.28^{bc}	5.78^{cd}
Mean	5.44	5.49
SE	0.157	0.163

There is no statistically significant difference between values.

The coleoptile classes of wheat inoculated with endophytic bacteria and *F. culmorum* pathogen were evaluated as short for classes of 20-30 mm, medium short for classes of 31-41 mm, medium for classes of 42-52 mm, medium long for classes of 53-63 mm, and long for classes of 64-74 mm (Table 7). In the application of only bacteria, the BMBA2 isolate had a coleoptile length of 69.8 mm from wheat and was classified as long, while the control was classified as short, with a length of 23.8 mm (Table 7).

The root lengths of wheat inoculated with endophytic bacteria and *F. culmorum* pathogen were evaluated as short for classes of 19-29 mm, medium short for classes of 30-40 mm, medium long for classes of 41-51 mm, medium long for classes of 52-62 mm, and long for classes of 63-73 mm (Table 7). In the bacterial application only, the BMBA2 isolate with a root length of 73 mm was classified as long and the control was classified as short with a root length of 21.5 mm (Table 7).

Table 7. Root and coleoptile lengths of wheat inoculated with endophytic bacteria and *Fusarium culmorum* pathogen

Isolates	Coleoptile Length (cm)	Root Length (cm)
Fusarium culmorum	2.38^{f}	2.15^{e}
BiBA1	3.45^{de}	3.13^{d}
BiBA2	2.85^{ef}	3.08^{d}
NDBA	4.53^{c}	4.13^{c}
ND2BA	3.10^{de}	3.48^{d}
ND3BA	5.80^{b}	4.23^{c}
YDBA	6.23^{b}	5.40^{b}
BMBA1	4.58^{c}	4.88^{b}
BMBA2	6.98^{a}	7.30^{a}
BMBA3	3.50^{d}	3.23^{d}
Mean	4.34	4.098
SE	0.13	0.11

There is no statistically significant difference between values.

Many studies are showing that bacteria that promote plant growth *in vitro* increase seed germination, root and shoot development in many plants such as wheat, corn, and sunflower, and our results are supported by these studies (Mishra et al., 2010; Öksel et al., 2022).

3.4. Determination of metabolic enzyme profiles of isolates with Bruker Maldi Biotyper

Nine isolates from the bacteria to be tested were selected by looking at their BiBA1, BiBA2, NDBA, ND2BA, ND3BA, and YDBA. BMBA1, BMBA2, and BMBA3 enzyme activities, carbohydrate test, and antagonistic activities, and were performed with a matrix-assisted laser desorption ionization-time-of-flight mass spectroscopy (MALDI-TOF MS) device (Bruker Microfleks LT Biotyper. Bruker Daltonics. Bremen. Germany).

As a result of in vitro studies with a matrix-assisted laser desorption ionization-time-of-flight mass spectroscopy (MALDI-TOF MS) device, it was determined that the BİBA1 and ND3BA isolates found to be effective were *B. amyloliquefaciens* subsp. *plantarum*. NDBA, ND2BA, BMBA1. It was determined that BMBA2 and BMBA3 isolates were *B. mojavensis*; BiBA2 and YDBA isolates were *B. subtilis* (Table 8). The fact that the most studied and used biological agent against various plant pathogens is Bacillus species is also supported by numerous studies on *B. subtilis, B. velezensis,* and *B. amyloliquefaciens* species (Shafi et al., 2017; Fira et al., 2018; Caulier et al., 2019).

Table 8. Species identification through metabolic enzyme profiles of isolates with Bruker Maldi Biotyper

Isolates	Isolated plant	Species
BiBA1	Rosemary (*Rosmarinus officinalis* L.)	*Bacillus amyloliquefaciens* subsp. *plantarum*
BiBA2	Rosemary (*Rosmarinus officinalis* L.)	*Bacillus subtilis*
NDBA	Olive (*Olea europaea* L.)	*Bacillus mojavensis*
ND2BA	Olive (*Olea europaea* L.)	*Bacillus mojavensis*
ND3BA	Olive (*Olea europaea* L.)	*Bacillus amyloliquefaciens* subsp. *plantarum*
YDBA	Loquat (*Eriobotrya japonica* (Thunb.) Lindl.)	*Bacillus subtilis*
BMBA1	Olive (*Olea europaea* L.)	*Bacillus mojavensis*
BMBA2	Olive (*Olea europaea* L.)	*Bacillus mojavensis*
BMBA3	Olive (*Olea europaea* L.)	*Bacillus mojavensis*

There are numerous reports on endophytic *Bacillus* strains used for the control of fungal pathogens in different crops. Pan et al. (2015) reported that endophytic *B. megaterium* and *B. subtilis* obtained from wheat grain inhibited the fungal growth of *F. graminearum*. In other studies, some strains of endophytic *B. mojavensis* were reported to protect plants against diseases (Bacon and Hinton, 1996 and 2007; Bacon et al., 2001).

4. Conclusion

In this study, three species of endophytic bacteria belonging to the genus *Bacillus* that were found effective were studied for their antagonistic activity against wheat root and coleoptile lengths and the *F. culmorum* pathogen. *Bacillus mojavensis* (BMBA2) isolates gave the most effective result in coleoptile and root length, followed by *B. subtilis* (YDBA) and *B. amyloliquefaciens* subsp. *plantarum* (ND3BA). This study emphasizes the importance of preparing bioinoculants of selected isolates that show antagonistic activity against pathogens that promote plant growth. *In vitro* conditions, nine potential isolates (BiBA1, ND3BA, NDBA, ND2BA, BMBA1, BMBA2, BMBA3, BiBA2, and YDBA) were determined to be antagonistic against fungal pathogens of endophytes that promote seed root and coleoptile growth in seed germination period characteristics. The results confirmed that endophytes have potential inoculant properties for effective colonization and use as antagonists against pathogens.

It is thought that it may have promising applications in terms of obtaining effective results in the parameters during the wheat seed germination period. Investigating the potential of environmentally friendly biological control agents and environmentally friendly biopesticides in controlling *N. dimidiatum* and *F. culmorum* pathogens may offer sustainable alternatives for disease management. It is also thought that it will contribute to the development of effective strategies for disease management and reduction of the *N. dimidiatum* pathogen.

Ethical Statement

Ethical approval was not required for this study, as the methods employed did not necessitate review by an ethics committee.

Conflict

The author declares that there are no conflicts of interest.

Funding Statement

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Author Contributions

The author takes sole responsibility for the conception, design, data collection, analysis, interpretation, and writing of this manuscript.

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Research Article

Unveiling Bioactive Profiles: Comparative Analysis of Soluble and Insoluble Compounds in Katokkon Chili Landraces of South Sulawesi, Indonesia

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Abstract: Katokkon chili is a type of local chili from Toraja, South Sulawesi, Indonesia, which has a unique character, both in shape and spicy taste. This research will reveal the bioactive compounds in three Katokkon chilies' landraces: Limbong Sampolo Sampolo, Leatung 1, and Leatung 2. Dissolved and undissolved bioactive compounds were identified using the gas chromatography-mass spectrometry (GC-MS) method. The findings were that these three Katokkon chili landraces had different bioactive compound compositions. However, the Limbong Sampolo Sampolo landrace has the most bioactive compounds, namely 58 types, compared to the Leatung landraces 1 (36 compounds) and 2 (32 compounds). The most soluble bioactive compound in the Limbong Sampolo Sampolo landrace is n-hexadecanoic acid, while in the Leatung 1 and 2 landraces, it is capsaicin. Then, the insoluble bioactive compounds were dominated.

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1. Introduction

Horticultural products are a crucial food source for the global population, providing a wide range of essential nutrients that are vital for maintaining human health and well-being. These products, which include fruits, vegetables, and spices, play an integral role in addressing nutritional needs and supporting food security worldwide. In addition to their nutritional significance, horticultural products hold substantial economic value, representing a dynamic market segment that benefits producers, distributors, and consumers. In Indonesia, horticultural products are a significant contributor to the agricultural economy, supporting livelihoods and driving economic growth at both local and national levels (Dewi et al., 2015).

Among the various horticultural products, chili stands out as one of the most prominent and widely consumed. Chili, belonging to the genus *Capsicum*, is an indispensable ingredient in both household and industrial food preparation, renowned for its ability to enhance flavor and provide a distinct spicy kick to dishes (Alonso-Villegas et al., 2023). The demand for chili is so significant in Indonesia that its unavailability in the market often triggers inflationary pressures, highlighting its critical role in the country's food supply chain (Nugrahapsari and Arsanti, 2019).

Chili's popularity stems from its unique and intense spiciness, a sensory characteristic that many consumers find highly appealing. The compound responsible for this spicy sensation is capsaicin, a bioactive component that not only defines chili's heat but also contributes to its health benefits. Chili is an excellent source of essential nutrients, including provitamin A, vitamins E and C, carotenoids, and various phenolic compounds such as capsaicinoids, luteolin, and quercetin, also flavonoid (Ergün, 2021). These compounds are widely recognized for their antioxidant properties and other beneficial biological activities, making chili a valuable addition to a healthy diet (Batiha et al., 2020).

Indonesia is home to a diverse range of chili species, both local and introduced, which are cultivated across the archipelago. Among these, the Katokkon chili is particularly noteworthy. Originating from the Tanah Toraja Regency in South Sulawesi, the Katokkon chili is a unique local variety known for its distinct shape, taste, and aroma. Resembling miniature bell peppers, these chilies possess a pungent, spicy, and savory flavor profile that sets them apart from other chili varieties. Due to their distinctive qualities, Katokkon chilies hold significant potential for the development of value-added products such as sauces, chili powders, and other processed ingredients, offering opportunities for business and industrial growth (Warisno and Dahana, 2010).

The spiciness of Katokkon chilies is attributed to their high capsaicin content, a compound characteristic of plants in the *Capsicum* genus. In addition to capsaicin, these chilies contain other valuable bioactive compounds, including alkaloids, carotenoids, flavonoids, and capsaicinoids, which contribute to their health-promoting properties (Basharat et al., 2021). Notably, among the *Capsicum* species, *Capsicum chinense* is recognized as the spiciest, containing the highest levels of capsaicin and related compounds (Chapa-Olivier and Mejia-Teniente, 2016).

Within Toraja, Katokkon chilies are further categorized into several landraces, each exhibiting unique morphological and biochemical traits. For instance, landraces from Limbong Sampolo Sampolo Village and Leatung Village differ in terms of leaf structure, fruit shape, and other physical characteristics. These variations reflect the rich genetic diversity of Katokkon chilies, which is a valuable resource for breeding, conservation, and agricultural development.

This research focuses on three distinct landraces of Katokkon chili to analyze the bioactive compounds present in their fruit. The findings are expected to provide critical insights for farmers, policymakers, and industry stakeholders, facilitating the sustainable development and commercialization of this unique local chili variety. By leveraging the genetic potential and unique properties of Katokkon chilies, Indonesia can further strengthen its position in the global horticultural market while preserving its agricultural heritage.

2. Material and Methods

2.1 Study location

The research was carried out from January to May 2020. Experimental materials were taken from farmers' gardens in Tao Pao Village, Toraja Regency. Bioactive compounds were analyzed at the Development Laboratory and Testing Laboratory of the Center for Agricultural Postharvest Research and Development, Bogor, Indonesia. The research used three (3) Katokkon chili landrace landraces: Limbong Sampolo Sampolo, Leatung 1, and Leatung 2. Limbong Sampolo Sampolo is a Katokkon chili landrace from Limbong Sampolo Village, while Letaung 1 and 2 come from Leatung Village.

2.2 Fruit collecting and sample preparation

The fruit sampled from each fresh Katokkon chili landrace was prepared in 50 g wet weight and cleaned of adhering dirt. The fruit flesh of the three Katokkon chili landraces was dried using the freeze-drying method. The drying aims to remove the water content from these materials. Drying using a freeze dryer starts by placing the ingredients in the freezer for 12 hours. Next, the material was freeze-dried

using a freeze dryer at -55°C for 24 hours. The ingredients resulting from the freeze dryer drying process are then floured using a mixer at a speed of 13,000 rpm until they become flour.

Figure 1. Fruit of three katokkon chili landrace.

2.3 Bioactive compounds analysis

At this stage, the content of the three landrace Katokkon chili bioactive compounds was analyzed using a GC-MS tool (Agilent Technologies 7890 B, USA). Then, the material was soaked using methanol and hexane solvents. Soaking with methanol solvent aims to determine the water-soluble content of the active ingredients in the three Katokkon chili landraces (polar compounds). Meanwhile, soaking with hexane solvent seeks to determine the active ingredient content in the three Katokkon chili landraces, which are not water-soluble (non-polar compounds) (Awotedu et al., 2020; Anwar and Lewar, 2023). The stages of the analysis process using a GC-MS tool followed the method of Maddo et al. (2021) with slight modifications. Approximately 5 g of Katokkon chili extract was soaked in methanol and hexane solvents for 24 hours. Then, 2 ml of the resulting extract was analyzed using a GC-MS tool to determine the bioactive compounds (both polar and non-polar) present in the three Katokkon landraces.

3. Results and Discussion

3.1 Limbong Sampolo landrace

A total of 58 bioactive compounds were discerned from the extraction of the chili flesh of the Katokkon landrace Limbong Sampolo (Table 1); however, only 14 of these compounds exhibited a peak area percentage exceeding 1%. The concentration of soluble compounds in the Limbong Simpulu landrace was more abundant than that of insoluble compounds. The soluble compound with a retention time of 83.098 minutes was identified as n-Hexadecanoic acid, showing an area percentage of 22.084%. The second-highest soluble bioactive compound appeared at a retention time of 98.839 minutes, with an area of 3 382 142.06 and a height of 509 472.88 resulting in an area percentage of 14.367%. Additionally, the compound capsaicin, an essential component in chili flesh, was detected with the third-highest area percentage at a retention time of 95.927 minutes, showing an area percentage of 13.998%. The insoluble compounds of 6-Hepten-3-one, and 5-hydroxy-4-methyl exhibited a significant area percentage of 76.167% and were detected at a retention time of 1.507 minutes.

Table 1. Soluble and insoluble bioactive compounds in Limbong Sampolo landrace

No	Compound	Molecular Form	Retention time (min)	Height	Area	Area (%)
			Dissolved			
1	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	83.098	248 412.73	2 450 461.62	22.084
2	9,12-Octadecadienoic acid (Z,Z)-	$C_{16}H_{32}O_2$ $C_{19}H_{34}O_2$	89.010 98.839	423 629.69 509 472.88	2 748 215.70 3 382 142.06	14.367
3	Capsaicin	$C_{18}H_{27}NO_3$	95.927	397 634.10	3 295 221.58	13.998
4	9-Octadecenoic acid, (E)-	$C_{18}H_{34}O_2$	93.827	111 912.63	1 116 624.76	4.744
5	1,1,6-trimethyl-3-methylene-2-(3,6,9,13tetramethyl-6-ethenyl-10,14-dimethylenepentadec-4-enyl) cyclohexane	-	111.748	339 58.88	1 114 468.41	4.734
6	9-Octadecenoic acid, (E)-	$C_{18}H_{34}O_2$	97.134	159 224.53	1 051 840.15	4.468
7	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	-	101.099	130,020.72	942 670.08	4.004
8	Palmitoleic acid	$C_{16}H_{30}O_2$	87.847	79 353.92	545 864.02	2.319
9	Oleic Acid	$C_{18}H_{34}O_2$	81.232	50 590.31	426 511.74	1.812
10	l-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	92.654	35 533.62	384 593.40	1.634
11	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	$C_{19}H_{34}O_2$	72.792	64 159.15	350 171.31	1.487
12	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	96.201	50 925.15	333 259.51	1.415
13	6-Octadecenoic acid	$C_{18}H_{34}O_2$	90.336	31 228.38	304 839.99	1.294
14	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	98.645	55 297.00	283 648.54	1.205
15	Others (44 compounds)	-				20.436
			Undissolved			
1	6-Hepten-3-one, 5-hydroxy-4-methyl-	$C_8H_{14}O_2$	1.507	13 153 583.86	26 420 287.27	76.167
2	Cyclopentane, methyl-	C_6H_{12}	1.616	6 764 521.91	6 172 592.08	17.793
3	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	98.862	76 618.79	621 288.63	1.790
4	Vitamin E	$C_{29}H_{50}O_2$	115.307	15 121.44	487 669.68	1.409
5	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	89.135	48 270.53	355 963.18	1.028
6	Others (14 compounds)	-				1.813

3.2 Leatung 1 landrace

A cumulative total of 36 bioactive compounds were identified from the extraction of the chili flesh belonging to the Katokkon landrace Leatung 1 (Table 2); nevertheless, merely 19 of these compounds demonstrated a peak area percentage surpassing 1%. The concentration of soluble compounds in the Leatung 1 landrace was significantly higher compared to insoluble compounds. Capsaicin, a soluble compound identified at a retention time of 98.851 minutes, exhibited the highest area percentage of 28.156%. In contrast, the Limbong Simpulu race contained n-Hexadecanoic acid as the compound with the highest area percentage. In the Leatung 1 chili landrace, n-Hexadecanoic acid had the second-highest area percentage at 17.843%, detected at a retention time of 89.117 minutes. Additionally, the compounds 6-Hepten-3-one and 5-hydroxy-4-methyl constituted an area percentage of 79.114% among the insoluble compounds.

Table 2. Soluble and insoluble bioactive compounds in Leatung 1 landrace

No	Compound	Molecular Form	Retention time (min)	Height	Area	Area (%)
			Dissolved			
1	Capsaicin	$C_{18}H_{27}NO_3$	98.851	155 922.85	1 216 874.82	28.156
2	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	89.117	112 653.40	771 082.72	17.843
3	Oleic Acid	$C_{18}H_{34}O_2$	97.134	44 134.13	326 000.95	7.543
4	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	101.111	40 455.53	294 536.91	6.814
5	Propanoic acid, butyl ester	$C_7H_{14}O_2$	27.199	79 93.35	57 677.62	4.860
		$C_7H_{14}O_2$	29.362	37 386.00	210 013.63	
6	Geranyl vinyl ether	$C_{12}H_{20}O$	87.870	27 628.22	171 951.78	3.978
7	Undec-10-ynoic acid, butyl ester	$C_{15}H_{26}O_2$	2.600	67 172.87	169 712.04	3.928
8	Methyl 12,13-tetradecadienoate	$C_{15}H_{26}O_2$	0.100	13 816.49	93 331.00	2.160
9	Pentanoic acid	$C_5H_{10}O_2$	23.686	96 35.53	87 077.63	2.016
10	Mono(2-ethylhexyl) phthalate	$C_{16}H_{22}O_4$	96.831	17 947.45	86 387.21	1.999
11	(R)-(-)-14-Methyl-8-hexadecyn-1-ol	$C_{17}H_{32}O$	72.804	13 464.54	73 553.44	1.701
12	n-Decanoic acid	$C_{10}H_{20}O_2$	81.604	6 784.89	65 026.55	1.504
13	15,15'-Bi-1,4,7,10,13pentaoxacyclohexadecane	$C_{22}H_{42}O_{10}$	96.218	9 193.31	60 051.34	1.388
14	2-Methoxy-3-methyl-butyric acid, methyl ester	$C_7H_{14}O_3$	2.823	25 455.66	58 928.95	1.363
15	Propanoic acid, butyl ester	$C_7H_{14}O_2$	27.199	7 993.35	57 677.62	1.335
16	9-Decenoic acid	$C_{10}H_{18}O_2$	80.220	7 895.48	51 928.04	1.202
17	3-Hydroxy-4-methoxybenzyl alcohol	$C_8H_{10}O_3$	84.368	7 645.75	50 673.67	1.171
18	Methyl 16-hydroxy-hexadecanoate	$C_{17}H_{34}O_3$	87.344	10 650.03	47 813.16	1.107
19	Methyl 8-methyl-nonanoate	$C_{11}H_{22}O_2$	61.354	6 345.77	47 476.14	1.098
20	Others (17 compounds)	-				8.853
			Undissolved			
1	6-Hepten-3-one, 5-hydroxy-4-methyl-	$C_8H_{14}O_2$	1.507	13 111 073.92	26 448 638.29	79.114
2	Cyclopentane, methyl-	C_6H_{12}	1.616	6 743 204.88	6 337 774.35	18.956
3	Others (11 compounds)	-				1.930

3.3 Leatung 2 landrace

The pulp of the Leatung 2 landrace of Katokkon chili contains 32 bioactive compounds, the lowest count compared to the Limbong Sampolo and other Leatung 2 landraces of Katokkon chili (Table 3). The capsaicinoid group makes up approximately 80.869% of the Leatung 2 Katokkon chili extracts, detected at a retention time of 99-103 minutes. Within this group, capsaicin accounts for 75.379%, and dihydrocapsaicin comprises 5.490%. The compounds capsaicin and dihydrocapsaicin are important compounds in chilies that affect the level of spiciness. Additionally, among the insoluble compounds, 6-Hepten-3-one, and 5-hydroxy-4-methyl represent an area percentage of 73.687%.

Table 3. Soluble and insoluble bioactive compounds in Leatung 2 landrace

No	Compound	Molecular Form	Retention time (min)	Height	Area	Area (%)
			Dissolved			
1	Capsaicin	$C_{18}H_{27}NO_3$	101.111	104 131.89	4 338 655.43	75.379
			103.016	111 740.78	3 066 625.42	
2	Dihydrocapsaicin	$C_{18}H_{29}NO_3$	99.680	21 933.71	539 276.29	5.490
3	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	98.862	60 985.60	425 075.65	4.328
4	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	89.134	47 694.69	353 311.92	3.595
5	2,5-Octadecadiynoic acid, methyl ester	$C_{19}H_{30}O_2$	2.594	53 760.87	157 136.76	1.599
6	3-(1,3-Dihydroxyisopropyl)-1,5,8,11,14,17 hexaoxacyclononadecane	$C_{16}H_{32}O_8$	97.151	19 777.55	128394.33	1.307
7	2-Buten-1-ol, propanoate	$C_7H_{12}O_2$	29.351	21 154.81	126 235.54	1.285
8	Others (25 compounds)	-				7.017
			Undissolved			
1	6-Hepten-3-one, 5-hydroxy-4-methyl-	$C_8H_{14}O_2$	1.507	13 133 816.24	26 482 443.46	73.687
2	Cyclopentane, methyl-	C_6H_{12}	1.616	6 514 654.91	6 189 363.43	17.221
3	7-Oxabicyclo [4.1.0] heptane, 1-(1,3-dimethyl1,3-butadienyl)-2,2,6-trimethyl-,	$C_{15}H_{24}O$	101.655	25 159.56	914 816.62	2.542
4	Others (14 compounds)	-				6.551

3.4 Comparative bioactive profile of Katokkon Chili Landraces

The radar charts illustrated hereinafter (Figures 2 and 3) serve to visually delineate these profiles. Figure 2 elucidates the dissolved compounds that are distinctive to each Katokkon landrace, thereby exemplifying the variations in concentration that characterize their prospective applications and health advantages, as similarly depicted in Figure 3, which highlights the undissolved compounds that are unique to each Katokkon landrace.

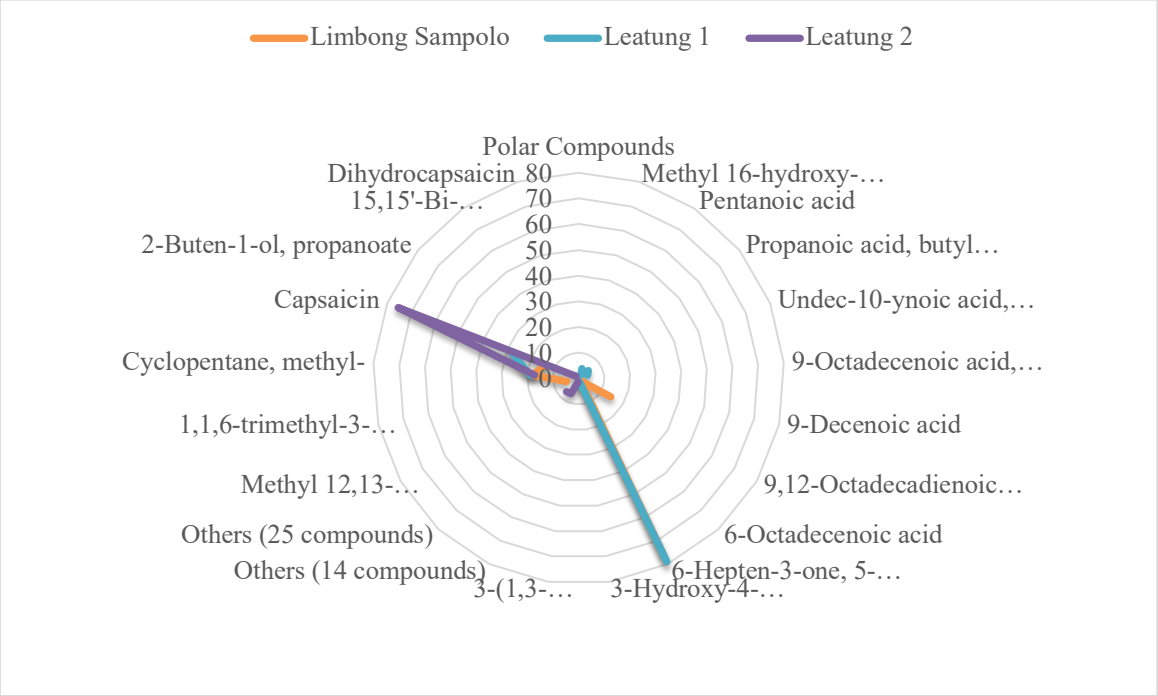

Figure 2. Radar chart for dissolved compound.

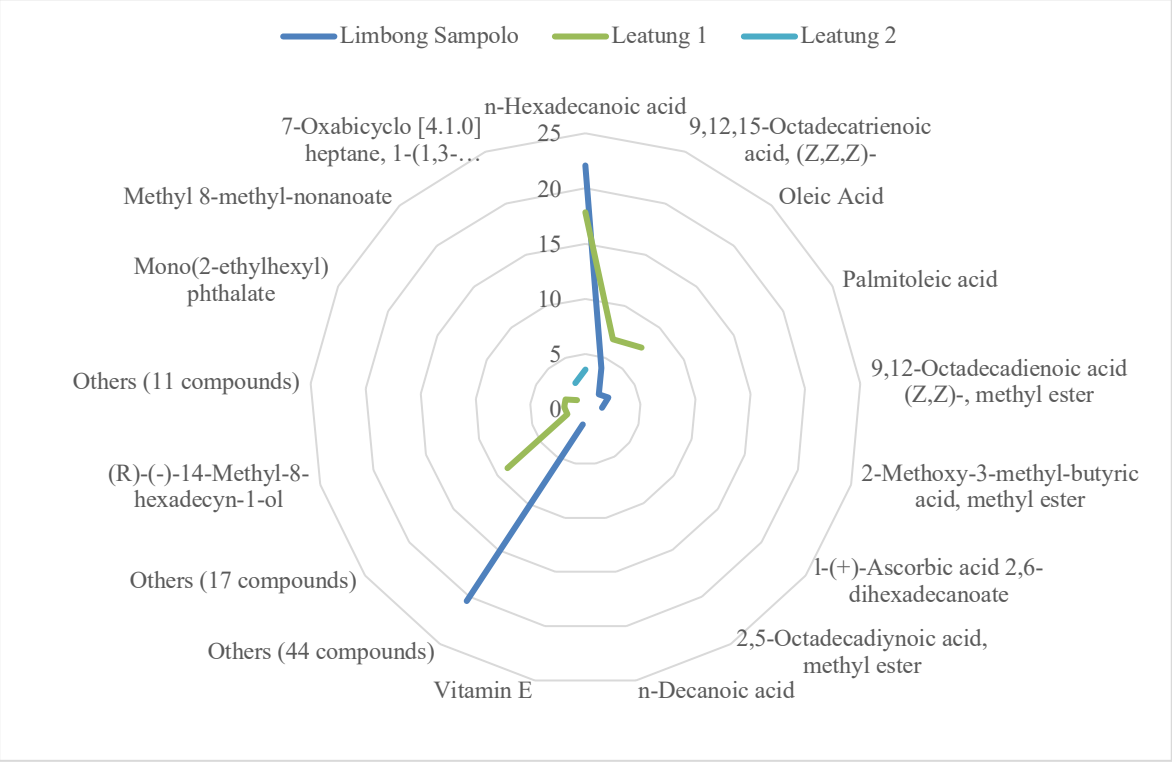

Figure 3. Radar chart for undissolved compound.

Discussion

The analysis of the bioactive compounds in the Katokkon chili landraces—Limbong Sampolo, Leatung 1, and Leatung 2—highlights their diverse chemical profiles and underscores their potential applications in health, nutrition, and industry. The Limbong Sampolo landrace was found to contain 58 bioactive compounds, with 14 exceeding 1% in peak area percentage. Among these, n-hexadecanoic acid was the most abundant. This compound, with the molecular formula $C_{16}H_{32}O_2$, this compound functions as an antioxidant, hypocholesterolemic, antiandrogenic, flavor, and nematicide hemolytic 5-alpha reductase inhibitor (Easwaran and Ramani, 2014). The compound with the second highest area percentage is 9, 12-Octadecadienoic acid. The compound 9, 12-Octadecadienoic acid is known as an omega-6 fatty acid that is important for average cell growth, for lowering cholesterol levels in the blood (Igwe and Okwu, 2013), and for supporting the quality of skin lubrication (Okwu and Morah, 2006). 9,12-Octadecadienoic acid (Linoleic acid) is known for its anti-inflammatory properties, linoleic acid is essential for heart health and skin barrier function. Studies have linked linoleic acid intake with reduced cardiovascular risks, which could enhance the culinary or nutraceutical applications of *Limbong Sampolo* extracts (Das, 2006; De Roos and Calder, 2018). 9-Octadecenoic acid (Oleic acid) is a primary component of olive oil and is beneficial for reducing inflammation and promoting cardiovascular health. It has been linked to improved blood lipid profiles and reduced risk of coronary heart disease (Poudyal and Brown, 2011; Carrillo et al., 2012). Oleic acid is also reported as an anti-inflammatory fatty acid that plays a role in activating competent immune cell pathways (Carrillo et al., 2012). Foods rich in oleic acid benefit inflammation-related diseases (Santa-Maria et al., 2023). Palmitoleic acid recognized for its anti-inflammatory and metabolic health benefits, palmitoleic acid may improve insulin sensitivity and support cardiovascular health. Its presence in Limbong Sampolo offers potential for use in skin care or wellness products (Mozaffarian and Wu, 2012; Yang et al., 2021). Vitamin E a well-known antioxidant can support immune health and protects cells from oxidative damage. Its presence in Limbong Sampolo could be advantageous for creating antioxidant-rich supplements (Traber and Stevens, 2011).

N-hexadecanoic acid, commonly known as palmitic acid, is a saturated fatty acid predominantly found in palm oil. Despite its limited nutritional benefits due to its saturated nature, palmitic acid holds significant value in the cosmetic industry, where it is widely appreciated for its moisturizing properties and its ability to improve skin hydration and texture (Raederstorff et al., 2015). Another notable compound is 9,12,15-octadecatrienoic acid, or alpha-linolenic acid, a well-known omega-3 fatty acid typically sourced from flaxseed. Renowned for its anti-inflammatory properties and its role in promoting cardiovascular health, the presence of alpha-linolenic acid in Leatung 1 highlights this chili variety's potential for nutraceutical applications. Although its concentrations in Leatung 1 are lower compared to conventional sources like flaxseed or fish oil, its inclusion underscores the diverse bioactive profile of this chili (Rodriguez-Leyva et al., 2010). Additionally, Leatung 1 contains unique esters and alcohols, such as methyl 8-methyl-nonanoate and 3-hydroxy-4-methoxybenzyl alcohol. These rare compounds are highly valued for their roles in enhancing fragrance and flavor, opening avenues for applications in food flavoring and cosmetic formulations. The distinct combination of these bioactive compounds not only enriches the functional profile of Leatung 1 but also positions it as a versatile ingredient for multiple industries.

Leatung 2 stands out due to its high concentration of capsaicin, a bioactive compound renowned for its diverse therapeutic properties. Capsaicin is widely recognized for its pain-relief capabilities, making it a key ingredient in topical creams designed to alleviate discomfort from conditions such as arthritis and neuropathy. Additionally, it exhibits potent anti-tumor, antioxidant, analgesic, alpha-amylase inhibitor, and anti-fungal. These well-documented benefits make Leatung 2 a promising candidate for use in both pharmaceutical and nutraceutical applications, including metabolic health supplements and anti-inflammatory products (Surh, 2002; Reyes-Escogido et al., 2011; Maokam et al., 2014; Levono and Prasad, 2017). Capsaicin is also an anti-microbial that plays a positive role in several mechanisms to kill microbes, like pepper extract can be used to inhibit bacteria growth (Taolin, 2019; Ergün et al., 2024). Complementing capsaicin is the presence of 2,5-octadecadiynoic acid, methyl ester, a compound with emerging potential as an anti-inflammatory agent (Alrabie et al., 2023). Although research on this compound is still in its early stages, its inclusion in Leatung 2 suggests synergistic possibilities when combined with capsaicin, particularly in the formulation of advanced anti-

inflammatory products. Together, these bioactive compounds highlight the unique therapeutic potential of Leatung 2, making it a valuable resource for the development of innovative health and wellness solutions. Leatung 1, Leatung 2, and Limbung Sampolo exhibit distinct biochemical characteristics, reflecting the genetic diversity among these genotypes. Similar findings were reported by Öntürk and Çürük (2019), who observed variations in growth and pungency levels across different local chili pepper populations. This highlights that both biochemical and morphological diversity are common in chili populations originating from different regions.

Conclusion

In conclusion, the study of these Katokkon chili landraces underscores their value as sources of bioactive compounds with diverse and significant applications. The presence of antioxidants, fatty acids, and capsaicinoids across the landraces highlights their potential to address various health challenges, from oxidative stress and inflammation to microbial infections and cancer. Moreover, the distinct chemical profiles of each landrace provide opportunities for targeted applications in food, pharmaceuticals, and nutraceuticals. Future research should explore the bioavailability and synergistic interactions of these compounds to fully harness their therapeutic potential. Additionally, the sustainable cultivation and selective breeding of these landraces could enhance their bioactive content, ensuring their continued relevance in modern health and industry applications. This study exemplifies the intersection of traditional agricultural practices and scientific innovation, offering a roadmap for leveraging indigenous crops in global markets.

Ethical Statement

This study was conducted ethically, adhering to all relevant guidelines and regulations, with no involvement of human participants, animal testing, or endangered plant species.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

Each author made an equal contribution to the article.

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Research Article

Effect of Different Additives and Ratios on Silage Quality Characteristics of Common Reed Growing in Drainage Channels

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Fermentation characteristics, Nutritional and chemical contents, *Phragmites australis*, Silage additives

Abstract: This study aims to determine some nutritional, chemical, and fermentation properties of Common reed silage using different additives (alfalfa grass, crushed corn, sugar beet molasses, and 1:1 alfalfa grass + sugar beet molasses) and their ratios (0%, 5%, 10% and 15%). Additives were added based on fresh weight to the Common reed grass, which was cut and chopped (1.0 cm sizes) at the beginning of the panicle. Then it was bagged in a vacuum device and left to ferment at 25±2 °C for 60 days. Analyse results showed that all additives increased crude protein, raw ash (RA), dry matter (except molasses), Fleig score, and lactic acid content (except alfalfa) of Common reed silage compared to the control but decreased the pH (except alfalfa), ammonia production, neutral detergent fiber, and acid detergent fiber contents. A similar situation (except RA) occurred as the levels of additives increased. On the other hand, molasses, crushed corn, and increasing levels of these additives increased the lactic acid content of the silage, while decreasing the acetic, propionic, and butyric acid contents. As a result, a quality silo material can be obtained from the Common reed by applying 5% alfalfa grass + 5% molasses.

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1. Introduction

In a healthy diet, animal products should be consumed in sufficient amounts as well as plant-based food sources. This can be achieved by providing sufficient and balanced nutrition for animals, too. However, due to the quality roughage deficit experienced (especially during transition periods and winter months when the herbaceous species in the vegetation dry up), animals cannot be fed adequately, and desired levels of animal products cannot be obtained (Temel and Şahin, 2011; Yavuz et al., 2020). In order to eliminate this problem, the use of corn, sorghum, alfalfa, and many other forage plants as silage has been seen as an important advantage in recent years. However, the amounts of silage obtained from known forage plant species are not at a level that can meet the needs of animals. In this sense, the use of plants that grow spontaneously in idle lands and have low nutritional content when used as green or dry grass has been seen as an alternative solution. Because coarse-structured plants, which are less preferred by animals, can be important feed materials rich in nutrients by ensiling. As a matter of fact, studies conducted, have revealed that silages prepared by adding additives in appropriate proportions to

low-quality feed materials are easily consumed by animals (Özinan, 2017; Güner and Temel, 2022; Keskin and Aksoy, 2024).

Phragmites australis can grow easily in heavy clayey, sandy, and salty soils, marshes and reeds, ponds, ditches, and all kinds of moist land (Mal and Narine, 2004). It can produce high amounts of biomass (16.32-28.35 tons ha^{-1}) per unit area during the growing season (Temel et al., 2023). In addition, the biomass it produces has a high nutritional content (in terms of nitrogen, NDF, potassium, manganese, and magnesium). Due to these properties, it has been shown that the plant can play an important role in closing the missing roughage gap and in the nutrition of ruminants as an alternative roughage source (hay, straw and silage) (Baran et al., 2002; El-Talty et al., 2015; Tanaka et al., 2016; Kadi et al., 2018; Aydoğan and Demiroğlu Topçu, 2022; Büyükkılıç Beyzi et al., 2022). However, the coarse structure of the plant can relatively limit the consumption preference and nutritional composition of the biomass it produces by animals. For this reason, it has been seen as an important advantage to evaluate the plant as silage rather than as fresh or dry grass. As a matter of fact, it has been shown that the common reed silage obtained without using any additives beforehand produces a higher quality feed material than its use as grass (Temel et al., 2023). However, it has been observed that the plant's pure silage is not at the desired quality level (Büyükkılıç Beyzi et al., 2022). For this reason, the idea of obtaining a higher quality silo feed from the plant by using different additives has been deemed important. For this purpose, studies were carried out to improve the nutritional, chemical, and fermentation properties of common reed silage by using different additives (glucose, lactic acid bacteria, urea, formic acid, NaOH, molasses, barley flour, enzyme, and mulberry leaves) and their ratios (Asano et al., 2018; Hussain, 2018; Saeed et al., 2019; Yeşil and Güney, 2023; Kazemi et al., 2024). In particular, alfalfa grass, crushed corn, and sugar beet molasses have been seen as preferred additives to increase the energy and nutrient content of silage feed (Gülümser et al., 2019). However, it has been observed that the studies carried out on the subject are limited.

The objective of the present study was to determine some nutritional, chemical, and fermentation properties of Common reed silage using alfalfa grass, crushed corn, sugar beet molasses, and 1:1 alfalfa grass + sugar beet molasses in different ratios.

2. Material and Methods

The samples were performed in Igdir located (at 39°59′21″N, 44°03′19″E) in the northeastern part of Türkiye in 2022. In the study, the Common reed plant that grows naturally in the drainage channels was used as silage material. Ground crushed corn and sugar beet molasses were preferred as carbohydrate sources to increase the fermentation quality of silage. In addition, to increase the crude protein content of the silage, alfalfa grass mowed and dried at 1/10 flowering stage was used.

The plants were mowed at a stubble height of 0.1 mm at the beginning of the panicle in the designated area (3 replications and each replication covered an area of 3 m^2) in August 2022 and were shredded with a silage machine to a diameter of 2-3 mm without withering. In a previous study conducted on the subject, although the dry matter ratio (64.80%) at the beginning of the panicle was very high compared to other development periods (I. development period; 44.80% and II. development period; 60.32%), it was determined that NDF and ADF content were low, and crude protein, dry matter digestibility, and relative feed value were high (Temel et al., 2023). For these reasons, the panicle beginning period was preferred. However, since the dry matter ratio of the common reed plant is high during the panicle beginning period, 30% water was added from the dry matter calculation to reduce the dry matter ratio and thus, the starting material (control) was created. Then, the determined additives (alfalfa, molasses, and crushed corn) and their ratios (5%, 10%, and 15%) were calculated on the wet material and added to the starting material, and the samples placed in airtight nylon bags were passed through a vacuum device and subjected to fermentation for 60 days. In the study, dry matter ratios of silage samples were determined according to AOAC (1990). The amount of 20 g silage sample and 180 mL pure water were added into the blender and mixed until the mixture became homogeneous. Then, the pH of the samples obtained from the filtrate was measured by a pH meter, and also the amount of ammonia by distillation and titration using the Kjeldahl method (AOAC, 1990). Fleig score was calculated using the equation developed by Kılıç (1986), and then the resulting values were used to reveal the quality class of the silage samples using the scale of "0-20: bad, 21-40: moderate, 41-60: satisfactory, 61-80: good and 81-100: very good".

$$Fleig\ score = 220 + (2 * DM\% - 15) - pH \quad (1)$$

Crude protein contents of ground silage samples were determined by the Micro Kjeldhal method (AOAC, 1997), and NDF and ADF ratios were analyzed in the ANKOM fiber analyzer using the method developed by Van Soest et al. (1991). 1.0 grams of ground silage samples were taken and kept in a raw ash furnace at 550 °C for 8 hours. The samples that turned gray ash were cooled in a desiccator until they reached room temperature and then the samples were weighed on a precision scale. Then, the weighing values were proportioned with a simple equation and the percentage raw ash ratios were calculated (AOAC, 1990).

The extraction methods determined by Canale et al. (1984) and Saad-Allah and Youssef (2018) were revised to determine organic acids. Then, the amounts of acetic, propionic, lactic and butyric acid in the silage extracts placed in the vial were determined by HPLC-DAD device (mobile phase 0.02 N H_2SO_4 in water, syringe volume 10 microliters (µl), flow rate 0.6 ml/min, column temperature 50 °C, detector wavelength 210/4 nm, column type HI-Plex H, 300 x 7.7 mm, column part no PL1170-6830, operating system isocratic and detector pressure 36.5 bar).

Since the additive applications were made under controlled conditions (laboratory), the data obtained from the study were subjected to analysis of variance (ANOVA) according to the completely randomized design in the IBM SPSS 17.0 statistical package program. The differences between the applications that were significant were determined by Duncan's multiple comparison test at 1% probability.

3. Results and Discussion

The effects of additives added at different rates on silage dry matter, pH, ammonia production, and Fleig score were found statistically significant ($p<0.01$) (Table 1).

3.1. Silage dry matter, pH, Ammonia production, and Fleig score

When Table 1 was examined, the highest dry matter ratio was measured in silo feed with 15% alfalfa and 15% alfalfa + 15% molasses addition, while the lowest ratio was measured in common reed silage with 5% molasses addition following 10% and 15% molasses applications. These results showed that other additives and applications except molasses increased the silage dry matter ratio compared to the control.

Table 1. The DM, pH, ammonia, and Fleig score of Common reed silage

Applications	DM (%)	pH	Ammonia (%)	Fleig score
0% Control	43.56 d	4.81 c	6.30 a	84.58 f
5% AG	48.40 b	5.08 b	5.68 ab	83.73 fg
10% AG	48.82 b	5.17 a	5.50 abc	80.83 g
15% AG	49.87 a	5.22 a	5.17 abc	81.07 g
5% SBM	42.40 e	4.11 g	4.99 abc	110.40 cd
10% SBM	42.84 de	4.08 g	3.94 cd	112.34 c
15% SBM	43.14 de	3.89 h	3.33 d	120.54 b
5% CC	48.11 b	4.72 d	5.34 abc	97.28 e
10% CC	48.17 b	4.42 ef	5.21 abc	109.42 cd
15% CC	48.72 b	4.06 g	4.99 abc	124.90 a
5% AG+ 5% SBM	46.72 c	4.38 f	4.52 bcd	108.23 d
10% AG + 10% SBM	48.31 b	4.41 f	4.08 bcd	110.08 cd
15% AG + 15% SBM	49.86 a	4.48 e	3.95 cd	110.65 cd
F value and significiant	174.46**	893.80**	5.12**	403.41**

Means shown by different letters in the same column are significantly different by Duncan at $p<0.01$. AG; Alfalfa grass, SBM; Sugar beet molasses, CC; Crushed corn, DM; Dry matter.

This may be due to the fact that alfalfa hay and crushed corn have higher dry matter content than molasses. In fact, the average dry matter ratio of alfalfa hay is 91.1%, corn crushed is 87.0% and beet molasses is 74.0% (Cacan et al. 2012). In addition, studies conducted with different feed materials have reported that molasses and crushed corn used as additives increased the dry matter ratio of the silage obtained (Keskin and Aksoy, 2024).

The pH value of the common reed silage varied between 3.89-5.22 (Table 1). When Table 1 is examined, only alfalfa grass increased the pH of the silage compared to the control, while other additives decreased the pH of the silage. Similar results were obtained in previous studies conducted with different species (Acar and Bostan, 2016; Kardes et al., 2023). This may be due to the high soluble sugar content of molasses and crushed corn, while the low content of alfalfa. On the other hand, to prevent proteolysis, which breaks down proteins into ammonia and causes silage deterioration in silo feeds, the pH of the silage is desired to fall below 4 (four) (Virtanen, 1993). In this study, it was observed that the application of 15% molasses added to common reed decreased the silage pH below 4 (four). In addition, considering the high dry matter ratios of the silages obtained, it can be said that other applications except alfalfa are suitable in terms of silage pH. As a matter of fact, in low moisture silages, the pH does not drop much and remains around pH 4.9. In addition, the fact that the pH does not decrease in this type of silo feed does not pose a problem in terms of silage quality (Açıkgöz, 2001).

Ammonia production of common reed silage varied between 3.33% and 6.30% (Table 1). The highest ammonia production (6.30%) was determined in common reed silage where no additives were used (control), and the lowest ammonia production was determined in 15% molasses application. On the other hand, it was observed that additives reduced ammonia production of common reed silage compared to control. In fact, in studies conducted with different species, it was reported that the additives used (molasses, corn grits, wheat bran, and barley grain) reduced the ammonia production of the silage obtained compared to the control (Bingol et al., 2009; Keskin and Aksoy 2024). In addition, increasing additive rates reduced the ammonia production of the silages. For example, while ammonia production in 5% alfalfa, 5% molasses, 5% crushed corn, and 5% alfalfa + 5% molasses applications was 5.68%, 4.99%, 5.34%, and 4.52%, respectively, these rates were measured as 5.17%, 3.33%, 4.99%, and 3.95%, respectively, in the highest dose of 15% applications. These results showed that the additive that reduced silage ammonia production the most was molasses following the alfalfa + molasses application.

When Table 1 was examined, it was seen that the Fleig score of the silages with control and alfalfa additive was good, and the Felig score of the silages with other additives was of the best quality. In general, while the alfalfa additive decreased the silage Felig score compared to the control, the other additives increased the silage Fleig score. In addition, increasing alfalfa additive doses decreased silage Fleig score, while increasing the doses of molasses, crushed corn, and alfalfa grass + molasses additives increased silage Felig score. This may be because the alfalfa additive increases silage pH compared to other additives. Indeed, in the current study, the highest Fleig scores were determined in the applications of 15% molasses and 15% crushed corn with low silage pH, while the lowest Fleig scores were calculated in the applications of 10% alfalfa and 15% alfalfa with high silage pH. Because the Fleig score is calculated by using the pH value and dry matter ratio (DM) of the silage material, and according to this equation, high silage pH decreases the Fleig score, and vice versa, increases it.

3.2. Crude protein, NDF, ADF, and Raw ash contents

Compared to the control, the additives used and their increasing doses caused significant increases in the crude protein content of common reed silage (Table 2). Accordingly, the highest silage crude protein content was measured in 15% alfalfa + 15% molasses application with 9.95%. The second significant increases in silage crude protein content were determined in 15% alfalfa (9.43%), 5% alfalfa + 5% molasses (9.28%), and 10% alfalfa + 10% molasses (9.75%) additive applications. These results showed that especially the alfalfa additive had a significant effect on the increase in silage crude protein content. This is an expected result. Because alfalfa grass has a high crude protein content (Keskin et al., 2021). As a matter of fact, in studies conducted with different forage materials, it has been reported that the presence of legume species in silage mixtures and increasing their proportions cause significant increases in the crude protein content of the silage obtained (Kaymak et al., 2021; Kardeş et al., 2023).

In the common reed silage, the NDF ratio varied between 51.31% and 70.74% and all additives applied decreased the silage NDF ratios compared to the control. In addition, as the additive ratios

increased, the silage NDF contents decreased (Table 2). Accordingly, the highest NDF ratio was determined in the control group where no additives were used (70.74%), and the lowest NDF content was determined in the 10% alfalfa + 10% molasses and 15% alfalfa + 15% molasses applications in the same statistical group. In a previous study, the NDF content of common reed silage harvested at the beginning of the panicle and without any additives was determined as 69.02% (Temel et al., 2023). Molasses is generally a material with a high water-soluble carbohydrate content and can provide the necessary energy for microorganisms that break down hemicellulose and cellulose, and as a result, it increases the activity of cellulolytic bacteria. On the other hand, NDF content is generally low due to the lack of cell wall substances (cellulose and hemicellulose) in alfalfa hay (Keskin et al., 2021; Erbeyli et al., 2022). For these reasons, the addition of alfalfa hay + molasses may have further reduced the NDF content of common reed silage. Indeed, Kazemi et al. (2024) stated that molasses addition and increasing doses compared to control decreased the NDF content of common reed silage. These results support the findings of the current study.

Table 2. The CP, NDF, ADF, and RA of Common reed silage

Applications	CP ratio (%)	NDF ratio (%)	ADF ratio (%)	RA ratio (%)
0% Control	6.82e	70.74a	47.98a	9.27f
5% AG	8.29b-e	68.26ab	45.69ab	9.81def
10% AG	8.82abc	65.06bcd	45.77ab	11.04ab
15% AG	9.43ab	64.72bcd	47.71a	11.06ab
5% SBM	7.77cde	62.14cb	45.53ab	11.14ab
10% SBM	8.28b-e	61.09cde	42.66bc	11.33ab
15% SBM	8.74a-d	61.05cde	40.47cde	11.63a
5% CC	7.27de	66.68abc	45.59ab	10.75abc
10% CC	7.57cde	63.30bcd	42.33cd	10.18cde
15% CC	7.71cde	60.09de	37.77ef	9.69ef
5% AG+ 5% SBM	9.28ab	56.41ef	36.88f	11.52a
10% AG + 10% SBM	9.75ab	54.58f	39.39def	11.35ab
15% AG + 15% SBM	9.95a	51.31f	40.31cde	10.55bcd
F value and significance	8.63**	18.22**	26.17**	14.88**

Means shown by different letters in the same column are significantly different by Duncan at $p<0.01$. AG; Alfalfa grass, SBM; Sugar beet molasses, CC; Crushed corn, NDF; Neutral detergent fiber, ADF; Acid detergent fiber, CP; Crude protein, RA; Raw ash.

When Table 2 was examined, the ADF content of common reed silage varied between 36.88% and 47.98%. In general, additives decreased the ADF content of silage compared to the control (except alfalfa). The highest ADF rate was determined in the control (47.98%) and 15% alfalfa (47.71%) additive applications in the same statistical group, while the lowest ADF rate in silage was determined in the 15% corn application followed by 5% alfalfa + 5% molasses application. On the other hand, increasing molasses and corn crushing doses decreased the silage ADF rates, while increasing alfalfa and alfalfa + molasses applications increased the silage ADF rates. This may be due to the higher ADF content of alfalfa compared to other additives. Because the alfalfa plant contains more lignin than carbohydrate-rich cereals. This is because alfalfa has a leaf steam and nerves rich in lignin content (Fales and Fritz, 2007).

The raw ash content of common reed silage varied between 9.27% and 11.63% and all applied additives increased the raw ash content of silage compared to the control (Table 2). On the other hand, increasing alfalfa and molasses additive doses increased the raw ash content of silage, while increasing doses of corn and alfalfa + molasses additive decreased it. Accordingly, the highest raw ash ratio was obtained in 15% molasses and 5% alfalfa + 5% molasses applications and these two applications were statistically in the same group. The lowest raw ash content was determined in the control group following 15% corn crushing.

3.3. Lactic acid, acetic acid, propionic acid and butyric acid

The lactic acid content of the silo feed obtained varied between 0.843% and 5.320%, and the highest lactic acid content was determined in the 15% molasses additive application (5.320%) followed

by the 10% molasses application (4.823%) (Table 3). The lowest lactic acid rate was determined in the 15% alfalfa additive application and this was followed by the control (%), 5% alfalfa, 10% alfalfa, and 5% molasses additive applications in the same statistical group. These results showed that additives, except for the alfalfa additive, generally increased the lactic acid content of silage. This may be due to the high soluble sugar content of molasses and crushed corn. Because molasses and corn have high water-soluble carbohydrate (WSC) content in dry matter (Canbolat et al., 2010). In general, in silages, the pH should fall between 3.5 and 4.0 for fermentation to develop healthily and the lactic acid content should increase to 2.0% and above for the silage material to be stored for a long time without spoiling (McDonald et al., 1991). According to this information, it was observed that other additives, except for the control and alfalfa applications, provided the desired lactic acid rate.

Table 3. The lactic, acetic, propionic, and butyric acid of Common reed silage

Applications	Lactic acid (%)	Acetic acid (%)	Propionic acid (%)	Butyric acid (%)
0% Control	1.383f	1.207c	0.093e	0.110c
5% AG	1.360f	1.317c	0.217d	0.220b
10% AG	1.220f	1.587b	0.377c	0.227b
15% AG	0.843g	1.747b	0.530a	0.280a
5% SBM	3.253c	0.743d	0.083ef	0.047d
10% SBM	4.823b	0.630de	0.070ef	0.020d
15% SBM	5.320a	0.447e	0.047f	0.020d
5% CC	1.483f	0.633de	0.060ef	0.023d
10% CC	2.347e	0.577de	0.047f	0.020d
15% CC	2.677d	0.553de	0.073ef	0.023d
5% AG+ 5% SBM	3.303c	1.210c	0.213d	0.193b
10% AG + 10% SBM	3.107c	1.753b	0.353c	0.123c
15% AG + 15% SBM	3.093c	1.973a	0.447b	0.113c
F value and significance	304.69**	97.03**	387.20**	62.85**

Means shown by different letters in the same column are significantly different by Duncan at $p<0.01$. AG; Alfalfa grass, SBM; Sugar beet molasses, CC; Crushed corn.

When Table 3 was examined, it was seen that molasses and crushed corn silage decreased the acetic acid content compared to the control and also that the acetic acid content decreased as the ratio of these two additives increased. This may be due to the high soluble sugar content of molasses and corn crushed. Accordingly, the acetic acid ratio in molasses applications varied between 0.447% and 0.743%, and in cracked corn applications it varied between 0.553% and 0.633%. These results showed that acetic acid was in the required range (0.3-0.8%) in a good silage (Menke and Huss, 1975). On the other hand, alfalfa and alfalfa + molasses additives and also the increasing doses of these additives generally increased the acetic acid content of the silage (Table 3). This increase may have been due to the alfalfa hay used as an additive. Because the water-soluble carbohydrate content in legume forage plants is low and the buffer capacity is high. This also slows down the pH drop (McDonald et al., 1991; Albrect and Muck, 1991).

When Table 3 is examined, it was determined that alfalfa and alfalfa + molasses additives and increasing doses of these additives increased the silage propionic acid content compared to the control. However, it was determined that crushed corn and molasses additives decreased the silage propionic acid content. According to these data, the highest silage propionic acid content (0.530%) was determined in 15% alfalfa additive, the lowest propionic acid rate was determined in 15% molasses and 10% crushed corn applications, and these two applications were statistically in the same group.

In the present study, the Butyric acid content of the silage obtained varied between 0.020% and 0.280% (Table 3). It has been observed that these rates are at the required levels in silo feeds. In fact, in a quality silo feed, the butyric acid content is desired to be between 0.1% and 0.7% (Weinberg and Ashbell, 2003). When Table 3 was examined, it was seen that alfalfa and alfalfa + molasses applications increased the butyric acid content of the silage compared to the control, and the highest butyric acid rate was determined in the 15% alfalfa additive application. However, crushed corn and molasses additives reduced the butyric acid content of silage compared to the control and the butyric acid content remained

at the lowest level in these applications. This may be because molasses and crushed corn with high soluble sugar content cause more lactic acid formation in the silage. Indeed, when the appropriate pH and dry matter ratio are provided in silo feeds, lactic acid production is high and the undesirable butyric acid content is low (Canbolat et al., 2010).

4. Conclusion

When compared with the control, it was determined that all additives used and increasing doses generally increased the crude protein and raw ash content of the silo feed and decreased the ammonia, NDF, and ADF content. On the other hand, molasses, crushed corn (except alfalfa), and their increasing doses increased the Fleig score and lactic acid content of the silage, while decreasing the acetic acid, propionic acid, and butyric acid contents compared to the control. As a result of the study, it was concluded that Common reed can be evaluated as an alternative silo feed in animal feeding.

Ethical Statement

This study does not require Ethics Committee Approval.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Author Contributions

Authors contributed equally.

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Research Article

Investigation of Virus and Viroid Diseases in Cucurbits

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Abstract: Cucurbita species are economically important vegetables, with Türkiye ranking second in the world for gherkin and cucumber production and sixth for pumpkin and squash production. However, diseases caused by viruses are a threat to global cucurbit production. Due to global warming, changes in agricultural practices, and advancements in virus detection, the number of viruses infecting cucurbits has significantly increased in recent years. In this study, to identify causal viruses (potyviruses and begomoviruses) and viroids (pospiviroids) that induced symptoms on cucurbits, leaves exhibiting virus-like symptoms were surveyed and collected from cultivation areas in Türkiye from July to September 2024, and subjected to detection of causal agents. A total of 150 plant samples were collected from three locations, with plants randomly selected and viral infection symptoms observed. RT-PCR assays for potyviruses identified watermelon mosaic virus (WMV) in 30 samples (20%), which included 11 snake melon, 3 pumpkin, and 16 zucchini samples. Additionally, PCR assays for pospiviroids successfully detected pepper chat fruit viroid (PCFVd) in six samples, including zucchini and snake melon. Amplified products of the expected size were sequenced and showed identities of over 95% with PCFVd isolates in NCBI. This study reports for the first time the identification of zucchini and snake melon as natural hosts of PCFVd.

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1. Introduction

Cucurbita species are among the 10 leading vegetable species (Ferriol and Pico, 2008). Türkiye is the world's second-largest producer of gherkins and cucumbers, with a production of 1.9 million tons. Additionally, it ranked sixth in the world for pumpkin, squash, and gourd production, with an estimated 744.30 tons produced (FAO, 2022). Cucurbit-producing countries must enhance production quality and quantity due to their economic significance. Cucurbits are threatened by various pathogens, including viruses, which vary greatly from country to country. In terms of changes in the viruses that infect cucurbit crops over the years, there were reports that there have been 23 different viruses in 1980, 55 in 2003, 70 in 2014, and 96 in 2023 (Lecoq, 2003; Lecoq and Katis, 2014; Ali, 2023). Climate change, particularly global warming, has led to an expansion in the distribution and population density of insect vectors, such as aphids and whiteflies, which are responsible for transmitting many plant viruses. In addition, intensified agricultural practices, including monoculture cultivation and longer cropping cycles

have created favourable conditions for viruses to emerge and spread. Moreover, advancements in molecular diagnostic tools, including high-throughput sequencing (HTS) and real-time PCR, have markedly increased the sensitivity, specificity, and throughput of virus detection, enabling the identification of previously unrecognized viral species. Cucurbit crops are infected by economically important viruses that belong to different families, such as Geminiviridae, Potyviridae, Bromoviridae, and Luteoviridae (Adams et al., 2012; Lecoq and Desbiez, 2012). The genus *Potyvirus* is the largest genus of the family Potyviridae (Berger et al., 2005). Some of the viruses are quite widespread and cause significant yield losses, while others cause infection in specific crops in limited geographical areas and do not cause significant economic damage. At least twenty potyviruses have been identified as significant threats to cucurbit crop production in the Mediterranean region (Sastry et al., 2019; Desbiez et al., 2020). Among these, zucchini yellow mosaic virus (ZYMV), papaya ringspot virus (PRSV) and watermelon mosaic virus (WMV) are the most common and economically important pathogens infecting cucurbits (Lecoq et al., 2001; Sharma et al., 2013). Potyviruses are efficiently transmitted in a non-persistent manner by many different aphid species and mechanically through plant-to-plant contact or pruning tools (Fauquet et al., 2005; Mishra et al., 2013). Potyviruses have a single-stranded, positive-sense RNA genome of approximately 10 kb characterised a 5' untranslated region (UTR), a single major open reading frame (ORF), and a 3' UTR ending with a poly adenine (Poly A) tail (Sharma et al., 2013). The *Potyvirus* genus encompasses over 180 species, yet only a limited number have been fully sequenced. While *Potyvirus* species exhibit considerable similarity in their genome organization, they display substantial genetic diversity at the nucleotide sequence level (Zhao et al., 2011). The host range of most *Potyvirus* species is relatively narrow; however, bean yellow mosaic virus, turnip mosaic virus, and WMV represent notable exceptions, each demonstrating the ability to infect a minimum of 12 plant families (Moury and Desbiez, 2020).

The family Geminiviridae is one of the largest family with circular single-stranded DNA genomes encapsidated in icosahedral particles (Zerbini et al., 2017). A large number of plant viruses that cause infection in economically important plants are included in this group. The genus *Begomovirus* is included in the family Geminiviridae and contains more than 320 species, accounting for approximately 88% of the total species in this family (Devendran et al., 2022). Begomoviruses are a group of plant viruses that are very important in crops due to their significant effects on agriculture and food production. They have been reported to be widespread in many regions of the world and are important for both developed and developing countries as they threaten the cultivation of economically important crops (Malathi et al., 2017). Begomoviruses, which primarily infect dicotyledonous plants, possess a single-stranded DNA (ssDNA) genome that may be organized either as a monopartite (DNA-A, approximately 2.8 kb) or bipartite (DNA-A and DNA-B, each approximately 2.5–2.7 kb) (Kumar, 2019). These viruses are persistently and non-propagatively transmitted by *Bemisia tabaci* (Hemiptera: Aleyrodidae) (Thompson, 2011). Tomato leaf curl New Delhi virus (ToLCNDV) is a member of *Begomovirus*, containing two circular single-stranded DNA molecules of about 2.5–2.7 kb, referred to as DNA-A and DNA-B (Moriones et al., 2017). ToLCNDV causes major yield reductions in a variety of crops belonging to the Solanaceae and Cucurbitaceae families (Hussain et al., 2004; Lopez et al., 2015). The presence of the virus has been increasing every year and is reported to cause significant economic losses in cucurbit production in many countries (Panno et al., 2016; Venkataravanappa et al., 2020; Siskos et al., 2022). Due to the wide host range of begomoviruses, their high recombination ability and effective transmission by their vector, and their synergistic interaction with different viruses in the host plant, they have a high risk of spreading over large areas in production areas in a short time and causing epidemics. Therefore, identifying the most commonly occurring and damaging viruses is critical for recommending management strategies.

Viroids are classified into two families: Pospiviroidae and Avsunviroidae, with more than 30 species identified worldwide (Di Serio et al., 2017; Matsushita et al., 2018). Among these viroid species, hop stunt viroid (HSVd) has been reported only in cucumbers under natural conditions (Sano, 2003; Lemmetty et al., 2011). Hence, the knowledge of viroids infecting cucurbit plants is limited. The present aimed to gain insight into the presence and evaluate the coexistence of viruses (potyviruses and begomoviruses) and viroids (pospiviroids) in the main cucurbit production areas of Türkiye.

2. Material and Methods

2.1. Field surveys and sampling

A survey was conducted on screening of leaves and fruits of different Cucurbitaceae species in the cities of Hatay, Kahramanmaraş, and Kayseri, Türkiye, from July to September 2024. All samples, including 45 snake melon, 45 pumpkin, and 60 zucchini leaf samples exhibiting virus-like symptoms such as mosaic, yellowing, mottle, leaf curling, and vein clearing (Figure 1) were randomly chosen from the fields.

2.2. Extraction of total RNA and total DNA

Following the cetyl trimethyl ammonium bromide (CTAB) method as described by Li et al. (2008), a singular nucleic acid extraction protocol was implemented for both DNA and RNA virus extractions. Briefly, 100 mg of leaf tissue ground in 1 ml of CTAB extraction buffer (100 Mm Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% CTAB, and 0.2% β-mercaptoethanol). The samples were transferred to eppendorf tubes and incubated at 65 °C for 15 min, and centrifuged at 10.000xg for 10 min. The supernatant was transferred to a microcentrifuge tube and mixed with an equal volume of chloroform/isoamyl alcohol (24:1), and the mixture was centrifuged at 15.000xg for 10 min at room temperature. The top aqueous phase was transferred to a new tube. About 0.7 volume of cold isopropanol was added, and the mixtures were centrifuged for 10 min at 15.000xg to collect the nucleic acid precipitate. The precipitated nucleic acid was washed with 70% cold ethanol. The ethanol was decanted and residual ethanol was removed by drying at room temperature. The pellet was dissolved in 50 µl sterile ddH_2O. The amount and quality of the RNA and DNA were assessed using a NanoDrop spectrophotometer (ThermoFisher Scientific, USA).

2.3. Complementary DNA synthesis and polymerase chain reaction

The complementary DNA was synthesized from the total RNA extracted as described above, by reverse transcription using the M-MLV RT reverse transcriptase (ThermoFisher Scientific, USA) and random hexamer primers. To identify the species of *Potyvirus* in the cucurbit samples RNA was used in reverse-transcription polymerase chain reaction (RT-PCR) using universal NIb2F and NIb3R primers (Zheng et al., 2010). To search for begomovirus infection, the samples were tested by polymerase chain reaction (PCR) with universal degenerate primers for begomoviruses Begomo-F and Begomo-R (Akhter et al., 2009), and ToLCNDV specific (DNA-A F/R) primer pairs (Kil et al., 2020) (Table 1). Additionally, PCR was performed with universal pospiviroid (Pospi1-F/RE) (Verhoeven et al., 2004) and PCFVd-specific primer (PCF-seq-F/R) (Yanagisawa and Matsushita, 2017) sets to investigate the *Pospiviroid* in cucurbits (Table 1). PCR products were separated by agarose gel electrophoresis and visualized using ethidium bromide staining. PCR products were sequenced in both directions using Sanger sequencing.

2.4. Sequence analysis and construction of phylogenetic trees

Multiple sequence alignments were made using the CLUSTAL W algorithm (Thompson et al., 1994) and BLASTn was performed to check the species homology. For phylogenetic analysis of WMV isolates, a total of 11 isolates were chosen considering different districts and host species. Sequences of WMV (accession numbers: PV582057-PV582067.1) and PCFVd (accession numbers: PQ741727-PQ741732.1) were deposited in GenBank (Supplementary Table 1). The phylogenetic trees were constructed using the MEGA software version 7 (Kumar et al., 2016) based on methods of neighbor-joining and Kimura 2-parameter. Bootstrap resampling (1000 replicates) was used to ensure the reliability of individual nodes in the phylogenetic tree. SDT (sequence demarcation tool) analysis was carried out by using the SDTv1.2 program (Muhire et al., 2014) with defult setting.

Table 1. List of universal and specific primers used to detect Potyvirus, Begomovirus, and Pospiviroid in this study

Primers	Pathogen	Primer Sequence (5'-3')	References
NIb2F NIb3R	*Potyvirus*	GTITGYGTIGAYGAYTTYAAYAA TCIACIACIGTIGAIGGYTGNCC	Zheng et al., 2010
Begomo-F Begomo-R	*Begomovirus*	ACGCGTGCCGTGCTGCTGCTGCCCCCA ACGCGTATGGGCTGYCGAAGTTSAGACG	Akhter et al., 2009
ToLCNDV DNA-A_F ToLCNDV DNA-A_R	*Begomovirus* tomato leaf curl New Delhi virus	GTGATGTACTCCCCTGTGCG ACAAGACAGATGCGTTAAAGGTT	Kil et al., 2020
Pospi1-FW Pospi1-RE	*Pospiviroid*	GGGATCCCCGGGAAAC AGCTTCAGTTGT(T/A)TCCACCGGGT	Verhoeven et al., 2004
PCF-seq-F PCF-seq-R	*Pospiviroid* pepper chat fruit viroid	CCGTCTTCTGACAGGAGTAATCCC ACCCGCACGGCGCTTCTC	Yanagisawa and Matsushita, 2017

3. Results

3.1. Surveys and detection of watermelon mosaic virus and pepper chat fruit viroid by polymerase chain reaction

A total of 150 plant samples were collected from 3 locations in Türkiye. Plants were collected randomly, and viral infection symptoms observed were recorded. The symptoms included severe to mild mosaic, especially newly growing apical leaves of pumpkin and snake melon samples showed severe mosaic symptoms (Figure 1). The squash leaf samples showed the symptoms of leaf curling, yellowing, mottling, mosaic, fan leaf appearance and vein clearing (Figure 1).

Figure 1. Field survey observations of virus-like symptoms in cucurbit crops and reverse-transcription polymerase chain reaction products of a) Potyvirus (NIb2F/NIb3R), b) Pospiviroid (Pospi1-RE/FW), c) pepper chat fruit viroid (PCF-seq-F/R).

Each cucurbit leaf sample was screened RT-PCR with NIb2F/NIb3R universal polerovirus primer pairs, resulting in the amplification of the expected 350 bp fragment from 11 snake melon, 3 pumpkin, and 16 zucchini samples (Figure 1a). Sequence analysis of these amplicons confirmed the out of 30 samples (20%) collected from three provinces was infected with watermelon mosaic virus (WMV) (Table 2). In addition, PCRs conducted with universal begomovirus and ToLCNDV-specific primers yielded no DNA amplification products.

Table 2. Sample numbers for each species, and numbers of infected samples by watermelon mosaic virus and pepper chat fruit viroid as assessed by reverse-transcription polymerase chain reaction

Viral pathogen	Snake melon	Zucchini	Pumpkin	Infected sample /Total number of sample
Watermelon mosaic virus	11/45	16/60	3/45	30/150
Pepper chat fruit viroid	2/40	4/50	Not detected	6/150

Moreover, RT-PCR was performed on the samples using the universal primer sets Pospi1-RE/FW (Verhoeven et al., 2004). Six samples produced amplicons of 189 bp with Pospi1-FW and Pospi1-RE primer pairs, which were then directly sequenced (Figure 1b). Sequencing of the amplicons showed identities of more than 96% identity with PCFVd isolates (KC762954.1, MW422292.1, and MW422290.1) in NCBI. To verify the presence of PCFVd in field samples, RT-PCR was performed using two pairs of PCFVd-specific primers (PCF-seq-F/R) (Yanagisawa and Matsushita, 2017). PCR products with the expected size (Figure 1c), obtained from 2 snake melon and 4 zucchini samples, were purified from agarose gel by the use of a Qiaquick gel extraction kit and subjected to bidirectional Sanger sequencing to confirm the presence of the viroid.

3.2. Sequence and phylogenetic analyses

Pairwise nucleotide sequence identities of the eleven WMV isolates obtained in the present study, determined by CLUSTAL W alignment, ranged from 91% to 100% (Figure 2a), whereas they shared the maximum nucleotide sequence identities of 91-99% with other isolates of WMV. Phylogenetic analysis of WMV isolates showed that sequences were grouped into several clusters (Figure 2b). Three of the WMV isolates (WMV21, WMV38, WMV49) were obtained from zucchini clustered with isolates from Iraq and Iran (MT780536.1, MT780537.1), France (EU660581.1, EU660586.1, JF273463.1, JF273467.1) and Spain (MH469650.1). Whereas four isolates (WMV9, WMV15, WMV18, WMV23), obtained from snake melon and zucchini, were clustered into an independent clade, shared the highest nucleotide identities at 95% to reference isolates. The other four isolates (WMV2, WMV19, WMV26, WMV44), obtained from zucchini and pumpkin, clustered separate clade the others with 99% bootstrap value (Figure 2b). Phylogenetic analysis show that the clustering WMV isolates were not associated with geographic origins or host species.

Pairwise sequence identity matrix from nucleotide (Figure 3a) sequences generated using the SDT 1.2 software, and six PCFVd-cucurbit isolates obtained in the present study were 100% identical to each other. The phylogenetic analysis of the PCFVd-cucurbit isolates was done to infer the relationship of the current isolates with the previously reported isolates in NCBI. Six PCFVd-cucurbit sequences were analysed, and the sequences were retrieved from the NCBI after BLASTn of these sequences. The BLASTn search of the NCBI database revealed high sequence identities (95% to 99.71%) with previously reported PCFVd isolates from different geographic regions. Furthermore, phylogenetic analysis showed that the six PCFVd-cucurbit isolates clustered together with isolates from tomato (KC762953.1, KC762954.1, MW422292.1) from Australia (originated in Israel) and the Netherlands, as well as isolates from pepper (MW012406.1, MW012415.1, MW422288.1) from Vietnam and the Netherlands (Figure 3b). Notably, this is the first report of PCFVd in zucchini and snake melon worldwide.

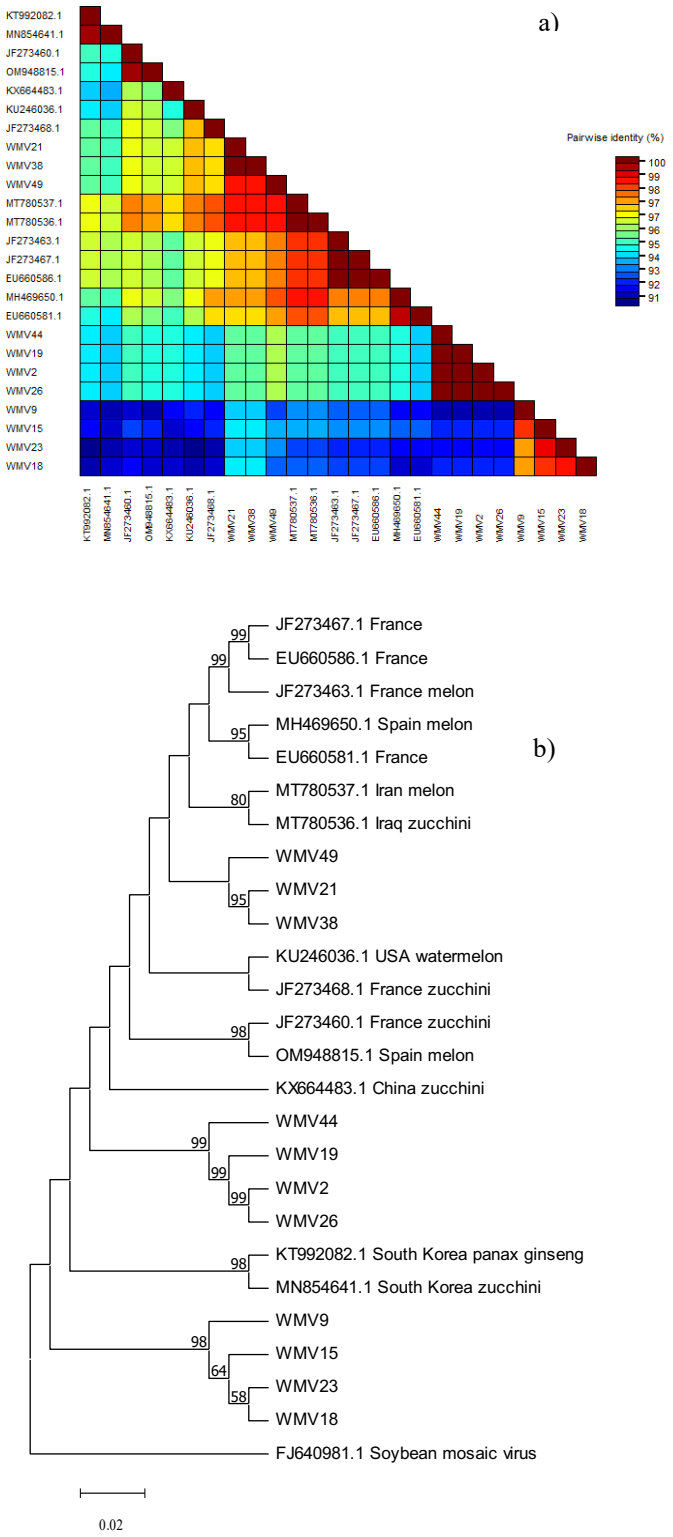

Figure 2. Sequence comparison and phylogenetic analysis of watermelon mosaic virus (WMV) isolates. The pairwise identity scores of WMV were generated using Sequence Demarcation Tool version 1.2 software (a). Phylogenetic tree showing relationship of WMV isolates in this study and from NCBI database (b). Soybean mosaic virus (FJ6440981.1) was used as the outgroup.

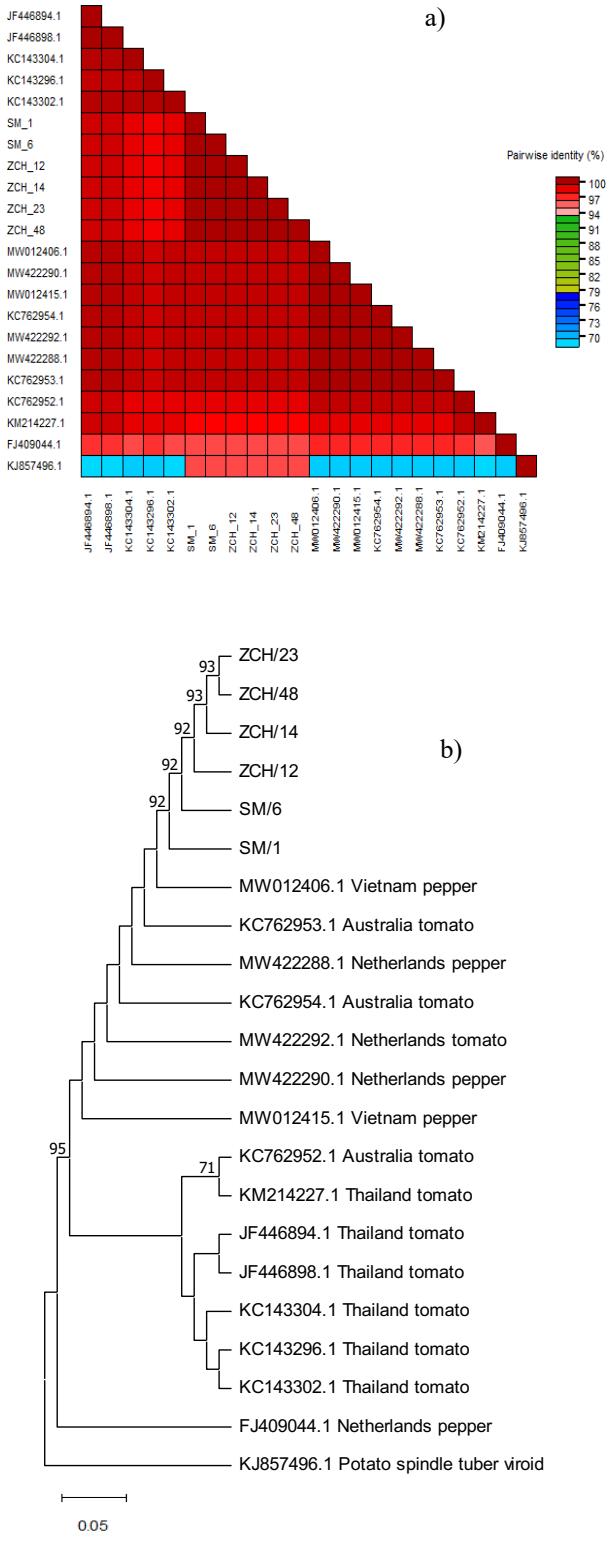

Figure 3. Sequence comparison and phylogenetic analysis of pepper chat fruit viroid (PCFVd) isolates. The pairwise identity scores of PCFVd isolates were generated using Sequence Demarcation Tool version 1.2 software (a). Phylogenetic tree showing relationship of PCFVd isolates in this study and from NCBI database (b). Potato spindle tuber viroid (KJ857496.1) was used as the outgroup.

4. Discussion

WMV, alongside with zucchini yellow mosaic virus (ZYMV) and papaya ringspot virus (PRSV), is one of the most destructive potyviruses which cause serious yield losses to cucurbit crops around the world (De Moya-Ruiz et al., 2023; Sharma, 2023). WMV have been commonly recorded in squash, watermelon, melon, pumpkin (Moreno et al., 2004; Yeşil, 2019; Pérez-de-Castro et al., 2020) and, has even been detected in snake melons and bottle gourds for the first time (Güller et al., 2024). Consistent with previous studies, we also detected WMV infection in 11 snake melon samples. Moreover, WMV is not seed-borne in cucurbits but can be transmitted non-persistently by at least 35 species of aphids, allowing the virus to spread rapidly among cucurbit plants. The geographical distribution of WMV is influenced by a range of factors, including the ecology of aphid vectors, the presence of wild plant host species, and the impacts of climate change (De Moya-Ruiz et al., 2023). Specifically in the Mediterranean basin, WMV has been reported in most countries as one of the dominant cucurbit viruses (Moreno et al., 2004; Pérez-de-Castro et al., 2020; De Moya-Ruiz et al., 2021). The high diversity of host plants and weeds in production areas contributes to the increased prevalence and infection rates of viral diseases (McLeish et al., 2017). Climate change, in particular, can extend growing seasons, allowing for more generations of vectors and increased opportunities for virus transmission. The interaction between aphid populations and the virus can lead to variations in the prevalence of WMV across different regions and cropping systems. Additionally, the global trade of vegetatively propagated plants has the potential to accelerate the spread of the virus, thereby increasing the molecular diversity within its population (Desbiez et al., 2020). Utilizing resistant cultivars is a recommended strategy for managing WMV, as it has been shown to effectively reduce both virus titers and symptom severity across various isolates (Díaz-Pendón et al., 2005). Besides, the use of silver reflective plastic mulches at planting is an effective management strategy to delay WMV infection in young cucurbit plants. These mulches repel aphid vectors by reflecting light, thereby reducing early-season virus transmission and promoting healthy initial plant growth until canopy closure diminishes their efficacy.

Whitefly-transmitted begomovirus infections on cucurbits has been recognized as an emerging disease in various regions worldwide, resulting in significant yield losses (Juárez et al., 2014; Yazdani-Khameneh et al., 2016; Cai et al., 2023; Troiano and Parrella, 2023). Fidan et al. (2023) reported the presence of ToLCNDV in cucurbits grown in greenhouses in Türkiye. However, in the present study, despite the observation of symptomatic plants exhibiting leaf curling, yellowing, and stunting, ToLCNDV was not detected in cucurbits. Pepper chat fruit viroid, *Pospiviroid parvicapsici*, belongs to the genus *Pospiviroid* of the family Pospiviroidae (Walker et al., 2021). PCFVd was initially identified as a novel pospiviroid species that infected sweet peppers (*Capsicum annuum* L.) (Verhoeven et al., 2009), later reported in tomato and pepper cultivars in Thailand, Vietnam and Australia (Yanagisawa and Matsushita, 2017; Reanwarakorn et al., 2011; Keyata et al., 2024). This is the first report of cucurbits being a natural host of PCFVd worldwide.

Conclusion

Cultivated under open field conditions in Türkiye indicating a natural occurrence of PCFVd in zucchini and snake melon. PCFVd was initially found in pepper and subsequently in tomato crops in various regions around the world. The detection of PCFVd in zucchini and snake melon represents a novel finding. The host range of viroids is narrow, but in recent years there have been studies showing that the host range of some agents has expanded. In most cases the origin of these infections remained unknown. The presence of WMV and PCFVd highlights the ongoing viral threats to cucurbit production, while the absence of begomoviruses in the surveyed samples is noteworthy.

Ethical Statement

Ethical approval is not required for this study.

Conflict of Interest

The author declares that there are no conflicts of interest.

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Author Contributions

The author confirms sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

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