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Research Article

Least Squares, Simple Linear Regression, and Pearson Correlation Analysis between the Lactation Milk Yield of Norduz Ewes and Their Lambs' Live Weight

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Correlation,
Growth,
Lactation milk yield,
Live weight,
Norduz sheep

Abstract: This study aimed to determine the lactation milk yield of Norduz ewes and to determine the effects of milk yield on their lambs' growth and development characteristics. Additionally, the correlations between the sheep's milk yield, lambs' live weight, and affecting factors were determined. The study was conducted at the Van University of Yüzüncü Yıl Agricultural Research Center. In the study, 61 Norduz ewes and 66 male and female lambs they bore were included. The sheep were milked at 30-day intervals until the 180th day. Similarly, the lambs were weighed at 30-day intervals from birth to 180th day. The means and standard errors of the milk yields were obtained on the 30, 60, 90, 120, 150th, and 180th days of milking. The live weight means and standard errors of the lambs on the 30th, 60th, 90th, 120th, 150th, and 180th days are given. According to the GLM analysis results, sex significantly influenced lactation length ($p<0.05$), and there was a difference in favor of female lambs. The impact of dam age was notably significant on live weight at days 60 and 90 ($p<0.05$). Additionally, birth type significantly affected live weight at day 30 and 60 ($p<0.001$), as well as at day 90 ($p<0.01$). Regarding the linear relationship between dam weight and live weight, a significant effect was observed at day 30 ($p<0.001$), day 60 ($p<0.01$), and day 90 ($p<0.05$). Notably, the linear effects of lamb birth weight on live weight remained significant across all control periods ($p<0.001$).

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1. Introduction

Norduz sheep are bred in the Gürpınar district (Van, Türkiye). They are white, ash, gray, and brown–white in color. Black spots can be found on the head, feet, and chest, particularly. Half of the ewes and most of the rams are horned. The sheep are combined productive. The sheep have a high-built body and are a fat-tailed breed (Anonymous, 2022a and 2022b; Çelikyürek, 2023). Norduz sheep are a newly registered sheep breed. There are very few studies on this breed of sheep. Studies are needed to determine the morphological, physiological, and other breed characteristics of these sheep breeds, which have 13 pairs of ribs (Çelikyürek, 2022).

The study focused on determining the live weights of Norduz lambs up to the 180th day, along with assessing lactation milk yield and lactation duration in Norduz sheep. The investigation aimed to uncover the impact of various factors including sheep lactation milk yield, lactation length, age, sex, birth type, dam weight, and lamb birth weight on the live weights of lambs.

Milk yield control determines how much milk an ewe gives in a day and during lactation. Milk yield controls can ensure that breeding candidates with good breeding value can be identified and that low-productive individuals in the flock can be identified and accurately culled so that the flock can be fed according to its actual performance level. Damage to the farm can be minimized by the early diagnosis and treatment of mastitis thanks to continuous controls (Şahin & Akmaz, 2004).

In current sheep farming, milk revenue is second only to lamb production. However, sheep milk is an important source of protein in terms of nutritional value and taste, despite little use in human daily life. With its high-fat content, sheep milk also has positive effects on physical development compared to goat and cow milk.

One of the most important features with economic value in sheep breeding is growth. Growth can be defined as the change that occurs in the weight and body dimensions of the living thing in a certain period of time (Eyduran et al., 2009; Yıldız et al., 2009; Kum et al., 2010). Changes in live weight and body measurements are directly related to meat production. Sheep meat production is directly related to the number of lambs produced per unit sheep and the meat production capabilities of lambs. Meat production capabilities of lambs can be defined by live weight and live weight increase rate (Karaca et al., 1990; Altın & Çelikyürek, 1996).

Since sheep are an important branch of animal husbandry in Turkey in terms of milk yield, many studies to determine milk yield have been conducted. However, there are almost no studies on Norduz sheep. Norduz sheep, as a breed, meet the region's milk and meat producers' requirements for regional sheep production and are well adapted to the specificities of the region. Norduz sheep are also an important potential resource in terms of their usability in the genetic breeding of livestock in the region.

2. Material and Methods

2.1. Animal materials

The study was conducted at the Agricultural Application and Research Enterprise in Van-Turkey in 2020. The sheep were mated using the natural method. The rams were always kept in the herd. Therefore, the birth of lambs began in February and continued until April. The sheep were kept in closed sheepfolds from November to May. This study included 61 Norduz ewes and 66 lambs. Twelve of the lambs were twins, and 54 lambs were singletons. The birth weights of lambs and ewes were taken using a 10-g scale within the first 24 hours after birth. On inspection days, the lambs were separated from their mothers at night. In the morning, they were weighed on empty stomachs. Live weights were weighed at 30-day intervals from birth to the 180th day. The interpolation method was used to determine the live weights of lambs born at certain intervals on the specified control days. Ewes were milked on the 30, 60, 90, 120, 150th, and 180th days, and milk yield was recorded. The milking process continued until the amount of milk obtained decreased to 0.10 ml. Ewes and their lambs were fed indoors for the first three months of the season and then grazed part-time on the pastureland of Van Yüzüncü Yıl University. In the sheepfold, dry-rough grass or silage feed is given to the sheep. Sheep are not given milk feed or fattening feed. The lambs were weaned at 90 days of age, on average.

2.2. Statistical analysis

The data underwent analysis through the least squares method and the general linear model procedure (GLM). Simple linear regression was employed to assess the impact of milk yield on days 30, 60, 90, 120, 150, and 180 on live weight. Pearson's correlation analysis was utilized to determine trait relationships, including the correlation between milk yield and body weight on each control day. Statistical analyses were conducted using R (2023) and SAS (2023) software programs.

2.3. Mathematical models

The lactation milk yield of ewes can be calculated using the Trapezoidal Method. Here's a general format of the formula used in such calculations:

$$X_{Trapez} = [(k_1.A) + \left(\frac{k_1+k_2}{2}\right).a_1 + \cdots + \left(\frac{k_{n-1}+k_n}{2}\right).(k_n.C)] \quad (1)$$

The following mathematical model was used for the milk yield.

$$MY_{ijk} = \mu + a_i + b_j + c_k + b_1\left(X_{1ijk} - \bar{X}_1\right) + b_2\left(X_{2ijk} - \bar{X}_2\right) + e_{ijk} \quad (2)$$

The following mathematical model was used for the live weight.

$$LW_{ijk} = \mu + a_i + b_j + c_k + b_1\left(X_{1ijk} - \bar{X}_1\right) + b_2\left(X_{2ijk} - \bar{X}_2\right) + e_{ijk} \quad (3)$$

3. Results and Discussion

The study was conducted in a sheepfold where sheep were housed in the winter and feeding was done indoors. In the sheepfold, dry-rough grass or silage feed is given to the sheep. Sheep are not given milk feed or fattening feed. Therefore, in this type of breeding system, sheep's milk (lactation milk yield; 53.934±2.31 l) is only enough to feed the lamb. It is thought that the milk obtained during milking is not enough to meet the labor and the workforce required for milking. However, according to the results of the literature reviewed, it was concluded that milking the milk obtained in rich pasture conditions together with lamb production could be economical. In such a breeding system, after the sheep's milk is milked, the remaining part is given to the lamb. As a result of this, the live weights of the lambs on the control days were found to be lower than the values in this study. After the lambs were taken to pasture, it was found that they closed this difference and had a higher average body weight.

3.1. Milk yield of Norduz ewes

The results of the data obtained are given in Table 1. Accordingly, 30, 60, 90, 90, 120, 150, and 180th day's average milk yields were 0.315±0.02, 0.465±0.02, 0.310±0.02, 0.232±0.01, 0.228±0.02 and 0.079±0.01 *l*, respectively. As per the trapezoidal calculation method (Berger & Thomas, 2013; Kaymakçı, 2013), the average lactation milk yield was 53.934±2.31 *l*. The lactation length was 178.24±1.24 days. From the results above, we see that the milk yield of sheep increased from the 30th to the 60th day and then decreased, and this decrease continued until the 180th day.

Bingöl (1998) determined a lactation milk yield of 132.78±2.70 *l* and a lactation length of 183.37±1.34 days in Norduz sheep. Yılmaz et al. (2004) determined lactation milk yield and lactation length of 125.09±0.93 *l* and 179.17±0.80 days, respectively, and Ocak (2009) 137.24±2.74 *l* and 182.55±1.33 days, respectively. Koncagül et al. (2012) determined a lactation milk yield of 130.9±3.24 *l*. The values obtained in these studies were higher than those obtained in this study, especially in terms of lactation milk yield. One of the main reasons for this difference is that the studies were conducted in village conditions and, in addition, the sheep were grazed in meadows and pastures with rich vegetation. Another reason could be that an adequate care and feeding program is not implemented for the sheep in the farm.

The lactation milk yield and lactation duration were 395.92 *l* and 185.01 days in Sönmez elite sheep (Kaymakçı et al., 2002), 98.92±3.85 *l* and 180.26±4.06 days in Kıvırcık sheep (Alarslan and Aygün, 2019) and 96.41±3.466 *l* and 198.76±0.981 days in Akkaraman crossbred sheep (Aşkan and Aygün, 2020). It was found that the obtained values were higher than the lactation milk yield and lactation duration in the study.

Lactation milk yield and lactation duration were 62.32±5.76 *l* and 173.3±10.7 days in Akkaraman sheep (Altın, 2001), 110.05 *l* and 165.46 days in Ivesi sheep (Gürsu & Aygün, 2014), 65.5±5.3 *l* and 167.9±7.4 days in Karakaş sheep (Altın & Çelikyürek, 1996), 103.08±3.354 *l* and 168.01±2.868 days in Karya sheep (Bayar, 2015), 192.76 *l* and 152.20 days in sheep grass flocks (Kaymakçı et al., 2002), 123.96 *l* and 158.65 days in Sakız x Akkaraman (F1) crosses (Esen & Özbey, 2002). These values were higher than lactation milk yield but lower in relation to lactation duration.

In terms of lactation milk yield and lactation duration, 50.65±6.36 *l* and 144.8±11.86 days were obtained for Hamdani x Akkaraman (F1) crosses (Altın, 2001), 56.9±7.4 *l* and 147.8±10.3 days for

Hamdani x Karakaş crosses (Altın & Çelikyürek, 1996). These values were lower than the values in this study.

The effects of dam age, birth type, and sex on milk yield in all control days and lactation milk yield were not significant in Norduz sheep ($p>0.05$). As a result of the analysis, it was found that dam age and type of birth had an insignificant effect ($p>0.05$), while sex had a significant effect ($p<0.05$) on lactation length. According to the results, the effect of sex was significant ($p<0.05$) in favor of female lambs. While this result supports this study, Bingöl (1998) reported in their study on Norduz ewes that the influence of dam age and birth type on lactation length was not significant but that dam age had a significant effect on lactation milk yield ($p<0.05$).

In a study conducted by Yılmaz et al. (2004) on Norduz sheep, it was reported that the effects of dam age and parturition type were significant ($p<0.001$). Koncagül et al. (2012) reported that the effect of dam age on milk yield in lactation was significant, while the effect of lamb birth type was insignificant in Norduz sheep. The linear effects ($p>0.05$) of ewe and lamb weights at birth on milk yield as determined on control days were found to be insignificant (except for the 60^{th} day).

Table 1. Factors affecting the milk yield of sheep in various periods, lactation milk yield, and lactation length (*l*)

Factors	n	30th day ($\bar{X} \pm S_{\bar{X}}$)	60th day ($\bar{X} \pm S_{\bar{X}}$)	90th day ($\bar{X} \pm S_{\bar{X}}$)	120th day ($\bar{X} \pm S_{\bar{X}}$)	150th day ($\bar{X} \pm S_{\bar{X}}$)	180th day ($\bar{X} \pm S_{\bar{X}}$)	Lactation m.y. ($\bar{X} \pm S_{\bar{X}}$)	Lactation length (*day*) ($\bar{X} \pm S_{\bar{X}}$)
Means	**66**	0.315±0.02	0.465±0.02	0.310±0.02	0.232±0.01	0.228±0.02	0.079±0.01	53.934±2.31	178.24±1.24
Dam Age									
2	**15**	0.303±0.06	0.485±0.05	0.305±0.05	0.201±0.03	0.187±0.04	0.040±0.03	50.562±5.85	179.84±3.06
3	**28**	0.266±0.04	0.484±0.03	0.319±0.03	0.204±0.02	0.257±0.03	0.109±0.02	52.879±3.85	174.51±2.01
4	**23**	0.333±0.05	0.385±0.04	0.306±0.04	0.230±0.03	0.219±0.04	0.089±0.02	52.021±4.89	178.75±2.55
Birth Type									
Single	**54**	0.331±0.03	0.470±0.02	0.305±0.02	0.242±0.02	0.228±0.02	0.068±0.01	54.996±2.69	180.01±1.41
Twin	**12**	0.270±0.06	0.433±0.05	0.315±0.05	0.181±0.04	0.214±0.04	0.091±0.03	48.645±6.11	175.39±3.19
Sex									*
Male	**38**	0.290±0.04	0.471±0.03	0.320±0.03	0.216±0.02	0.190±0.03	0.082±0.02	50.897±3.86	174.88±2.02
Female	**28**	0.312±0.04	0.431±0.04	0.301±0.03	0.208±0.02	0.252±0.03	0.077±0.02	52.745±4.23	180.52±2.21
Regression (*Lin.*)									
Dam Weight at birth		0.003±0.00	0.006±0.00*	0.003±0.00	0.004±0.00	0.002±0.00	0.000±0.00	0.624±0.33	0.156±0.17
Lamb Birth Weight		0.043±0.03	-0.01±0.02	0.010±0.02	0.000±0.02	0.010±0.02	0.015±0.01	2.883±2.79	0.133±1.46

$\bar{X}$ = Means, $S_{\bar{x}}$ = Standard error, ($p<0.05$): *.

3.2. Live weights of Norduz lambs on control days

The results of the data obtained are given in Table 2. Accordingly, live weights of 11.663±0.25, 17.342±0.36, 24.635±0.52, 31.999±0.64, 34.482±0.70, and 36.225±0.79 *kg* were determined for the 30, 60, 90, 120, 150 and 180th day, respectively. In a study on Norduz lambs conducted by Bingöl (1998), the live weights obtained were higher than the values from day 30 to 120 (9.20±0.10, 14.58±0.26, 20.27±0.21 and 29.93±0.28 *kg*, respectively) but lower than the values from day 150 and 180 (37.44±027 and 40.92±0.28 *kg*, respectively). The reason for the lower values could be that the lambs were given 3 meals per day after weaning and grazed on pastures with rich flora.

The values obtained in the study were higher than the mean values for live weight on the 30, 60, 90, 120, 150th, and 180th day (10.99±1.15, 18.10±0.29, 22.09±0.41, 25.34±0.42, 29.19±0.47, and 32.23±0.47 *kg*, respectively) as determined by Yılmaz et al. (2018) in their study (Norduz lambs).

It was also higher than the values at 90th and 180th day (21.78±1.30 and 35.09±1.62 *kg*) obtained by Bingöl & Bingöl (2015) in their study on Hamdani lambs. It was higher than the values (60th day 13.42, 90th day 17.51, 120th day 23.35, 150th day 26.48, 180th day 28.69 *kg*) obtained by Altın & Çelikyürek (1996) in their study on Akkaraman and Akkaraman x Hamdani lambs. It goes without saying that the values (7.51±0.94, 10.84±1.36, 15.13±1.89, 19.91±2.49, 25.03±3.13, 30.09±3.76 *kg*) obtained by Bingöl & Aygün (2014) in their study on Karakaş lambs are much higher.

The analyses revealed that dam age had a significant effect ($p<0.05$) on live weight on the 60th and 90th days. On the other inspection days, the effect was insignificant ($p>0.05$). A significant effect of maternal age was found for live weight at days 60 and 90 (Bingöl et al, 2007; Sarı et al., 2013), as well as researchers who reported that there was no effect of maternal age on all study ages; Bingöl & Aygün (2014) (Karakaş lambs); Altın & Çelikyürek (1996) (Akkaraman and Hamdani x Akkaraman lambs); Yılmaz et al. (2017) (Norduz lambs); Bingöl & Bingöl (2015) (Hamdani lambs); Alarslan & Aygün (2019) (Kıvırcık lambs, out of birth weight).

It was determined that the linear effects of lamb birth weight on live weights in all inspection days were highly significant ($p<0.001$). The linear effects of the dam's weight at birth on the 30th, 60th, and 90th day live weights were ($p<0.001$), ($p<0.01$) and ($p<0.05$), respectively.

The influence of birth type on live weight was significantly pronounced ($p<0.001$) on the 30th and 60th days and remained significant ($p<0.01$) on the 90th day. Studies on Norduz lambs revealed the following impact of birth type on live weight:

Karakuş et al. (2009) (highly significant ($p<0.001$) on the 30th, 60th, and 90th days and significant ($p<0.01$) on the 120th, 150th, and 180th days; Demirel et al. (2004): significant ($p<0.001$); and Bingöl (1998): significant on all inspection days. Thus, the results obtained in our study are comparable with those of the abovementioned studies.

Furthermore, there have also been studies that have shown a correlation between live weight; Yılmaz et al. (2007) reported significant ($p<0.01$) on live weight of Norduz lambs on the 90th and 180th days, while Daşkıran et al. (2010) reported insignificant effect on live weight of Norduz lambs.

It was found that the effect of sex on the live weight of lambs on control days was insignificant ($p>0.05$). In studies conducted on lambs of the same breed, Karakuş et al. (2009) reported that the effect of sex on live weight was significant on the 30 and 60th day ($p<0.001$) and on the 90th, 120th, and 180th days. For the specific day it was significant ($p<0.01$), Daşkıran et al. (2010) reported that it was significant from birth to day 198, while Bingöl (1998) reported that it was significant on all control days ($p<0.01$). In the studies conducted on live weights in different periods, Ceyhan et al. (2007) found that the effect of sex on live weights of Kıvırcık, Gökçeada and Sakız lambs was significant in the fourth, sixth and twelfth months ($p<0.01$), Altın et al. (2003) found that the effect of sex on live weight of Kıvırcık and Karya type sheep was significant at day 103 ($p<0.05$) and day 117 ($p<0.01$), but was not significant at the other periods. The linear effects of lamb birth weight on live weight were found to be highly significant ($p<0.001$) on all inspection days in our study. The linear effects of sheep birth weight on live weight on the 30th, 60th, and 90th days were ($p<0.001$), ($p<0.01$), and ($p<0.05$), respectively.

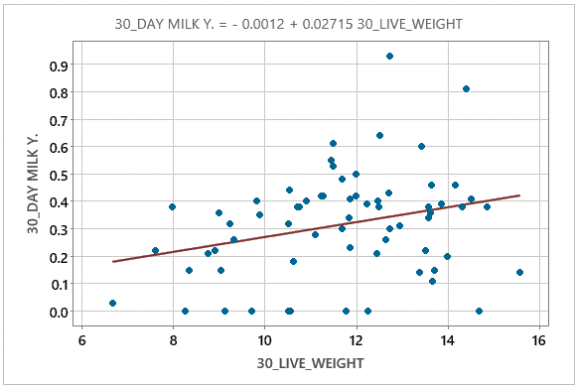
Table 2. Factors influencing on live weight of lambs on specified control days (kg)

Factors	n	30^{th}day ($\bar{X} \pm S_{\bar{X}}$)	60^{th}day ($\bar{X} \pm S_{\bar{X}}$)	90^{th}day ($\bar{X} \pm S_{\bar{X}}$)	120^{th}day ($\bar{X} \pm S_{\bar{X}}$)	150^{th}day ($\bar{X} \pm S_{\bar{X}}$)	180^{th}day ($\bar{X} \pm S_{\bar{X}}$)
Means	**66**	11.663±0.25	17.342±0.36	24.635±0.52	31.999±0.64	34.482±0.70	36.225±0.79
Dam Age			*	*			
2	**15**	11.191±0.33	17.108±0.62[a]	24.627±0.98[a]	32.331±1.31	34.339±1.44	35.078±1.65
3	**28**	11.093±0.22	16.898±0.41[a]	24.173±0.65[a]	31.543±0.86	34.275±0.95	36.345±1.09
4	**23**	10.530±0.28	15.375±0.52[b]	21.872±0.82[b]	29.857±1.09	32.249±1.20	33.491±1.38
Birth Type		***	***	**			
Single	**54**	12.093±0.15	17.841±0.28	25.254±0.45	32.416±0.60	34.879±0.66	36.716±0.76
Twin	**12**	9.782±0.35	15.080±0.64	21.861±1.02	30.072±1.37	32.363±1.50	33.227±1.72
Sex							
Male	**38**	10.998±0.22	16.783±0.41	24.097±0.65	32.023±0.86	34.265±0.95	35.320±1.09
Female	**28**	10.878±0.24	16.138±0.45	23.017±0.71	30.465±0.95	32.977±1.04	34.623±1.19
Regression (*Lin.*)							
Dam Weight at Birth		0.071±0.02 ***	0.102±0.04 **	0.106±0.06 *	0.079±0.07	0.063±0.08	0.067±0.09
Lamb Birth Weight		1.384±0.16 ***	1.779±0.30 ***	2.339±0.47 ***	3.092±0.63 ***	3.577±0.69 ***	3.936±0.79 ***

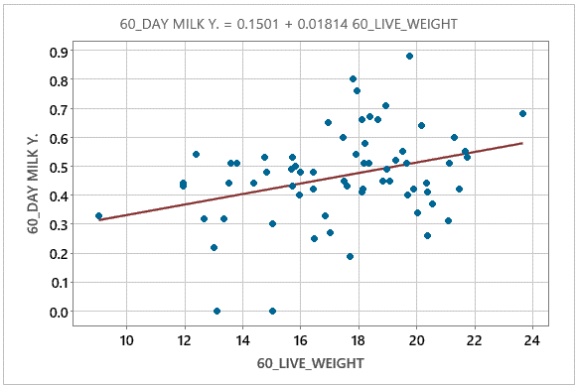
a, b, c= Values within a row marked with different superscripts are significantly different at the following levels: *=(P<0.05), **=(P<0.01), and ***=(P<0.001). $\bar{X}$ = Means, $S_{\bar{x}}$ = Standard error.

3.3. Simple linear regression analysis to determine the effect of milk yield on live weight

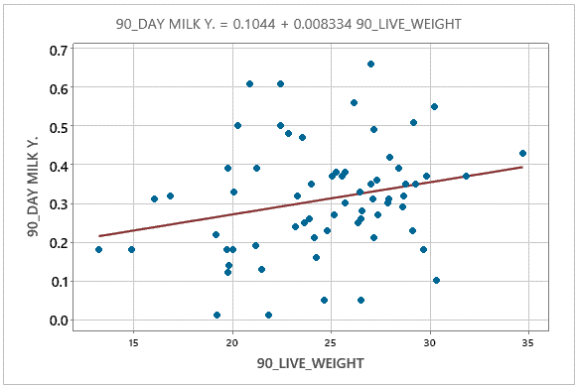
Simple linear regression analysis was performed to determine the effects of milk yields of ewes at 30, 60, 90, 120, 150th, and 180th inspection days on the live weights of their lambs (Figure 1). As can be seen from the graphs A, B, C, D, E, and F in Figure 1, the effects of milk yield on live weight were found to be positive and statistically significant ($p<0.05$). The highest contribution of ewes' milk yield to the live weight of lambs was found on the 30th day inspection. It is understood from the graphs that this effect gradually decreased until the 180th day.



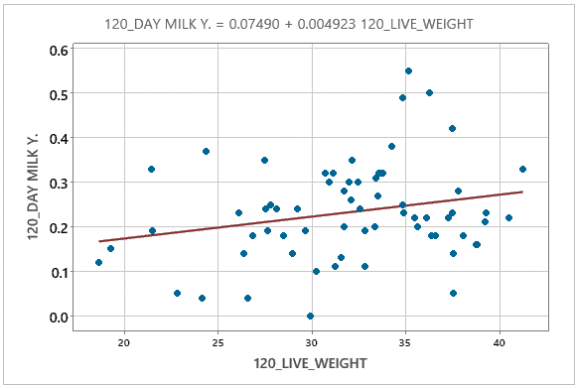
A: 30th DMY = -0.0012 + 0.02715 (30th LW)



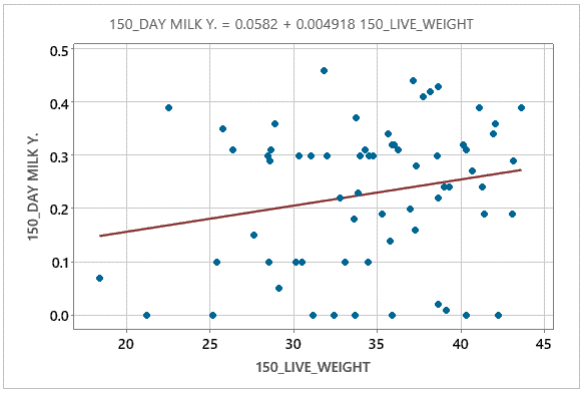
B: 60th DMY = 0.1501 + 0.01814 (60th LW)



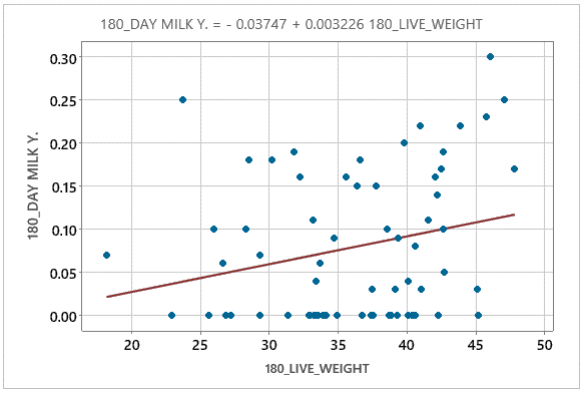
C: 90th DMY = 0.1044 + 0.008334 (90th LW)



D: 120th DMY = 0.07490 + 0.004923 (120th LW)



E: 150th DMY = 0.0582 + 0.004918 (150th LW)



F: 180th DMY = 0.03747 + 0.003226 (180th LW)

Figure 1. Simple linear regression analysis was used to evaluate the impact of milk yield on the live weight of Norduz lambs across different inspection days (Note: DMY: Daily Milk Yield, LW: Live Weight).

3.4. Correlation between all animal yields

Figure 2 illustrates a significant correlation between ewe live weight at birth and milk yield on the 90th, 120th, and 150th days. Moreover, the birth weight of the lamb shows a notable association with live weight on the 30th, 60th, 90th, 120th, 150th, and 180th days, contributing significantly to the overall live weight on control days.

Significant correlations were found between ewe milk yield on the 30th day and lamb live weight on the 30th and 120th days. Similarly, a significant correlation was observed between ewe milk yield on the 60th day and lamb live weight on the 60th, 90th, 120th, 150th, and 180th days. Furthermore, correlation tests indicated significant relationships between ewe milk yield on the 90th day and lamb live weight on the 90th day; ewe milk yield on the 120th day and lamb live weight on the 120th, 150th, and 180th days; and ewe milk yield on the 150th and 180th days and lamb live weight on the 90th and 180th days.

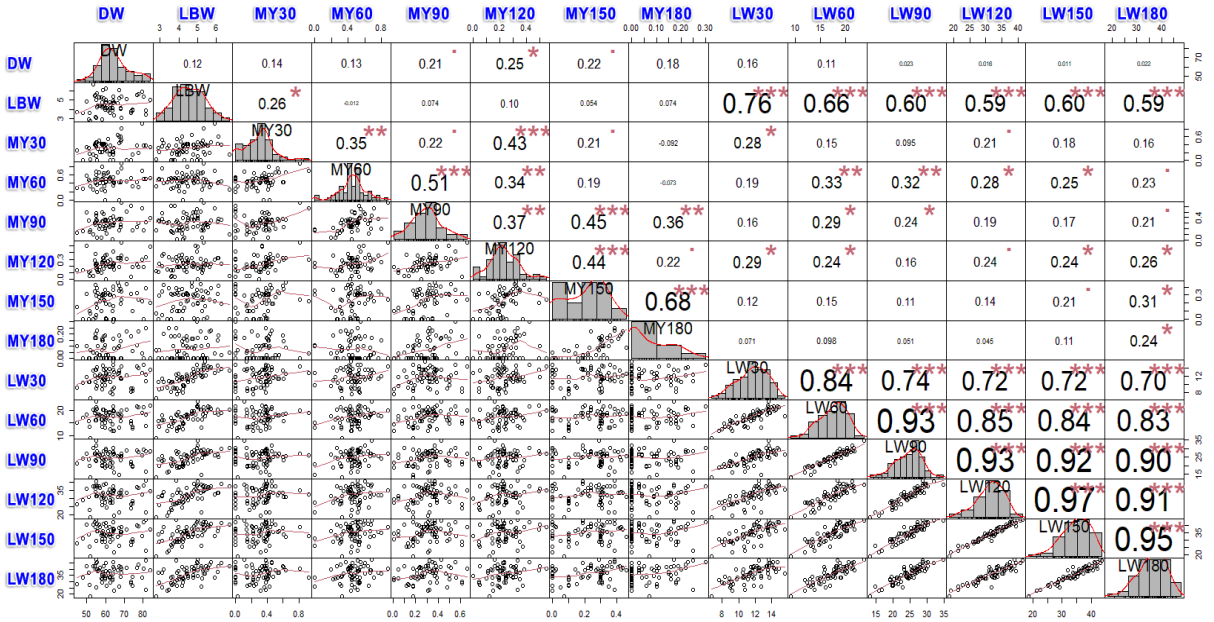


Figure 2. Pearson correlation coefficients of the factors affecting milk yield of Norduz ewes and the live weight of their lambs in different control days (DW: Dam weight, LBW: Lamb birth weight, LW: Live weight, MY: Milk yield, ($p<0.05$);* ($p<0.01$);** ($p<0.001$);***).

Figure 2 displays the correlation values as follows: between milk yield on the 30th day and live weight on the 30th day ($r=0.28$, $p<0.05$), between milk yield on the 60th day and live weight on the 60th day ($r=0.33$, $p<0.01$), between milk yield on the 90th day and live weight on the 90th day ($r=0.24$, $p<0.05$), between milk yield on the 120th day and live weight on the 120th day ($r=0.24$, $p<0.05$), between milk yield on the 150th day and live weight on the 150th day ($r=0.21$, $p<0.05$), and between milk yield on the 180th day and live weight on the 180th day ($r=0.24$, $p<0.05$). Analysis of these results reveals that the influence of ewe milk yield on lamb live weight increases progressively from birth and reaches its peak on the 60th day. This increase could be attributed to the lambs being weaned by the 90th day and subsequently being separated from the ewes, leading to distinct grazing patterns.

Conclusion

The study was conducted in a sheepfold where sheep were housed in the winter and feeding was done indoors. In the sheepfold, dry-rough grass or silage feed is given to the sheep. Sheep are not given milk feed or fattening feed. Therefore, in this type of breeding system, sheep's milk (lactation milk yield; 53.934±2.31 l) is only enough to feed the lamb. It is thought that the milk obtained during milking is not enough to meet the labor and the workforce required for milking. However, according to the results of the literature reviewed, it was concluded that milking the milk obtained in rich pasture conditions together with lamb production could be economical. In such a breeding system, after the sheep's milk is

milked, the remaining part is given to the lamb. As a result of this, the live weights of the lambs on the control days were found to be lower than the values in this study. After the lambs were taken to pasture, it was found that they closed this difference and had a higher average body weight.

Through a simple linear regression analysis of the dams' milk yield on the live weight on control days as determined in the research, it was found that the milk yield of the dams had a positive and statistically significant effect on the live weights of the lambs ($p < 0.05$).

The correlation analysis between yields reveals a significant positive relationship between the birth weight of lambs and their live weights across all age periods. Additionally, the correlation table indicates a significant relationship between the average milk yield on milk inspection days and the live weights of the lambs at various inspection ages.

According to the GLM results of the data obtained in the study, it is seen that the effect of factors such as dam age, birth type, and sex of the lambs on milk yield in Norduz sheep is generally not statistically significant. Statistical analysis indicates that dam age significantly affects the live weight of lambs on the 60th and 90th days. Furthermore, the linear effect of birth type and dam weight consistently decreases until the 90th day from birth, as observed through statistical analysis. Notably, the linear effect of the live weight of the lamb at birth on the live weight at other ages remains significant ($p<0.001$).

In conclusion, it was determined that milk yield had a significant positive impact on the live weights of lambs in Norduz ewes bred under a sheepfold system without additional milk or fattening feed. Additionally, the birth weight of lambs showed a significant effect on their live weights at other ages.

Ethical Statement

The study was approved by the Republic of Turkey Ministry of Agriculture and Forestry, Van Directorate of Provincial Agriculture and Forestry, under permission number E-44762815-325.04.02[325.04.02]-3175480, indicating that it falls within the scope of a study not requiring ethics committee approval.

Conflict of Interest

The Authors declares that there are no conflicts of interest.

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Author Contributions

The first author designed the project and conducted official correspondence and interviews with support departments. The second author assisted the first author in data collection and helped to determine the methodology of the study. The first author tabulated the statistical analysis of the study, followed the official procedures and wrote the first version of the manuscript. The first author sent the manuscript to the second author for checking. After checks and corrections, it was uploaded to the journal.

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Research Article

Assessment of Durian Diversity and Its Wild Relatives (*Durio* spp.) Based on Leaf Morphology and Molecular Marker

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Conservation,
DNA barcoding,
Genetic diversity,
Phylogeny

Abstract: Durian (*Durio* spp.) is native to Southeast Asia and has potential for development. However, some species are threatened due to deforestation and extensive land conversion. This study aimed to determine the genetic diversity and relationships of durian and wild relatives (*Durio* spp.) on the Indonesia Island Borneo using a leaf morphology and DNA barcoding (*mat*K) marker. In this study, 15 durian samples from this region were used, excluding 'Monthong' (*Durio zibethinus*) and 'Bengang' (*Neesia strigosa*) as the outgroups from the GenBank database. The leaf morphology was analyzed descriptively, whereas the genetic diversity was by the nucleotide diversity index (π%). The relationship of durians was revealed by the maximum likelihood (ML) method and examined with the bootstrap statistics for 1000 replicates, also confirmed by the PCA (principal component analysis). Based on the leaf morphology, the durians are divided into five forms, i.e., obovate-lanceolate, elliptic, ovate, oblong, and linear-oblong. 'Pampaken' and 'Pampaken Burung Kecil' indicated the earliest form (obovate-lanceolate), whereas the linear-oblong was by 'Kamundai.' Following the molecular marker, it was seen that the durians have low genetic diversity (π%) with only 0.015. However, phylogenetically, the durians were separated into four similar clades or groups for ML and PCA. In this instance, it has appeared that most of the durians evaluated in the current study have close relationships, except for the taxa with the farthest relationship. The results provide valuable information for the local and global durian conservation mission, including future breeding programs.

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1. Introduction

Durian, belonging to the genus *Durio*, is essential for economic and ecological purposes (Aziz and Jalil, 2019). For example, nine durian species, e.g., *Durio lowianus*, *D. graveolens*, *D. kutejensis*, *D. oxleyanus*, *D. testudinarum*, *D. grandiflorus*, *D. dulcis*, *D. excelcus*, and *D. zibethinus*, are edible

fruits with various tastes (Aziz and Jalil, 2019). The *D. zibethinus* is an essential commodity for export demands today (Cheon et al., 2017). In 2020, Indonesia, for example, one of the highest world durian producers, was prosperous in exporting this commodity to many other nations, in Asia (China, Hongkong, Singapore, and Malaysia), Middle Eastern (Saudi Arabia and Qatar) and Europe (Netherlands, Portugal, and Russia) with a total transaction of US$ 232,000 (Rizaty, 2021). In contrast to edible fruit, 14 species of durian also generate wood that can be useful as interior materials. It can also be used for medicinal and pharmaceutical purposes, particularly in malaria treatment (Feng et al., 2016).

Ecologically, this germplasm is native to Southeast Asia, particularly Indonesia and Malaysia (Mursyidin and Daryono, 2016). In Indonesia, 18 of 27 durian species worldwide have been reported, and most are recognized as endemic. Importantly, Borneo Island is part of the Indonesian region, which has a tremendous genetic diversity of durian (Uji, 2005). Indonesia is also known as the center of the world's durian diversity. However, most of them are threatened. Deforestation and extensive land conversion are the main factors causing the problem (Wilcove et al., 2013). The International Union for Conservation of Nature or IUCN (2023) reported several threatened durians, e.g., *D. acutifolius*, *D. dulcis*, *D. grandiflorus*, *D. kutejensis*, *D. testudinarium*, and *D. lanceolatus*.

Consequently, preservation or conservation, including cultivation and breeding tasks, is necessary. In general, conservation is an activity directed at saving and preserving the existence of threatened species (Wintle et al., 2019). Meanwhile, cultivation and breeding activities aim to explore and utilize functional genes to develop new superior cultivars in the future (van Huylenbroeck, 2018). According to Mursyidin and Daryono (2016), most germplasm (wild durian relatives) has beneficial traits or genes that aid in the preservation and breeding efforts, such as a high tolerance to environmental stresses and specific (patch canker) diseases; their existence has been disturbed. In other words, the collection and identification of germplasm are critical for facilitating future durian preservation and breeding tasks (Acquaah, 2015).

So far, genetic characterization of durian germplasm is commonly done using morphological markers (Mursyidin and Daryono, 2016; Mursyidin, 2023). While this marker has many shortcomings, such as being time-consuming and strongly impacted by environmental influences, including multiple gene inheritance (Wu et al., 2021), it is still commonly used to evaluate germplasm because it is strongly related to gene expression results (Mursyidin and Daryono, 2016). Recently, the genetic diversity and relations of durians have been studied using sequencing-based DNA barcoding markers (Mursyidin and Daryono, 2016; Santoso et al., 2017). According to Lee et al. (2017), these markers often exhibit great accuracy and repeatability. Hence, its application is faster, more effective, and more efficient, which can complement the available morphological data.

Our study is focused on determining the genetic diversity and relationship of durian and wild relatives (*Durio* spp.) from Borneo Island, Indonesia, by the leaf morphology and a DNA barcoding (*mat*K) marker. According to Barthet et al. (2020), this molecular marker has a high phylogenetic signature and a moderately rapid mutation rate, which is usable in determining the evolutionary relationships between different plant taxa. Thus, the results of this study will help assist durian preservation and breeding efforts in the future, both locally and globally.

2. Material and Methods

2.1. Plant materials

For the study, fifteen durian samples (*Durio* spp.) were collected from Borneo Island, Indonesia, using the purposive sampling method (Figure 1). The two others, ‘Monthong’ (*Durio zibethinus*) and ‘Bengang’ (*Neesia strigosa*), were collected from the GenBank and used as outgroups (Table 1). All were prepared for morphological and molecular assays.

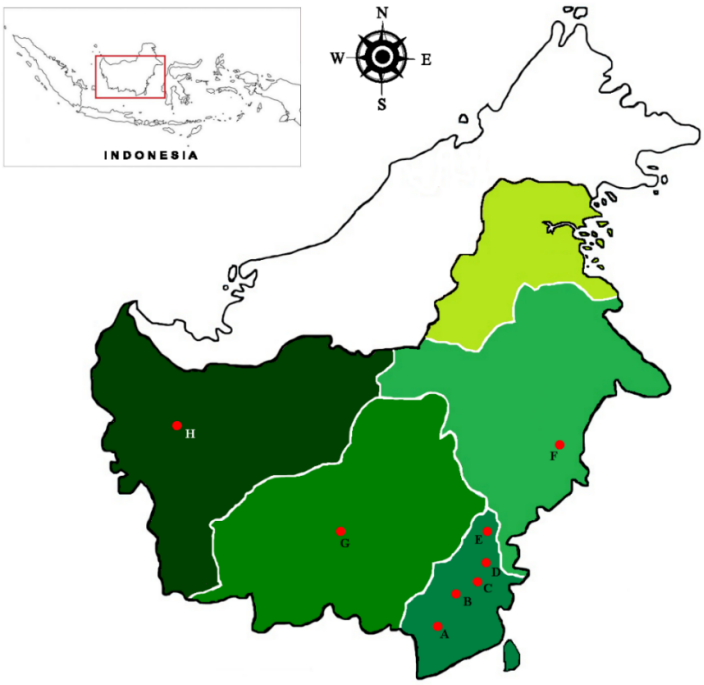

Figure 1. Sampling locations on the Borneo Island of Indonesia (red marks), where fifteen durians (*Durio* spp.) samples were collected and used in this study, i.e., A=Banjar, South Kalimantan; B = South Hulu Sungai, South Kalimantan; C = Central Hulu Sungai, South Kalimantan; D = Balangan, South Kalimantan; E = Tabalong, South Kalimantan; F = Kutai, East Kalimantan; G = Katingan, Central Kalimantan; H = Sekadau, West Kalimantan (see Table 1 for details).

Table 1. Fifteen samples of durian (*Durio* spp.) were used in the study, including their province origin

Name of species	Name of accession	Code	Province origin
D. kutejensis	'Kalih Haliyang'	4	Balangan, South Kalimantan
	'Pampaken Burung Kecil'	1	South Hulu Sungai, South Kalimantan
	'Pampaken'	8	Tabalong, South Kalimantan
	'Kamundai'	14	Tabalong, South Kalimantan
	'Lai Lidung'	2	Kutai, East Kalimantan
D. lowianus	'Malutu'	6	South Hulu Sungai, South Kalimantan
	'Lahung Alang'	10	Balangan, South Kalimantan
D. oxleyanus	'Maharawin Hamak'	9	Banjar, South Kalimantan
	'Karantungan Besar'	12	Katingan, Central Kalimantan
D. excelsus	'Burung Besar'	7	Balangan, South Kalimantan
	'Mantuala Batu Hayam'	13	Central Hulu Sungai, South Kalimantan
D. testudinarium	'Kura-Kura'	15	Sekadau, West Kalimantan
D. zibethinus	'Likol'	5	Tabalong, South Kalimantan
	'Sahang'	3	Tabalong, South Kalimantan
	'Si Japang'	11	Banjar, South Kalimantan
	'Monthong*'	-	Thailand
Neesia strigosa	Bengang*	-	USA

Note: *outgroup, obtained from the GenBank database with the accession numbers MT321069.1 and AY321189.1, respectively.

2.2. Morphological assay

Morphological characteristics of leaves were observed according to durian (*Durio zibethinus* Murr.) descriptors (Bioversity International, 2007).

2.3. Molecular assay

A DNA isolation kit (GP100, Geneaid Biotech Ltd.) was used to prepare the young leaf samples of durian. The quantification of DNAs was employed using the UV-VIS spectrophotometry method (GE Healthcare, UK). Amplification of DNA was employed using a Multigene Optimax PCR (Labnet International Inc., USA) and the universal primers of *mat*K: *mat*K-F (5'-CGTACAGTACTTTTGTGTTTACGAG-3'); *mat*K-R (5'-ACCCAGTCCATCTGGAAATCTTGG TTC-3') (Le et al., 2020). Amplification (PCR) was done with 25 µL of a total volume reaction, consisting of MyTaq HS Red PCR Mix (22 µL), ten µM primers (2.0 µL), ten ng DNA template (1 µL). The reaction was programmed by initial denaturation (94 ºC, 5 min); denaturation (94 ºC, 30 sec), annealing (48 ºC, 30 sec), extension (72 ºC, 45 sec) for 35 cycles; and final extension (72 ºC, 7 min) (Mursyidin et al., 2021). The DNA targets were examined using a UV transilluminator after being separated using 2% agarose gel electrophoresis in a 1X TBE buffer solution and GelRed staining (SMOBiO, Taiwan). At 1st Base Ltd. in Malaysia, it was subsequently purified and bidirectionally sequenced using the Sanger method.

2.4. Data analysis

The forward and reverse sequence of *mat*K of durians were assembled and analyzed manually using the MEGA11 to generate consensus (Tamura et al., 2021). All were then aligned using ClustalW (Thompson et al., 2002). The genetic diversity was measured by the nucleotide diversity index (π%), using the criteria of low (0.1 - 0.4), moderate (0.5 - 0.7), and high (0.8 - 2) (Nei and Li, 1979). The genetic relationship was employed by ML (maximum likelihood), using MEGA11 (Tamura et al., 2021), and the PCA (principal component analysis), with the assistance of MVSP ver. 3.1 (Kovach, 1999). The bootstrap methodology with 1000 replicates was used to examine the phylogenetic trees (Lemey et al., 2009).

3. Results and Discussion

3.1. Leaf diversity

The durians show different characteristics of leaf morphology, both in shape and size (Figure 2). The complete traits of the leaves can be seen in Table 3, whereas their length and width are presented in Table 2. Based on its size (Table 2), 'Kura-Kura' (*D. testudinarum*) is a durian sample that has the lengthiest leaf size (23.3 cm), whereas the 'Pampaken Burung Kecil' was the shortest with 9.3 cm. In this study, 'Pampaken' (*D. kutejensis*) has a leaf length of 16.0 cm. In contrast, the shortest width (2.6 cm) was pointed out by 'Pampaken Burung Kecil,' and the most comprehensive (8,0 cm) was by 'Mantuala Batu Hayam' and 'Kamundai.'

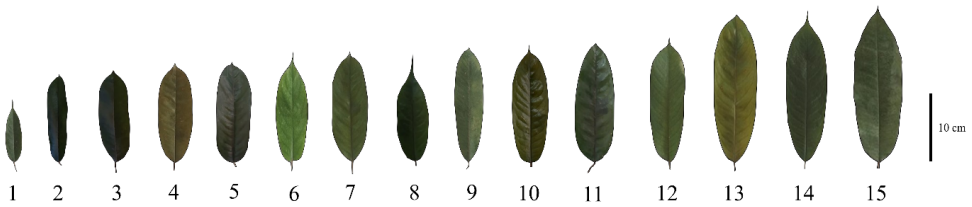

Figure 2. The leaf morphology of durian and its wild relatives (*Durio* spp.) samples used in this study shows differences in shape and size. The name of each sample is provided in Table 2.

Based on their shape (see Table 3), the durians are divided into five forms, i.e., obovate-lanceolate, elliptic, ovate, oblong, and linear-oblong. 'Pampaken Burung Kecil' and 'Pampaken' indicated the obovate-lanceolate form, whereas the linear-oblong was by 'Kamundai.' Based on the shape of the leaf apex, the acuminate, long-acuminate, and cuspidate were presented. In this case, the latest (cuspidate) by 'Pampaken.' Following the leaf base trait, round and cuneate are the dominant forms, whereas the two others (acute and obtuse) are not, as shown by 'Kalih Haliyang,' 'Kamundai,' and 'Si Japang.' Concerning the adaxial (upper) and abaxial (lower) leaf surfaces, durian was divided

into smooth-shiny, slippery, slightly rough, and rough. For the last character (leaf margin), most durian leaves are entire, except 'Kamundai', which is undulate.

Table 2. The leaf length and width of fifteen durian (*Durio* spp.) samples used in the study

Local name	Code	Species	leaf length (cm)	leaf width (cm)
'Pampaken Burung Kecil'	1	*D. kutejensis*	9.3	2.6
'Lai Lidung'	2	*D. kutejensis*	13.0	4.1
'Sahang'	3	*D. zibethinus*	13.4	3.5
'Kalih Haliyang'	4	*D. kutejensis*	14.3	4.2
'Likol'	5	*D. zibethinus*	14.8	4.4
'Malutu'	6	*D. lowianus*	15.7	4.1
'Burung Besar'	7	*D. excelsus*	16.0	5.0
'Pampaken'	8	*D. kutejensis*	16.0	4.7
'Maharawin Hamak'	9	*D. oxleyanus*	16.5	4.5
'Lahung Alang'	10	*D. lowianus*	17.2	5.6
'Si Japang'	11	*D. zibethinus*	17.4	6.2
'Karantungan Besar'	12	*D. oxleyanus*	18.5	5.6
'Mantuala Batu Hayam'	13	*D. excelsus*	21.8	8.0
'Kamundai'	14	*D. kutejensis*	22.1	8.0
'Kura-Kura'	15	*D. testudinarium*	23.3	7.0

Table 3. Morphological characteristics of durian (*Durio* spp.) leaves

Local name	Code	Leaf blade shape	Leaf apex shape	Leaf base shape	Adaxial (upper) leaf surface	Abaxial (lower) leaf surface	Leaf margin
'Pampaken Burung Kecil'	1.	Obovate-lanceolate	Acuminate	Cuneate	Smoot shiny	Slightly rough	Entire
'Lai Lidung'	2.	Elliptic	Long acuminate	Cuneate	Smoot shiny	Slippery	Entire
'Sahang'	3.	Ovate	Acuminate	Round	Smoot shiny	Rough	Entire
'Kalih Haliyang'	4.	Oblong	Acuminate	Acute	Slippery	Rough	Entire
'Likol'	5.	Ovate	Acuminate	Round	Smoot shiny	Smoot shiny	Entire
'Malutu'	6.	Oblong	Long acuminate	Round	Smoot shiny	Rough	Entire
'Burung Besar'	7.	Elliptic	Long acuminate	Round	Smoot shiny	Smoot shiny	Entire
'Pampaken'	8.	Obovate-lanceolate	Cuspidate	Cuneate	Slightly rough	Slippery	Entire
'Maharawin Hamak'	9.	Oblong	Long acuminate	Cuneate	Smoot shiny	Smoot shiny	Entire
'Lahung Alang'	10.	Ovate	Acuminate	Round	Smoot shiny	Smoot shiny	Entire
'Si Japang'	11.	Ovate	Acuminate	Obtuse	Smoot shiny	Smoot shiny	Entire
'Karantungan Besar'	12.	Oblong	Long acuminate	Round	Smoot shiny	Rough	Entire
'Mantuala Batu Hayam'	13.	Oblong	Acuminate	Cuneate	Smoot shiny	Slightly rough	Entire
'Kamundai'	14.	Linear-oblong	Acuminate	Acute	Smoot shiny	Rough	Undulate
'Kura-Kura'	15.	Ovate	Acuminate	Round	Smoot shiny	Slightly rough	Entire

According to Dkhar and Pareek (2014), the emergence of leaf diversity in plants may be caused by genetic and environmental factors. For example, leaf size will shrink as altitude decreases, rainfall increases, and soil nutrient content increases (Ke et al., 2022). Light and temperature also sometimes affect the shape and size of the leaves, although this requires further explanation (Dkhar and Pareek, 2014). Genetically, gene regulatory networks (GRNs) and signaling pathways play an essential role in bringing out the diversity of leaf shapes (Dkhar and Pareek, 2014), for example, the *KANADI* gene family (Zumajo-Cardona et al., 2019).

Referring to Tsukaya (2017), the diversity of leaves in shape and size is closely related to their role as the location of photosynthesis. Photosynthesis requires efficient absorption of light energy and the exchange of CO_2 for O_2, as well as water content and temperature. Consequently, the most high-yielding form can differ according to environmental conditions, and leaf shape also varies (Tsukaya, 2017).

3.2. Sequence characteristics, genetic diversity, and phylogenetic relationship

In this study, we used the *maturase* K (*mat*K) gene, a part of the chloroplast genome (cpDNA), to determine the genetic identity, including diversity and relationship, of this germplasm from Borneo Island, Indonesia. Conceptually, *mat*K is an intron-encoded gene in chloroplast with unique features. It has a variety of lengths, both partial and complete regions. According to Mustafa et al. (2018), the total length of this sequence is 1,536 bp. Based on Table 4, the part of *mat*K of durian germplasm ranged from 829 to 865 bp.

Table 4. Genetic information for the *mat*K sequence of durian (*Durio* spp.) germplasm

Parameter	***mat*K**
Range of sequence (bp)	829 to 865
Total length sequence observed (bp)	810
Parsimony informative sites (Pi)	18
Singleton sites (S)	61
Variable sites (V)	80
Insertion-deletion (indels) sites	20
Transition/transversion (Ti/Tv) bias value (*R*)	0.94
Nucleotide diversity (π%)	0.015
Guanine-cytosine/GC content (%)	33.18
Maximum likelihood value (ln*L*)	-1736.270
Akaike information criterion (AICc)	3532.677
Bayesian information criterion (BIC)	3758.150

However, it is different with a similar gene from several other plants, mainly Angiosperms, e.g., *Ficus* (Li et al., 2012), *Lycopersicum* (Căprar et al., 2017), *Tetrastigma* (Habib et al., 2017), *Theobroma* (Immanissa et al., 2020), and *Zanthoxylum* (Suriani et al., 2021), with a range of 830 to 857 bp (Tosh et al., 2016). The different lengths of *mat*K in germplasm are related to substitution and single indels (insertions-deletions) mutations (Chen and Shiau, 2015). In this study, 20 indels are present in the *mat*K sequence of durians, including transversion and transition (Table 4). In this case, the last (12) is higher than the transversion (6.5) (Table 5).

Table 5. The nucleotide substitution pattern on the *mat*K sequence of durians (*Durio* spp.)

Nucleotide	Code	A	T	C	G
Adenine	A	-	6.50[a]	6.50[a]	12.00[b]
Thymine	T	6.50[a]	-	12.00[b]	6.50[a]
Cytosine	C	6.50[a]	12.00[b]	-	6.50[a]
Guanine	G	12.00[b]	6.50[a]	6.50[a]	-

Note: a = transversions; b = transition

Following Aloqalaa et al. (2019), transversion occurs in this sequence more frequently than transversions. Therefore, it is typical in the evolution of molecules (Stoltzfus and Norris, 2016). As a part of the cpDNA genome, *mat*K has a relatively high mutation rate (Kar et al., 2015; Barthet et al.,

2020). However, the rhythm and type of its evolution differ from one another. According to Kar et al. (2015), the mutation rate of *mat*K is three times higher than the *rbc*L, so it is called a fast- or rapidly-evolving gene.

Referring to Suriani et al. (2021), a long-lasting single nucleotide polymorphism causes genetic variation in cpDNA, including *mat*K. As a result, polymorphism has generated a strong phylogenetic signal that may be used to resolve evolutionary connections among plants at all taxonomic levels (Kar et al., 2015). Further, phylogenetically informative features are changeable and not the result of homoplasy in phylogenetic analysis (parallel evolution). These phylogenetically informative features are not so variable that they can not be aligned across taxonomic levels. According to Kar et al.(2015), in locations with minimal variability and conserved sequence, *mat*K possesses many critical features that can be aligned to demonstrate evolutionary links from species to divisional or even higher taxonomic levels.

The *mat*K sequence of durians has a low level of polymorphism, with only 80 variable sites of 810 bp (see Table 4). As a result, this germplasm has a low-level nucleotide diversity ($\pi\% = 0.015$). Natural selection and founder effects may affect this genetic diversity, including genetic isolation and inbreeding (Gao et al., 2017). In this case, inbreeding is the most probable because it may reduce genetic diversity (Mursyidin et al., 2017) and decrease disease resistance and resilience to extreme conditions or environmental depression (Lloyd et al., 2016). In addition, a few samples used in this study may also generate a low level of genetic diversity. Then, for further studies, it is suggested that we use a larger sample size to confirm our results.

Moreover, natural selection and evolutionary processes require genetic variation to produce a core population (Govindaraj et al., 2015). It is, therefore, genetic variation is a crucial factor in the evolution or necessity of upcoming adaptive modifications. Consequently, genetic variation significantly affects conservation-related tasks (Lloyd et al., 2016). To increase the efficacy and efficiency of this attempt, it is imperative to comprehend genetic variation in this context. Since large-scale population genetic studies are the only way to address certain conservation elements, like the loss of gene diversity (Luan et al., 2006).

Plant breeding can benefit from genetic diversity information as well. To develop new, superior cultivars with desired traits or associated with a variety of abiotic and biotic stress tolerance, breeders, in this case, use all available plant genetic resources or genetic diversity (Swarup et al., 2021). Furthermore, it increases the genetic diversity of the population that will follow to adapt to future changes. Stated differently, only the present population needs a significant degree of gene variety to react rapidly to environmental changes (Lloyd et al., 2016).

Apart from the genetic diversity, the threatened durians of Borneo Island, Indonesia, showed unique phylogenetic relationships. This germplasm was grouped into four clades following ML (Figure 3) and PCA (Figure 4). Further, the genetic divergence revealed that 20 pairs of durian have the closest relation. At the same time, the farthest is shown by four durians, e.g., 'Kura-Kura' (*D. testudinarium*) and 'Burung Besar' (*D. excelsus*) (Figure 5).

In this instance, the phylogenetic trees showed a monophyletic divergence of germplasm. Slobodan and Pastana (2020) define it as a group of taxa descended from a single taxon or a common ancestor. It is noteworthy that two unique durian germplasms from Borneo Island, Indonesia, named 'Likol' (*D. zibethinus*) and 'Lai Lidung' (*D. kutejensis*), are closely linked to 'Monthong' as an outgroup (Figure 5). Because the outgroup can affect ingroup relationships and polarizing characteristics when determining the root's location, the outgroup is crucial in phylogenetic analyses (Wilberg, 2015).

However, future conservation efforts will benefit from this phylogenetic information (Flint-Garcia, 2013), mainly when calculating the genetic diversity of the progeny (Acquaah, 2015). Turner-Hissong et al. (2020) state that a wide range of genetic variants may be present in the offspring of individuals with distant ties who cross. In contrast, when individuals have a closely related cross, their progeny may be homozygous genetically. Once more, this knowledge is helpful for future durian management, preservation, and breeding efforts (Fernández-García, 2017).

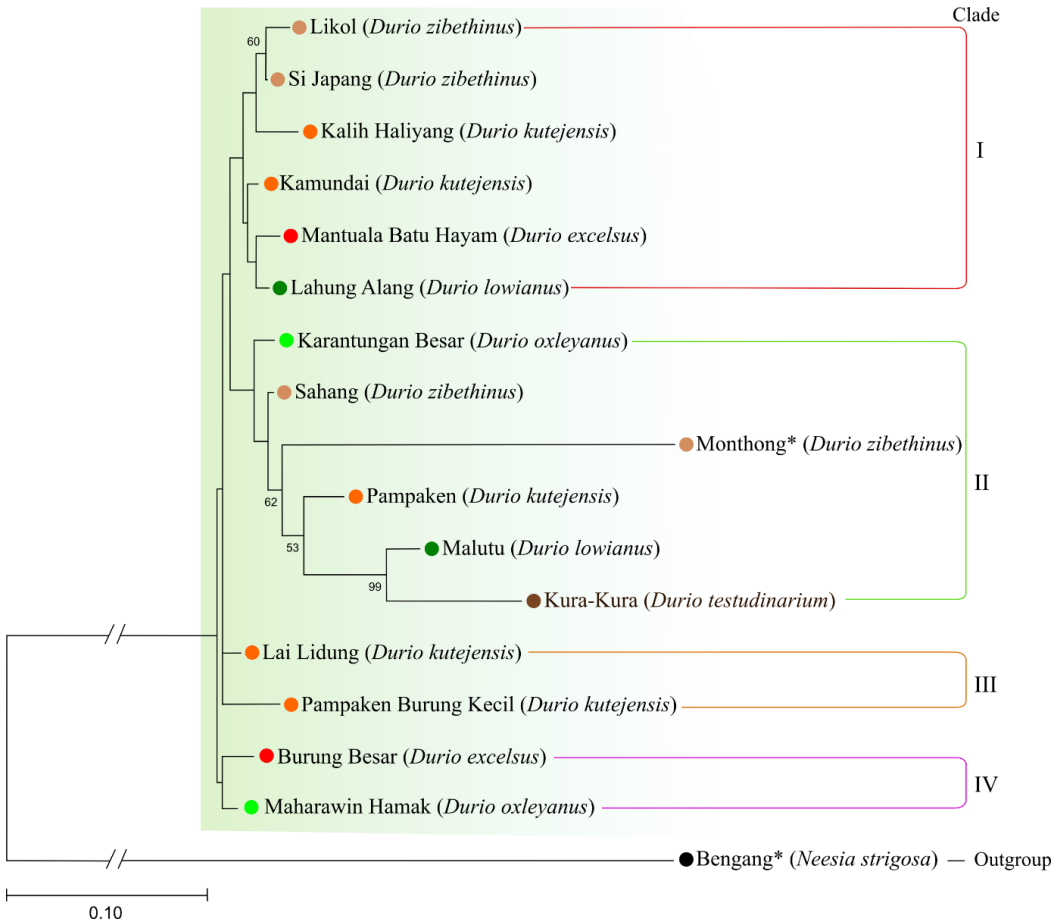

Figure 3. The genetic relationship of durian and its wild relatives (*Durio* spp.) from Borneo Island, Indonesia, were grouped into four clades based on the ML (maximum likelihood) with a bootstrap of 1,000 replicates.

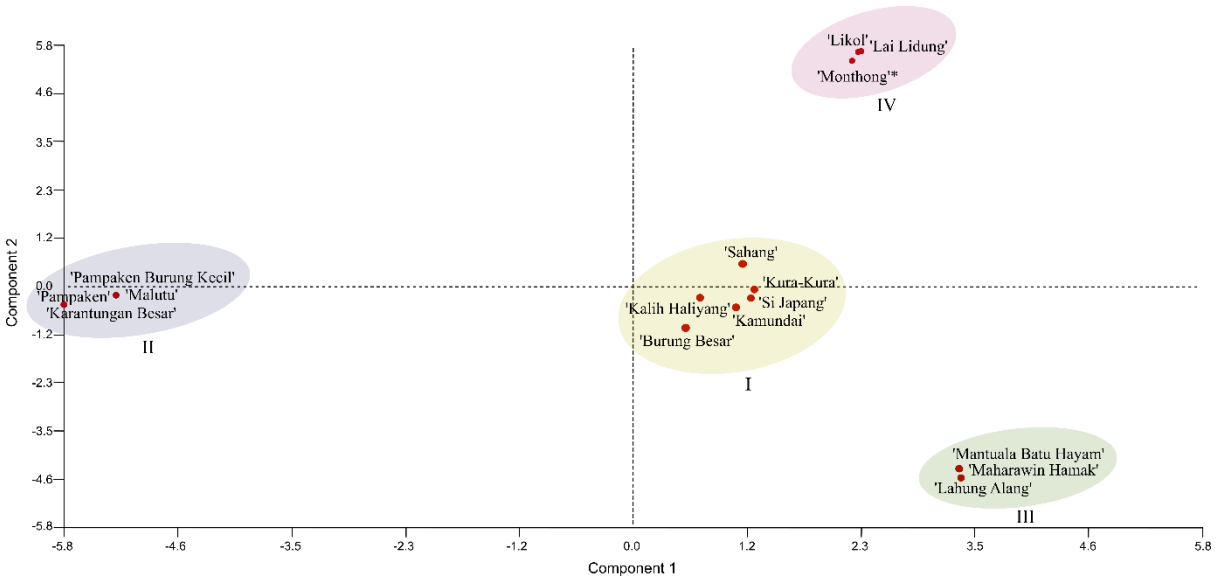

Figure 4. The durians (*Durio* spp.) from Borneo Island of Indonesia were grouped into four clusters based on the principal component analysis (PCA).

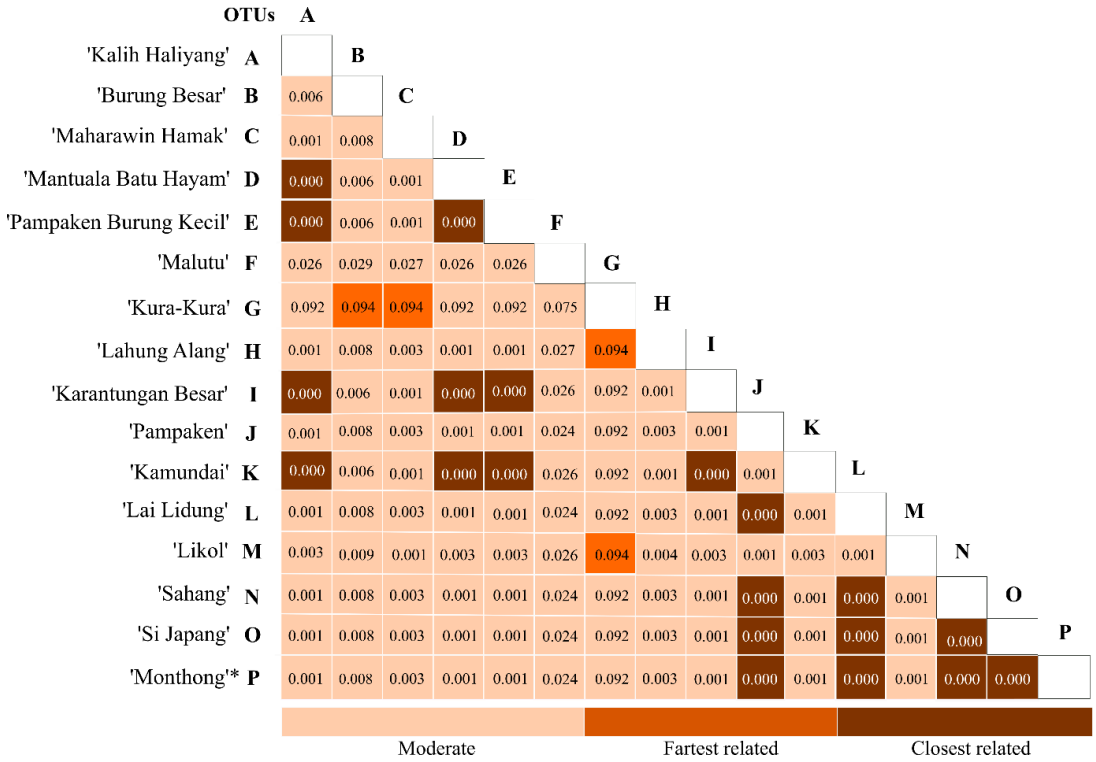

Figure 5. Genetic differentiation among durian (*Durio* spp.) accessions, revealed by maximum likelihood model. OTUs= Operational taxonomic units; * = Outgroup.

Conclusion

Based on the leaf morphology, the durians are divided into five forms, i.e., obovate-lanceolate, elliptic, ovate, oblong, and linear-oblong. 'Pampaken Burung Kecil' and 'Pampaken' indicate the obovate-lanceolate, while the linear-oblong is by 'Kamundai.' Molecularly, the durian germplasm of Borneo Island, Indonesia, has a low-level nucleotide diversity ($\pi\% = 0.015$). The phylogenetic analysis revealed that the durians are grouped into four clades, ML and PCA. In this case, most durian has the closest relationship, whereas the farthest by four durians, e.g., 'Kura-Kura' (*D. testudinarium*) with 'Burung Besar' (*D. excelsus*). Thus, this information is helpful for future durian preservation and breeding efforts.

Ethical Statement

Ethical approval is not required for this study because plant samples are collected freely and are not included as protected ones.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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Author Contributions

DHM and YAN conceptualized and designed the study. YAN is responsible for the sample collection and laboratory analysis conducted by MRF. DHM and MRF are involved in data analysis. DHM drafted the initial article. All authors finalized the article.

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Research Article

Effect of Different Metals on Synthesis of Siderophores by Endophyte Bacteria Isolated from Various Annual Plants

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Abstract: Endophyte bacteria are microorganisms that pass all or part of their life cycle in the tissues of healthy plants without causing any obvious signs of disease. Most siderophore-producing endophytic bacteria could improve the plant growth. Here, the effect of metals, iron (Fe), nickel (Ni), and cobalt (Co), on the growth and siderophore production profiles of 30 endophyte bacterial isolates were investigated. The results of the Minimum Inhibition Concentration (MIC) tests showed that endophytes exhibit varying degrees of tolerance to heavy metals and the metal tolerance decreased in the order Fe^{3+}>Ni^{2+}>Co^{2+}. It was revealed that while 10 isolates could not produce siderophores under any circumstances, 20 isolates produced siderophores at different degrees, and siderophore molecules synthesized and secreted by these 20 isolates had affinities for all three metals ($Fe^{3+,}$ Co^{2+}, and Ni^{2+}). In addition, siderophore production profiles of isolates under each heavy metal stress were investigated by adding these metals to the Chromium Azurol Sulfonate (CAS) medium at optimum concentration. The results suggested that siderophore synthesis could be one of the coping mechanisms of only two isolates with Co^{2+} and Ni^{2+} heavy metals. In the final stage of the study, molecular identification of a certain number of isolates selected according to their siderophore production values was carried out by 16S rRNA sequencing. As a result of the sequence analysis, 2 *Pseudomonas* sp., 4 *Bacillus* sp., 1 *Chryseobacterium* sp., 1 *Staphylococcus* sp., and 1 *Peribacillus* sp. were revealed.

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1. Introduction

Endophyte bacteria are microorganisms that spend all or part of their life cycle in/between plant tissues without causing disease symptoms in their host (Kumar et al., 2014). Endophytic bacteria (EBs) have been isolated and characterized from different plant parts, including roots, stems, leaves, seeds, fruits, and nodules of a wide variety of plants (Afzal et al., 2019). EBs can directly or indirectly contribute to plant health and development by improving nutrient and mineral cycles such as phosphate, nitrogen, and other nutrients (Santoyo et al., 2016). They are important for a variety of biotechnological

applications as they have the potential to produce a variety of secondary metabolites such as alkaloids, steroids, terpenoids, and flavonoids (Singh et al., 2017).

On the other hand, siderophores are extracellular secondary metabolites found in microorganisms, fungi, and plants that can contain very different chemical structures, generally known for their iron-binding properties. In other words, siderophores can be defined as iron-binding chelating agents synthesized by organisms in order to obtain iron from environments surrounded by iron starvation conditions (Hussein and Joo, 2014). It is also known that siderophores have the capacity to bind other metals besides iron (Hofmann et al., 2020). Previous studies have shown that siderophores can bind to metals other than iron such as silver, aluminum, cadmium, nickel, and mercury. Bacteria have developed various resistance mechanisms such as physical sequestration, exclusion, complexation, and detoxification, thus reducing their toxicity to tolerate heavy metal ion uptake (Rajkumar et al., 2010). There is also increasing evidence that metals other than iron can activate the synthesis of siderophores by bacteria, thereby implicating siderophores in the homeostasis of metals and especially heavy metal tolerance protecting bacteria against metal toxicity. (Schalk et al., 2011).

Biotechnological application areas of bacterial and fungal siderophore include medicine, pharmacology, bioremediation, biodegradation, and food industries besides agriculture. They can be used in agricultural applications to promote plant growth and also as biocontrol agents against plant pathogens (Rout et al., 2013). Heavy metals released from industrial plants to the environment pose an environmental threat if processes are not properly managed (Verma and Sharma, 2017). The use of siderophore-producing microorganisms in bioremediation practices, known as the use of organisms to clean polluted areas such as soil, water, and oceans, is gaining increasing attention (Braud et al ., 2009; Edberg et al., 2010). In addition, siderophores have the potential to be used to treat various diseases or improve human health. The first and most studied aspect of siderophore biotechnology is the treatment of iron overload during transfusion due to non-hemorrhagic conditions (Ribeiro et al., 2022). When siderophores combine with components such as metal ions, antibiotics, targeting ligands, and nanoparticles, they acquire many functions in imaging, sensors, or therapeutics (Fan and Fang, 2021).

The unique ability allows siderophores to remove heavy metals from contaminated environments, thereby facilitating their bioremediation. Therefore, it is important to screen and find the microorganisms producing siderophores from nature and to reveal the relations of these siderophores with non-ferrous metals. In this study, it was aimed to investigate the tolerance of 30 endophyte bacterial isolates, which were previously isolated from some cultivated and wild cereal plants (Poaceae family) against cobalt (Co^{2+}), nickel (Ni^{2+}), and iron (Fe^{3+}); to reveal the siderophore production profiles and capacities of these bacteria under cobalt (Co^{2+}) and nickel (Ni^{2+}) stress other than iron (Fe^{3+}).

2. Material and Methods

2.1. Bacterial isolates

In this study, endophyte bacteria isolated from some cultivated and wild cereal plants (Poaceae family) in and around Van province, which are in the stocks of Van Yuzuncu Yil University, Faculty of Agriculture, Department of Plant Protection, Bacteriology laboratory were used.

2.2. Determination of minimum inhibition concentration (MIC) values for cobalt (Co), nickel (Ni), and iron (Fe) elements of isolates

The Minimum Inhibitory Concentration (MIC) values for each metal ion of the isolates were determined by gradually increasing the heavy metal concentrations in the solid media medium until the isolates could not grow. For this, $CoCl_2$, $NiSO_4$, and $FeCl_3$ stock solutions were prepared and sterilized by filtration method. Nutrient Agar (NA) media containing $CoCl_2$, $NiSO_4$, and $FeCl_3$ increasing in 0.1 mM intervals were prepared by using these stocks. In order to prepare NA nutrient media containing the relevant concentrations of metals, the required volume of the relevant metal stock was added to the nutrient media sterilized by autoclave, cooled to 60-70 ºC, and mixed and poured into petri dishes under aseptic conditions. Suspensions of EB isolates were prepared at a density of 10^6 cfu/ml. 20 µl of these suspensions were taken and inoculated in the NA plates by dripping at points equidistant from each other. MIC values were noted when isolates did not grow in petri dishes even after 10 days of incubation.

2.3. Determination of siderophore activities

2.3.1. Chrome Azurol S (CAS) test

Before starting the experiment, the glassware was rinsed with 3 M hydrochloric acid (HCl) to remove iron and then washed in deionized water (Cabaj and Kosakowska, 2009). CAS reactive dye was prepared according to Schwyn and Neilands (1987). The ability of bacterial isolates to produce siderophores was determined using a modified version of the original universal CAS-siderophore test developed by Schwyn and Neilands (1987) (Arora and Verma, 2017). CAS agar plates were prepared by mixing 100 ml of separately sterilized CAS reagent solution into 900 ml of sterilized Luria Broth (LB) agar medium. The final pH of the CAS reactive dye and LB agar medium was slowly brought to 6.8 using NaOH and HCl before autoclaving.

The isolates obtained as a pure culture were transferred to CAS Agar medium by spot inoculation technique and incubated at 25ºC for 7 days. Orange colored zones formed around the colonies were evaluated as positive isolates for the CAS test (Payne, 1994). All analyses were performed in four replicates.

2.3.2. Determination of the ability of siderophores to bind Co^{2+} and Ni^{2+} metals other than Fe^{3+}

Chrome azurol S (CAS) reagent solution containing different metals (Co and Ni) was prepared to screen the ability of siderophores to bind cobalt and nickel metals other than iron (Mehnert et al., 2017; Hofmann et al., 2021). CAS reactive dye was prepared according to Schwyn and Neilands (1987), but instead of 1 mM $FeCl_3$ in the mixture, the same concentration (1 mM) $CoCl_2$ or $NiSO_4$ was used. Chrome Azurol Sulfonate (CAS) dye forms a complex with Co^{2+} or Ni^{2+} instead of $Fe^{3+,}$ and in the presence of a metal-chelating agent (siderophore), the specified reaction takes place, free dye is released and the blue color turns orange.

The isolates obtained as a pure culture were transferred to CAS Agar medium by spot inoculation technique and incubated at 25 ºC for 7 days. Orange colored zones formed around the colonies were evaluated as positive isolates for the CAS test (Payne, 1994). All analyses were performed in four replicates.

2.3.4. Determination of siderophore production profiles of isolates under metal stress

In order to examine the siderophore production profiles of the isolates under metal stress, the final concentrations of these metals in CAS media were determined by considering the MIC values of the isolates against each metal. Final concentrations in the media were determined as 0.3 mM for $CoCl_2$, 0.8 mM for $NiSO_4$, and 1 mM for $FeCl_3$. In order to prepare CAS-LB agar media containing metals at the specified concentrations, CAS-LB agar media were prepared by adding metals from previously prepared sterile stock solutions to the media to the final concentrations specified. In addition, CAS-Fe reactive dye was used for standardization in all the media prepared in this part of the study.

The isolates obtained as a pure culture were transferred to CAS Agar medium by spot inoculation technique and incubated at 25 ºC for 7 days. Orange colored zones formed around the colonies were evaluated as positive isolates for the CAS test (Payne, 1994). All analyses were performed in four replicates.

For isolates, the siderophore production index (SI) was calculated using measurements taken at the end of the incubation periods. Siderophore production indices were expressed as the ratio of the mean zone diameters measured in the relevant test to the mean colony diameters, based on the method first used as the extracellular enzyme production index (Carrim et al., 2006; Doğan and Taşkın, 2021).

2.4. Genotypic characterization of selected isolates

After determining siderophore activities, 10 isolates were selected for diagnosis processes, giving successful and different SI values. The selected strains were identified by 16S rRNA gene sequencing. DNA isolation was performed by the method modified from Govindarajan et al. (2007) and 16S rRNA was amplified in polymerase chain reaction (PCR) using genomic DNA as a template and bacterial universal primers, 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'- TAC GGT TAC CTT GTT ACG ACT T-3') (Frank et al., 2008). A 50 µL reaction mixture contained 2.5 U Taq polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 0.3 mM dNTPs, 25 mM $MgCl_2$, 20 pmol

of each primer, 5 µL of 10 x reaction buffer (Thermo Fisher Scientific), and 20 ng of template DNA. The step-up PCR procedure included denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, with a final extension at 72 °C for 10 min. Amplification products were electrophoresed on a 1.5% agarose gel in 1 x TBE buffer.

The 16S rRNA gene sequencing was performed by BM Labosis Biotechnology Company (Türkiye) using the Sanger platform. The sequences obtained were analyzed using the database on the website "https://www.ezbiocloud.net/", and then the sequences were logged in to the GenBank site and accessed "Accession" numbers (Table 3).

2.5. Statistical Analysis

All enzyme measurement experiments were performed in four replicates and each petri measurement was repeated twice. Statistical Analysis System (SAS version 9.4 SAS, Cary, NC) was used to analyze the data. General linear model (GLM) analysis was used to determine differences between the averages of the groups, and Duncan multiple comparison test was used to determine differences between the groups. P values <0.05 were considered statistically different.

3. Results

3.1. Determination of minimum inhibition concentration (MIC) values for cobalt (Co), nickel (Ni), and iron (Fe) elements of isolates

The Minimum Inhibitory Concentration (MIC) values for each metal ion were investigated in an LB solid nutrient medium for a total of 30 isolates grown at 25 °C. Heavy metal concentrations in the solid media were determined by increasing them gradually in the range of 0.1 mM increments for each metal until the isolates could not grow. For all the isolates, the MIC values for each metal are given in Appendix A. The results revealed that no isolates could grow in the media containing 0.9 mM and above $CoCl_3$. Although the isolates showed different MIC values for cobalt metal, only one isolate was observed with the highest value of 0.9 mM, and 10 isolates with the lowest value of 0.4 mM. According to the results, 20 isolates could not grow at 1.1 mM and higher $NiSO_4$ concentrations. Only 2 isolates grew up to 2.6 mM, making them the most resistant to nickel. While the lowest inhibition value for iron belonged to only one isolate with 1.1 mM, 18 isolates could not grow at iron concentrations of 2.9 mM and above (Appendix A).

3.2. Determination of the ability of siderophores to bind Co^{2+} and Ni^{2+} metals other than Fe^{3+}

At this stage of the study, three types of solid LB media containing CAS reactive dye prepared with $FeCl_3$, $CoCl_2$, and $NiSO_4$ metals were prepared and the isolates were inoculated into these media. Yellow-orange zones formed around the colonies, which were incubated for 7 days at 25 ºC, were evaluated as siderophore formation and as positive isolates for the CAS test (Payne, 1994). At this stage, no metal was added to the nutrient media, except for the CAS reactive dye. Therefore, no metal stress was created.

Here, it was aimed to test the ability of the siderophore molecules produced by the isolates to meet their Fe^{3+} needs, to bind to Co^{2+} and Ni^{2+} heavy metals instead of Fe^{3+}. Instead of Fe^{3+}, Co^{2+} or Ni^{2+} metals form a complex with CAS dye and then the siderophore molecules secreted by the isolates bind the metals, resulting in the release of the free dye, which creates a yellow-orange zone around the isolate. (Figure 1). According to the siderophore production index (SI) data created using the zone and colony diameters obtained at the end of incubation, it was observed that 10 isolates with code numbers G119S1, G25K3, G79Y2, G35S1, G6Y2, G9Y2, G12S1, G101K4, G53Y2 and G135Y4 did not produce siderophores in all three media (Table 1). It was revealed that 20 isolates other than these isolates produced siderophores with different SI values (Table 1). In addition, it was seen that the siderophore molecules synthesized and secreted by these 20 isolates have affinities for all three metals ($Fe^{3+,}$ $Co^{2+,}$ and Ni^{2+}).

Table 1. Average siderophore production index (SI) values of isolates in LB medium containing Chrome azurol S (CAS) reagent solution (dye) prepared with $NiSO_4$, $CoCl_2$, and $FeCl_3$

		CAS-Fe	CAS-Co	CAS-Ni
Isolate No	**Codes of the Isolates**	**SI**	**SI**	**SI**
1	G119Y2T	1.51 ± 0.67^{g}	1.31 ± 0.02^{i}	1.52 ± 0.02^{hi}
2	G88K1	2.47 ± 0.02^{d}	3.33 ± 0.00^{ab}	3.21 ± 0.13^{abc}
3	G119S1	-	-	-
4	G120S3	1.55 ± 0.01^{g}	2.57 ± 0.08^{cdef}	3.76 ± 0.12^{a}
5	G32S2	2.00 ± 0.00^{ef}	2.83 ± 0.07^{bcd}	3.20 ± 0.02^{abc}
6	G25K3	-	-	-
7	G30S1	1.53 ± 0.03^{g}	1.33 ± 0.05^{i}	1.39 ± 0.06^{i}
8	G47K1	1.62 ± 0.13^{g}	2.88 ± 0.00^{bcd}	1.76 ± 0.19^{ghi}
9	G56Y1	2.96 ± 0.20^{ab}	3.05 ± 0.23^{bc}	2.31 ± 0.07^{efg}
10	G33Y3	3.18 ± 0.33^{ab}	3.07 ± 0.14^{bc}	3.15 ± 0.19^{abc}
11	G88S1	1.50 ± 0.00^{g}	2.30 ± 0.12^{efg}	2.45 ± 0.05^{def}
12	G20Y3	1.38 ± 0.01^{g}	1.67 ± 0.11^{hi}	1.40 ± 0.10^{i}
13	G115S1	2.33 ± 0.06^{de}	2.91 ± 0.09^{bcd}	3.23 ± 0.23^{abc}
14	G24Y1	1.66 ± 0.08^{fg}	2.62 ± 0.16^{cdef}	2.35 ± 0.49^{efg}
15	G79Y2	-	-	-
16	G35S1	-	-	-
17	G111K3	1.63 ± 0.04^{g}	1.67 ± 0.04^{hi}	1.86 ± 0.00^{fghi}
18	G37K1	2.23 ± 0.05^{de}	2.67 ± 0.17^{cde}	2.81 ± 0.38^{cde}
19	G6Y2	-	-	-
20	G45K1	1.56 ± 0.06^{g}	2.39 ± 0.11^{defg}	2.47 ± 0.13^{def}
21	G9Y2	-	-	-
22	G15S1	3.24 ± 0.10^{a}	3.59 ± 0.28^{a}	3.29 ± 0.10^{abc}
23	G12S1	-	-	-
24	G101K4	-	-	-
25	G99K3	3.00 ± 0.00^{ab}	2.00 ± 0.00^{gh}	3.00 ± 0.00^{bcd}
26	G53Y2	-	-	-
27	G111K1	2.83 ± 0.17^{bc}	3.00 ± 0.50^{bc}	3.33 ± 0.33^{abc}
28	G105Y1B	2.55 ± 0.22^{cd}	2.12 ± 0.13^{fgh}	2.01 ± 0.10^{fgh}
29	G45Y1	1.27 ± 0.14^{g}	2.05 ± 0.09^{gh}	3.55 ± 0.10^{ab}
30	G135Y4	-	-	-
p values		<0.001	<0.001	<0.001

* Siderophore Production Indices (SI) were calculated as the ratio of the mean zone diameters measured in the relevant test to the mean colony diameters. All measurements were made in triplicate.
**Within the same column, the difference between groups expressed with different letters is statistically significant ($p<0.05$) (Mean ± Std. Error).
***- means no siderophore production.

3.3. Determination of siderophore production profiles of isolates under metal stress

At this stage of the study, it was aimed to examine the siderophore production profiles of the isolates under metal stress. For this purpose, considering the MIC values of the isolates against each metal, the final concentrations in the nutrient media were determined as 0.4 mM for $CoCl_2$, 0.8 mM for $NiSO_4$, and 1 mM for $FeCl_3$. Siderophore production index (SI) data were created using the zone and colony diameters obtained at the end of the incubation (Table 2).

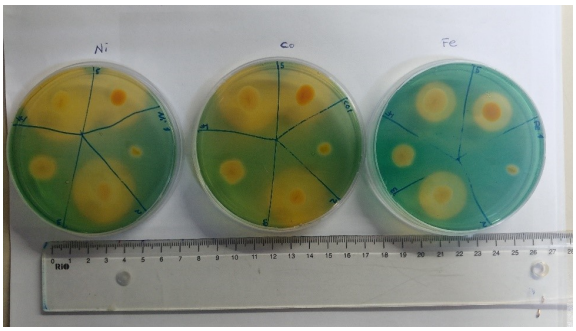

Figure 1. The sample photograph of the siderophore CAS test made with isolates with the same code number, from left to right, in a solid LB medium containing CAS reactive dye prepared with $NiSO_4$, $CoCl_2$, and $FeCl_3$, respectively.

1 mM $FeCl_3$ in CAS-LB+Fe, CAS-LB+Co+Fe, and CAS-LB+Ni+Fe nutrient media was used to eliminate siderophore production in the presence of ready, usable Fe^{3+} in the nutrient media for the isolates. In this way, siderophore production in these nutrient media would only be associated with heavy metals, Ni^{2+} and Co^{2+}. Interestingly, only two isolates, G56Y1 and G33Y3, were not affected by this rule and produced very high zones in all three media containing 1 mM usable Fe^{3+}, giving considerable SI values (Table 2). On the other hand, isolates with code numbers G111K1 and G105Y1B could not produce siderophores as expected in CAS-LB+Fe nutrient medium containing only 1mM $Fe^{3+,}$ but they gave significant SI values in CAS-LB+Co+Fe and CAS-LB+Ni+Fe medium containing the same amount of Fe^{3+} (Table 2). The isolates other than these did not produce siderophores, although they formed colonies in iron-containing media as expected.

3.4. Genotypic characterization of selected isolates

After the determination of the siderophore production indices (SI) given under heavy metal stress, 8 promising isolates (G32S2, G56Y1, G33Y3, G115S1, G111K1, G105Y1B, and G45Y1) and 1 non-siderophore producing isolate (G53Y2) were analyzed with 16S rRNA sequencing selected for identification. As a result of the comparative data analysis using the database, 16S rRNA gene region sequences of 8 isolates, 2 *Pseudomonas* sp. (G115S1, G45Y1), 3 *Bacillus* sp. (G15S1, G56Y1, G33Y3, G105Y1B), 1 *Chryseobacterium* sp. (G32S2), 1 *Staphylococcus* sp. (G111K1) and 1 *Peribacillus* sp. (G53Y2) gave results belonging to the genus (Table 3).

Table 2. Average siderophore production index (SI) values of isolates in LB media under different metal stress

		CAS-LB	CAS-LB+Co	CAS-LB+Ni	CAS-LB+Fe	CAS-LB+Co+Fe	CAS-LB+Ni+Fe
Isolate No	**Codes of the isolates**	**SI**	**SI**	**SI**	**SI**	**SI**	**SI**
1	G119Y2T	1.44 ± 0.06^{kl}	1.29 ± 0.08^{h}	1.42 ± 0.08^{hij}	-	-	-
2	G88K1	2.62 ± 0.09^{ef}	1.88 ± 0.37^{defgh}	3.71 ± 0.21^{abc}	-	-	-
3	G119S1	-	-	-	-	-	-
4	G120S3	2.01 ± 0.01^{ghi}	-	1.22 ± 0.06^{j}	-	-	-
5	G32S2	1.89 ± 0.03^{ghijk}	3.32 ± 0.19^{ab}	1.85 ± 0.14^{ghij}	-	-	-
6	G25K3	-	-	-	-	-	-
7	G30S1	1.73 ± 0.18^{hijkl}	1.47 ± 0.04^{fgh}	1.34 ± 0.08^{ij}	-	-	-
8	G47K1	2.17 ± 0.31^{gh}	2.21 ± 0.20^{cde}	2.00 ± 0.31^{fghi}	-	-	-
9	G56Y1	3.07 ± 0.04^{cd}	3.23 ± 0.29^{ab}	2.93 ± 0.61^{de}	3.49 ± 0.08^{a}	2.55 ± 0.31^{a}	2.97 ± 0.06^{a}
10	G33Y3	3.83 ± 0.21^{b}	3.67 ± 0.42^{a}	3.29 ± 0.42^{bcd}	3.31 ± 0.19^{a}	2.66 ± 0.11^{a}	2.57 ± 0.16^{ab}
11	G88S1	1.54 ± 0.00^{jkl}	1.51 ± 0.00^{fgh}	1.57 ± 0.00^{ghij}	-	-	-
12	G20Y3	1.66 ± 0.04^{ijkl}	2.05 ± 0.23^{defg}	1.34 ± 0.03^{ij}	-	-	-
13	G115S1	2.34 ± 0.04^{fg}	1.55 ± 0.32^{efgh}	3.89 ± 0.20^{ab}	-	-	-
14	G24Y1	1.71 ± 0.11^{hijkl}	2.15 ± 0.05^{def}	1.48 ± 0.11^{ghij}	-	-	-
15	G79Y2	-	-	-	-	-	-
16	G35S1	-	-	-	-	-	-
17	G111K3	1.72 ± 0.17^{hijkl}	1.88 ± 0.15^{defgh}	1.87 ± 0.08^{ghij}	-	-	-
18	G37K1	1.94 ± 0.21^{ghij}	2.27 ± 0.30^{cd}	2.07 ± 0.15^{fgh}	-	-	-
19	G6Y2	-	-	-	-	-	-
20	G45K1	2.02 ± 0.14^{ghi}	1.86 ± 0.07^{defgh}	1.83 ± 0.10^{ghij}	-	-	-
21	G9Y2	1.37 ± 0.01^{l}	-	1.61 ± 0.19^{ghij}	-	-	-
22	G15S1	3.46 ± 0.07^{bc}	2.84 ± 0.06^{bc}	3.23 ± 0.27^{cd}	-	-	-
23	G12S1	-	-	-	-	-	-
24	G101K4	-	-	-	-	-	-
25	G99K3	5.00 ± 0.00^{a}	2.33 ± 0.00^{cd}	4.00 ± 0.00^{a}	-	-	-
26	G53Y2	-	-	-	-	-	-
27	G111K1	3.00 ± 0.29^{de}	2.40 ± 0.10^{cd}	2.61 ± 0.39^{ef}	-	1.80 ± 0.00^{b}	2.03 ± 0.26^{bc}
28	G105Y1B	2.33 ± 0.15^{fg}	1.86 ± 0.24^{defgh}	2.11 ± 0.10^{fg}	-	1.51 ± 0.05^{b}	1.60 ± 0.24^{c}
29	G45Y1	2.06 ± 0.10^{ghi}	1.41 ± 0.06^{gh}	1.26 ± 0.03^{j}	-	-	-
30	G135Y4	-	-	-	-	-	-
p values		<0.001	<0.001	<0.001	0.442	0.002	0.005

* Siderophore Production Indices (SI) were calculated as the ratio of the mean zone diameters measured in the relevant test to the mean colony diameters. All measurements were made in triplicate.

**Within the same column, the difference between groups expressed with different letters is statistically significant ($p<0.05$) (Mean ± Std. Error).

***- means no siderophore production.

Table 3. Comparative analysis of sequence analysis results using the EzBioCloud database and GenBank accession numbers

Code of the Isolates	Top-hit reference species	Top-hit reference strain	Similarity (%)	Coverage (%)	GenBank Accession Numbers
G32S2	*Chryseobacterium shigense*	DSM 17126	97.23	70.2	ON571627
G56Y1	*Bacillus siamensis*	KCTC 13613	99.03	77.0	ON571630
G33Y3	*Bacillus siamensis*	KCTC 13613	98.59	28.9	ON571628
G115S1	*Pseudomonas orientalis*	CFML 96-170	99.36	75.4	ON571626
G53Y2	*Peribacillus simplex*	NBRC 15720	100.00	75.4	ON571625
G111K1	*Staphylococcus pasteuri*	ATCC 51129	99.91	76.1	ON571624
G105Y1B	*Bacillus halotolerans*	ATCC 25096	99.91	76.4	ON571623
G45Y1	*Pseudomonas kilonensis*	DSM 13647	99.82	76.7	ON571629

4. Discussion

In case of iron starvation, many microorganisms secrete at least one type of siderophore to make the limited amount of iron soluble in their habitat and take it into the cell (Haas, 2003). These secondary metabolites produced by bacteria, fungi, and plants are chelating agents that provide iron uptake. Iron exists as an insoluble oxide hydrate compound in many habitats where oxygen is present (Schalk et al., 2011).

In this study, siderophore production profiles of 30 endophyte bacterial isolates under iron (Fe^{3+}), cobalt (Co^{2+}), and nickel (Ni^{2+}) stress were revealed for the first time. In order to create a stress environment in the nutrient media by using the relevant heavy metals, it was first aimed to determine the minimum inhibition concentration (MIC) values for the Co^{2+}, Ni^{2+}, and Fe^{3+} elements of the isolates. According to the MIC values, while the isolates were most negatively affected by cobalt, they tolerated the increasing concentrations of iron the most among the metals (Appendix A). Although there is currently no acceptable standard concentration value, bacteria that can grow at concentrations of metal ions of 1.0 mM and above can be considered resistant to the relevant metal to distinguish metal resistivity (Malik and Jaiswal, 2000; Tomova et al., 2015). According to this inference, while none of the isolates were resistant to cobalt, all of them were resistant to nickel and iron.

Determining the ability of siderophores synthesized by the isolates and secreted out of the cell to bind Co^{2+} and Ni^{2+} metals other than Fe^{3+}, before creating heavy metal stress *in vitro*, was the next goal of this thesis study. For this, the universal CAS reactive dye was prepared separately for each of the three metals, using the same concentration of the relevant metals, and added to the solid nutrient media. No other metal additions were made to the prepared nutrient media. The results are consistent with data from studies showing that the universal CAS test originally developed by Schwyn and Neilands (1987) can be used not only to detect the siderophores with Fe-CAS solution but also to test siderophores for their ability to bind other metal ions (Mehnert et al., 2017; Hoffman et al., 2021). At this stage of the study, it was observed that the SI values of isolates such as G88K1, G32S2, and G88S1 with CAS reagents prepared with Co^{2+} and Ni^{2+} were higher than those with Fe^{3+} (Table 1). However, these data are insufficient to conclude that siderophores bind to Co^{2+} and Ni^{2+} ions with higher affinity. Since the siderophore molecules bound to these two metals cannot meet the iron requirement, the possibility of the cells synthesizing and secreting more siderophores into the environment should be considered. The hypothesis that the isolates synthesized a metal chelating agent other than the siderophore and that the SI values were high for this reason was also evaluated as low probability since metal was not added to the nutrient medium at this stage and did not create a stress environment.

In the third stage of the study, siderophore production profiles of isolates under metal stress were investigated. For this purpose, the maximum metal concentrations that caused stress but did not have a toxic effect on the isolates were determined by taking into account the MIC values against each metal, and CAS-LB agar media were prepared. As seen in Table 2, the reason for adding 1 mM iron in addition to cobalt and nickel to CAS-LB+Co+Fe and CAS-LB+Ni+Fe media was to keep the usable iron ion concentration high and thus to stop the production of siderophores. In this context, G111K1 and G105Y1B coded isolates gave extraordinary results; while the production of siderophores was stopped in CAS-LB+Fe medium, they showed the ability to produce a remarkable CAS reaction in CAS-

LB+Co+Fe and CAS-LB+Ni+Fe nutrient media (Table 2). These results suggest that one of the coping mechanisms of these two isolates with Co^{+2} and Ni^{+2} heavy metals may be siderophore synthesis.

Another interesting result of this experiment is the SI values of isolates with code numbers G56Y1 and G33Y3. These isolates continued to show CAS reaction without being affected by 1 mM Fe+3 metal ion added to CAS-LB broth and gave very high SI values (Table 2). However, there are many studies in which the addition of Fe^{3+} at much lower concentrations to the medium stopped/suppressed the synthesis of siderophores in many microorganisms. For example, in a study examining the effects of growth conditions on siderophore-producing bacteria, it was revealed that Fe^{3+} concentration increased up to 50 µM inhibited siderophore synthesis without affecting the growth rate in bacteria (Sinha et al., 2019). In a study conducted by Machuca and Milagres (2003), they observed that only *Aspergillus niger* fungi formed a CAS reaction (zone formation) in solid media even in the presence of 4 mM Fe^{3+} concentration in experiments conducted with various fungal species. It is known that the biosynthesis of siderophores is regulated by the iron content of the medium and inhibited in the presence of excess iron (Neilands, 1993; Machuca and Milagres, 2003). Positive results at high iron concentrations suggest the presence of a non-siderophore compound or a chelator other than a siderophore that reacts with the CAS reagent. For example, since *A. niger* is known to be a good producer of citric and oxalic acids, these organic acids might likely have reacted with CAS at high iron concentrations (Machuca and Milagres, 2003). In addition, the release of organic acids in response to iron-deficiency stress conditions has been documented for Neurospora crassa. It was thought that the acids secreted by these fungi interact with the iron concentrated on the cell surface, making the iron soluble, making it suitable for use by fungi (Winkleman, 1979; Guerinot et al., 1990). It should be taken into account that a similar scenario may also be valid for our isolates with code numbers G56Y1 and G33Y3.

At the last stage of our study, 8 isolates that we successfully molecularly identified were grouped into three main branches *Firmucutes* (*Bacillus* sp., *Peribacillus* sp., *Staphylococcus* sp.), *Proteobacteria* (*Pseudomonas* sp.), and *Bacteroidetes* (*Chryseobacterium* sp.). 16S rRNA gene sequences alone may not be sufficient to identify a new species, but it is the first and strongest indicator that a new species has been isolated (Tindall et al., 2010). Depending on the taxonomic group investigated, 16S rRNA sequence similarity between 98.2% and 99.0% seems reasonable as a threshold for discovering a new species (Meier-Kolthoff et al., 2013). It is well known that digital DNA hybridization (DDH) and 16S rRNA gene sequence similarities are not linear, and DDH values obtained for a given 16S rRNA gene sequence similarity value can differ significantly (Keswani and Whitman, 2001). In line with all these data obtained in recent years, it is a possibility that our G32S2 isolate, which matches *Chryseobacterium shigense* with a 97.23% similarity rate, may be a new species, and therefore polyphasic studies will be needed.

Conclusion

The detoxifying effect of siderophores or siderophores-producing MOs has been used for bioremediating metal pollution. After chelated by siderophores, metals can be sequestered through different extracellular mechanisms, such as biosorption and bioaccumulation. Also, siderophores have received much attention in recent years because of their potential roles and applications in various areas of environmental research such as biocontrols, biosensors, and bioremediation. Their ability to bind various metals in addition to iron makes siderophores important in a wide variety of biotechnological fields.

This study is the first study to characterize endophyte bacterial isolates isolated from some cultivated and wild cereal plants (Poaceae family) from certain regions of the Van Lake basin in terms of siderophores, which have important application areas in the biotechnological and health sector.

Ethical Statement

Ethical approval is not required for this study.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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Author Contributions

BT designed the research. BT and ŞA conducted experiments, analyzed data, wrote and revised the manuscript. Both authors read and approved the manuscript.

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Research Article

The Impact of Ventilation System Type on the Microclimate of Boar's Pen and Their Clinical Triad Parameters

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Physiological parameters,
Ventilation system

Abstract: The purpose of the study was the impact of different types of ventilation systems in boar`s pen on the microclimate and their physiological parameters. The control group of boars was kept in a house with a transverse ventilation system, and the animals of the experimental group were kept in a geothermal air supply. It was found that, regardless of the season, transverse ventilation provides a significantly higher air velocity and relative humidity: in Winter - 0.15 m s^{-1} and 5.4%; in Spring - 0.35 m s^{-1} and 5.3%; in Summer - 0.41 m s^{-1} and 0.7%; in Autumn - 0.28 m s^{-1} and 8.1%. Maintaining a stable temperature by the normative values in the boar housing was due to geothermal ventilation, regardless of the season, especially the "basement effect" was observed in the summer months, where the air temperature was cooled to 4.5°C ($P < 0.001$), compared to the transverse ventilation system. Compared with the boars in the experimental group, under the influence of the temperature increase in Summer, the boars in the control group increased significantly the respiratory rate to 50.9 ppm ($P < 0.001$) and heartbeat rate of 45.7 ppm ($P < 0.001$). An increase in rectal temperature in boars at elevated ambient temperature under both air ventilation systems was not found. The obtained results make it possible to introduce the use of cost-effective geothermal air supply technology in pig farms to harmonize the physiological parameters of boars to meet their biological needs, even in closed housing to improve their welfare.

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1. Introduction

According to the United Nations, as of November 15, 2022, the world's population reached 8 billion people, and the population growth forecasts from the same global organization are as follows: 8.5 billion in 2030, 9.7 billion in 2050, and 10.4 billion in 2100 (United Nations Department of Economic and Social Affairs, Population Division, 2022). Based on the above prerequisites, global population growth by 2050 will increase the food demand by about 60% (Mun et al., 2021). This will mean that livestock producers will need to spend more energy soon to increase the number of animals to meet the needs of a developing population and balance food and nutrition security (Ofuoku and Ekorhi-robinson, 2020; Wijaya et al., 2023). In addition, animal proteins belong to some foods with an amino acid composition similar to that of human proteins, so their digestibility in the human digestive system is 90-98%, which is the main reason why the world's population uses up to 25% of animal proteins (Mottet et al., 2017). For this reason, raising animals in enclosed spaces with regulated humidity, temperature, and other parameters, also known as livestock houses, is essential. According to Costantino et al. (2021), the outcome is that contemporary livestock complexes are highly automated systems for producing pork that run with the least amount of expenses, the highest level of technology, and the greatest possible production capacity to comply with legal needs for housing pigs from various technical groups.

These facts are contributing to a profound transformation of energy systems in livestock buildings, which are gradually shifting from fossil fuels to more sustainable and low-carbon energy sources such as photovoltaic, solar thermal, and geothermal energy (Kim et al., 2023). As noted by Krommweh et al. (2014), the use of renewable energy is an important alternative to fossil resources in the agricultural sector, particularly in pig production. At the same time, the use of alternative energy in pork production is necessary to reduce the industry's environmental impact, as well as to ensure the welfare of pigs to normalize the biological needs of pigs by keeping them at industrial complexes (Krommweh et al., 2014).

Energy has become increasingly important to agriculture from an economic and environmental standpoint throughout the past year, particularly amid the aggressor country's huge shelling of Ukraine's energy facilities. In light of this, renewable energy sources like geothermal energy are gradually replacing fossil fuels, as mandated by EU legislation.

The meteorological conditions of the southern region of Ukraine, in particular Zaporizhzhya region, are temperate continental with mild winters with little snow and particularly hot summers. In this regard, the temperature, humidity, and quality of the air pool in pig housing are important care parameters, and temperature control is crucial for pigs, as a slight change in internal temperature has a negative impact on behavior, health, growth, performance parameters and, as a result, welfare (Lacetera, 2019; Gody et al., 2020; Gourdine et al., 2021; Costantino et al., 2022). Even with complete feeding, obtaining high-grade protein from premium pork is unachievable without guaranteeing balanced microclimatic factors. In Ukraine, temperature fluctuations are quite pronounced throughout the year (Figure 1), and therefore there are still problems with the air temperature inside the pig housing.

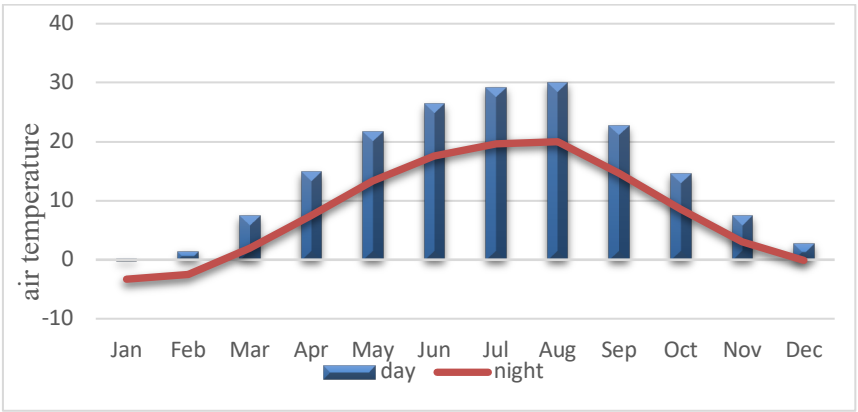

Figure 1. Day and night air temperature in the Zaporizhzhya region in 2021.

Source: data from the Ukrainian Hydrometeorological Center of the State Emergency Service of Ukraine as of 2021 in Zaporizhzhya region.

With changes in ambient temperature, the metabolism of pigs increases, which leads to a decrease in the efficiency of feed resources and an increase in the time to reach slaughter weight. Thus, the geothermal ventilation system is suggested as a way to enhance the pig housing's microclimate, boost the pigs' performance traits, avoid pig infections, and reduce the emission of noxious gases and odors during high or low ambient temperatures. Geothermal ventilation systems, in which the supply air travels via stones placed beneath the chamber and basement air supply ventilation systems. The temperature of the ground, which can reach up to 10 °C in the summer and 5 °C in the winter, causes the supply air to heat or cool in subterranean ducts or as it travels through the stones. Additionally, geothermal ventilation with stone cushions is used for the boars. Beneath the structure are three-meter-deep pits filled with slabs, and the floor is covered in broken stones. Thus, the cooled air passes through the trench, cools down to 12 °C, and then goes to the boars. As a result, the temperature does not rise above 24 °C indoors, even though it can be over 40 °C outside.

In connection with the above, the aim of the study was to investigate the effect of different types of ventilation systems on microclimate and physiological parameters in pig barns.

2. Material and Methods

2.1. Ethics

Regulations on the protection of animals and their comfort (Council Directives 2008/120 / EU, 2010/63/EU) and the Order of the Ukrainian Ministry of Economy governed the conditions for feeding, watering, housing, care, prevention, and treatment. Boars were handled in the tests in a way that complied fully with bioethical guidelines for the humane treatment of animals. October 28, 2021 (007/2021) saw the approval of the experimental protocol by the Bioethics Commission of the National University of Life and Environmental Sciences of Ukraine.

2.2. Experimental design

Experimental studies were carried out at the period of 2021, at the farm of Ukraine - Private Joint Stock Company “Plemzavod “Stepnoy” Zaporizhzhya region. A total of 18 boars of Large White, Landrace, and Duroc breeds were used in the experiment (Figure 2).

Figure 2. Breeds of boars kept on the farm.

Source: photo by the authors.

The average live weight of boars aged 24 months was: Large White - 325 kg, Landrace – 330 kg, Duroc - 326 kg. They were kept on litter in individual pens with an area of 7 m^2, on a concrete floor with thermal and moisture isolation. The boars of different breeds selected for the experiments were clinically healthy. The pig housing used forced transversal and geothermal ventilation with electronic control. Boars were fed individually with a pelleted complete feed «Eber» 2.8-3.0 kg of feed per head/day of nutritional value: a crude protein content of 202.630 g/kg and an exchange energy of 12.406

MJ/kg. The composition of 1 kg of granulated feed «Eber» produced by "Private Company "Alternative" Limited Liability Company contains the following ingredients (%): corn (20.000), wheat (18.355), wheat bran (25.000), soybean cake (22.645), sunflower meal (10.000), AminoMix Eber (4.000), (the certificate of quality according to the Technical Conditions of the State Standard of Ukraine 4508:2005). Feed was provided twice a day, at 8:00 a.m. and 4:00 p.m. The boars had constant access to drinking water from the nipples of watering devices.

Microclimate parameters during the keeping of boars corresponded to the Departmental Norms for Technological Design - Agro-Industrial Complex - 02.05 "Pig-breeding enterprises (complexes, farms, small farms)", 2005. The boars were divided into 2 groups of 9 heads each (3 heads of each breed). The control group of boars was kept in a house ventilated by a transverse ventilation system with wall air intake valves (1), exhaust wall fans (2), and an automated microclimate control system (3) (Figure 3). A computerized microclimate control system controlled the opening of the wall valves, which let air into the room, as well as the exhaust fans' speed.

1 **2** **3**

Figure 3. Structural elements of transverse ventilation for keeping boars of the control group (Source: photo by the authors).

The design features of the ventilation system in the room where the boars of the experimental group were kept are the organization of air circulation by a geothermal system: air inflow from the environment is carried out through the air intake shaft (1), then the air flows through an underground tunnel-air duct (2), where it is additionally heated in winter or cooled in summer by soil energy before entering the room directly through the lower air racks (3), which are evenly located near the Exhaust fans of the shafts located on the ceiling extract air outside, and the functioning of the entire system is organized and controlled by a microclimate control device (4), Figure 4.

1 **2** **3** **4**

Figure 4. Structural elements of geothermal ventilation for keeping boars of the experimental group (Source: photo by the authors).

Both experimental groups of boars kept in houses with different ventilation systems had identical structures, were made of the same building materials, and were equally spatially located relative to the prevailing wind rose. The number of cages in both buildings was the same, with the same area, and a similar system of watering and feed transportation and distribution, manure removal was carried out by horizontal conveyors TSN-3 and remote conveyors on tractor trailers.

2.3. Measurement of microclimate parameters

The microclimate parameters were measured every month of the corresponding season of the year at the same time three times a day (at 7:00 am, 02:00 pm, and 10:00 pm). During the experiment in 2021, in both experimental groups of boars, indoor microclimate parameters were measured using certified devices: air temperature - pyrometer «Testo 810» manufacturer – «Testo AG» (Germany) with a range of non-contact surface temperature -30...+300 °C and an error of ±2 °C and air temperature -- 10...+50 °C with an error of ±0.5 °C; air velocity – thermo-anemometer «Testo 425» manufacturer – «Testo AG» (Germany) with a temperature measurement range: -20...+70 °C with an accuracy of ±0.5 °C, flow rate measurement range: 0...20 m $s^{-1}$and accuracy ±(0.03 m s^{-1}+ 5% of the measured value). Relative humidity was measured using a thermo-hygrometer «Testo 605» manufacturer – «Testo AG» (Germany) with a measuring range of 5-95% RH (±3.0% RH). The air content of ammonia (NH_3), hydrogen sulfide (H_2S) and carbon dioxide (CO_2) was measured using gas analyzer «DOZOR-SM» manufacturer – «SPE Orion» (Ukraine) with a measuring range for CO_2 of 0-10,000 ppm (±2,500 ppm), for NH_3 of 0-28.18 ppm (±7.05 ppm), and for H_2S of 0-21.12 ppm (±3.52 ppm). The equipment is certified in Ukraine and complies with DSTU 3377-96.

An electronic microclimate analyzer was used to record the dynamics of temperature swings in August, the hottest month of the summer. Throughout the course of the week, three independent sensors recorded variations in the microclimate's temperature every 60 minutes and stored the data on each sensor's internal electronic storage. Additionally, all sensor values were transferred to the central console via Wi-Fi switching to create a shared database. From there, a duplicate recording of the memory card was done. Three different locations were assessed for air temperature: 1. outdoors; 2. the boars' resting place, which is situated 25–30 cm above the ground; and 3. the standing area, which is situated 60–70 cm above the ground.

The degree of heat stress in pigs was determined by the temperature-humidity index (THI) developed by Thom (1959), which is a combination of two variables (air temperature and relative humidity) and allows for assessment of the need for cooling of animals and take the necessary measures to avoid heat stress. THI gradations: Suitable THI < 74; Mild: 74 ≤ THI < 78; Moderate: 78 ≤ THI < 82; Severe: THI ≥ 82.

2.4. Physiological data

Respiratory rate (RR) counted the number of uninterrupted flank movements (bpm) per minute (60 seconds) using a stopwatch. The heartbeat rate (HR) was measured in pigs using a portable veterinary pulse oximeter UT100V for pulse rate with a saturation interval of 25-350 bpm with an accuracy of ±2 bpm by fixing the device on the ears of the animal. The rectal temperature (RT) was measured using a digital thermometer inserted 50 mm into the rectum until the reading was constant.

2.5. Data analysis

Data were analyzed using Statistica 12.0 (StatSoft Inc., 2014, www.statsoft.com). Results are presented as mean ± standard deviation (X ± SD). The following significance levels were used for the study: $P < 0.05$, 0.01, and 0.001.

3. Results and Discussion

The analysis of the temperature in the boar housing in winter indicates a significant excess of its values in the room with geothermal ventilation by 3.6 °C ($P < 0.01$) (Table 1). It should be noted that the measurement of air velocity in a room with transverse ventilation in winter resulted in higher values of this microclimate parameter by 0.15 m s^{-1} ($P < 0.001$) and higher relative humidity by 5.4% ($P < 0.001$).

The study of temperature changes in the spring made it possible to establish that the air temperature in the boar housing under the influence of the geothermal ventilation system was significantly higher by 2.5 °C ($P < 0.01$) than in the house with a transverse climate control system. Regarding the parameters of air velocity and relative humidity, the buildings with a transverse type of

ventilation system had significantly higher values by 0.35 m s^{-1} ($P < 0.001$) and 5.3% ($P < 0.001$), respectively, than those with an underground type of air supply.

Because Summer ambient temperatures in the south of Ukraine have been and remain extremely high, with peak temperatures of 38.0 °C in the sun and average values of 29.9 °C, the air temperature in the boar housing also increases significantly with a transverse ventilation system - 28.9 °C, and with an underground tunnel type of air supply, its temperature was 24.4 °C, which is 4.5 °C significantly lower ($P < 0.001$). If there is a high ambient temperature, which in pigs exceeds the zone of their temperature neutrality, it becomes more difficult for animals to maintain temperature comfort due to their limited ability to sweat (Hörtenhuber et al., 2020; Lykhach et al., 2022; Hu et al., 2023).

Table 1. Microclimate parameters in a boar housing with different types of air ventilation systems throughout the season of the year, X ± SD

Parameter	Normative value	Season of the year			
		Winter	Spring	Summer	Autumn
Transverse ventilation					
Air temperature (AT)	17.0-19.0	15.6±0.74	16.3±0.56	28.9±0.48***	17.2±0.83
Relative humidity (RH)	40.0-65.0	63.6±0.52***	67.5±0.46***	43.4±0.34	70.5±0.39***
Air velocity (AV)	0.30-1.00	0.30±0.011***	0.45±0.008***	0.60±0.012***	0.45±0,010***
THI	< 74	59.6	60.7	75.8	62.1
Geothermal ventilation					
Air temperature (AT)	17.0-19.0	19.2±0.69**	18.8±0.49**	24.4±0,62	19.4±0,45*
Relative humidity (RH)	40.0-65.0	58.2±0.41	62.2±0.54	42.7±0,72	62.4±0,61
Air velocity (AV)	0.30-1.00	0.15±0.012	0.10±0.011	0.19±0.042	0.17±0.011
THI	< 74	64.5	64.2	70.2	65.0

Notes: Significant: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (in comparison with the parameter of the different types of ventilation systems).
Source: author's measurement data and calculations.

As a livestock production and processing technologist, it is important to keep in mind that animals undergo heat stress when the air temperature in the boar-breeding room rises to +26 °C or higher. This stress directly impacts the primary reproductive function, the quality of the ejaculate produced by the boars, and causes morphological changes in the sperm (Forcada and Abecia, 2019; Gody et al., 2020; Gourdine et al., 2021; Kondracki et al., 2021). As a result, the quality of embryos decreases, embryonic mortality and abortions in the early stages of sow pregnancy are more common, and litter weight decreases (Mc Glone et al., 2019).

Therefore, the control of the microclimate in the housing allows to ensure the optimal temperature for boars, however, at extremely high external temperatures, which have been observed more and more often recently, it requires the use of an air-cooling system from the ground through underground shafts (channels), as in our case. Fixing the air velocity revealed a tendency to a significant excess of this value in the boar's housing with a transverse ventilation system relative to the same parameter with a geothermal ventilation system by 0.41 m s^{-1} ($P < 0.001$). It is noteworthy that throughout the Summer of the examined year, the relative humidity in boar housing with both ventilation systems ranged from 42.7 to 43.4%, meeting hygienic and sanitary norms. Regarding the Autumn period of the year, it should be noted that the temperature in the room at the boar's standing level with geothermal ventilation was recorded at 19.4 °C, which is within the norms of "VNTP-APK-02.05 - Pig enterprises (complexes, farms, small farms)", as well as the recommendations of PIC on the organization of the artificial insemination station. The temperature in the boar housing room with the transverse system in autumn was measured at 17.2 °C, which is significantly lower ($P < 0.05$) by 2.2 °C than ventilation with an air supply. The parameters of air velocity and relative humidity are higher by 0.28 m s^{-1} ($P < 0.001$) and 8.1% ($P < 0.001$), respectively, than those set for the transverse ventilation system.

Our research is consistent with the experiments conducted by Mykhalko et al. (2022), who found that a geothermal ventilation system normalizes the air temperature in pig farms, especially in Summer, reducing it by 3.9 °C ($P < 0.01$), which allows for maintaining thermal indifference in pigs at an

appropriate level. However, these researchers compared different air ventilation systems in a farrowing shed, where the object of study was lactating sows and piglets.

In general, the TNI in the boar housing with both ventilation systems was in line with the norm and did not exceed 74. However, in the Summer the TNI in the boar housing was 75.8 with the transverse ventilation system, which clearly indicates the presence of a mild degree of heat stress in animals, which is a consequence of increased heart rate and respiratory rate compared to the norm. Niu et al. (2024), in the manuscript "Impacts of climate change-induced heat stress on pig productivity in China" measured the effect of heat stress on the production and output of the pig industry and found a significant negative relationship between the THI (a characterization of heat stress) and pig production. End up authors projecting the loss of production and output value of the pig industry under heat stress levels.

Both microclimate systems provided different gas compositions of the air in the boars' housing (Table 2). According to the legal requirements for the content of harmful gases in pig farms, the critical value of CO_2 is set at 2000 ppm. In our experiment, most often the level of carbon dioxide was below the critical value, depending on the ventilation flow rate and the outside air temperature. Thus, in a boar's housing under the influence of geothermal ventilation, the concentration of carbon dioxide in the air ranged from 1200 ppm (in Summer) to 2000 ppm, with $P < 0.001$ (in Winter). The latter value corresponded to the lowest ambient air temperature of 1.2 °C. Significant high concentrations of CO_2 in the air in the boar's housing were recorded with the geothermal microclimate system throughout the year, where the difference in Winter - 300 ppm ($P < 0.001$), in Spring - 200 ppm ($P < 0.001$) and in Autumn - 100 ppm ($P < 0.001$) compared to the same parameter of the transverse ventilation system. The results of our experiment are in line with the findings of Wenke et al. (2018), where the concentration of carbon dioxide in the air ranged from 1130 ppm (in Summer) to 4363 ppm (in Autumn) in a pig fattening room. According to Krommweh et al. (2014), Islam et al. (2016), and Mun et al. (2021), the geothermal air ventilation system has the resource capacity to reduce carbon dioxide and other harmful gases in pig housing. However, our experimental results do not coincide with the opinion of these authors and demonstrate that when using an underground air supply, the carbon dioxide content in the boar's housing was significantly higher in all seasons except Spring than in the transverse ventilation microclimate system, which is consistent with the reports of Mykhalko et al. (2022) in the lactating sow housing.

Table 2. Contents of gases content in boar housing throughout the year with various air ventilation systems, X ± SD

Parameter	Normative value	Season of the year			
		Winter	Spring	Summer	Autumn
Transverse ventilation					
CO_2, ppm	2000	1700±19.0	1400±12.2	1200±21.4	1900±16.1
NH_3, ppm	20.0	11.1±0.18	7.4±0.25	6.8±0.16	8.2±0.21
H_2S, ppm	10.0	1.2±0.06	1.5±0.12	1.8±0.21*	1.2±0.09
Geothermal ventilation					
CO_2, ppm	2000	2000±27.2***	1600±42.0***	1200±19.5	2000±16.6***
NH_3, ppm	20.0	12.4±0.34**	9.8±0.17***	7.4±0.24	8.7±0.21
H_2S, ppm	10.0	1.2±0.07	1.4±0.12	1.3±0.11	1.1±0.08

Notes: Significant: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (in comparison with the parameter of the different types of ventilation systems).
Source: author's measurement data and calculations.

According to Michiels et al. (2015), and Mun et al. (2021), ammonia and hydrogen sulfide are typical gaseous compounds in pig housing, which are produced by animals and, as a result of manure biotransformation, have a negative impact on the health of both pigs and staff by penetrating the respiratory system. The threshold limit for ammonia is 20.0 ppm. The ammonia concentrations measured during the experiment in the air of the boar's housing with geothermal ventilation were 7.4-12.4 ppm and with transverse ventilation - 6.8-11.1 ppm. With the underground air supply, the highest values of ammonia in the air for boars were recorded in Winter - 12.4 ppm ($P < 0.01$) and in Spring - 9.8 ppm ($P < 0.001$), compared to the transverse ventilation system. Forcada and Abecia (2019) found higher ammonia levels in the air with a valve ventilation system in a pig housing. According to Rong and Aarnink (2019) and Jo et al. (2020), ammonia content of 50 to 100 ppm causes pathologies in the

tissues and organs of pigs, reducing their average daily weight gain by 10%. In our experimental case, the ammonia content in the air in the boar's housing, depending on both air ventilation systems, did not exceed the standard values in different seasons of the year.

Hydrogen sulfide concentrations of 50-100 ppm cause chronic and acute intoxication. According to Szabo (2018), people with toxicological effects of high doses of hydrogen sulfide in the air experience irritation of the mucous membranes of the respiratory system, visual analyzer, olfactory paralysis, loss of consciousness, pulmonary edema, and even death. Mykhalko et al. (2022) reported that higher temperatures in the pig housing and lower air velocity led to an increase in the concentration of hydrogen sulfide in the air. In our case, a similar significant increase in hydrogen sulfide content of 1.8 ppm ($P < 0.05$) was observed in Summer with the transverse ventilation system due to a higher air temperature for boars - 28.9 °C, compared to geothermal ventilation. It should be noted that in different seasons of the year, the hydrogen sulfide content did not exceed the standard values regardless of the air supply system.

In August, measured the oscillation of air temperature outside, in the area where boars lie at a level of 25-30 cm from the floor, in the standing area at a level of 60-70 cm from the floor, depending on the air supply system in the boar housing (Figure 5, Figure 6).

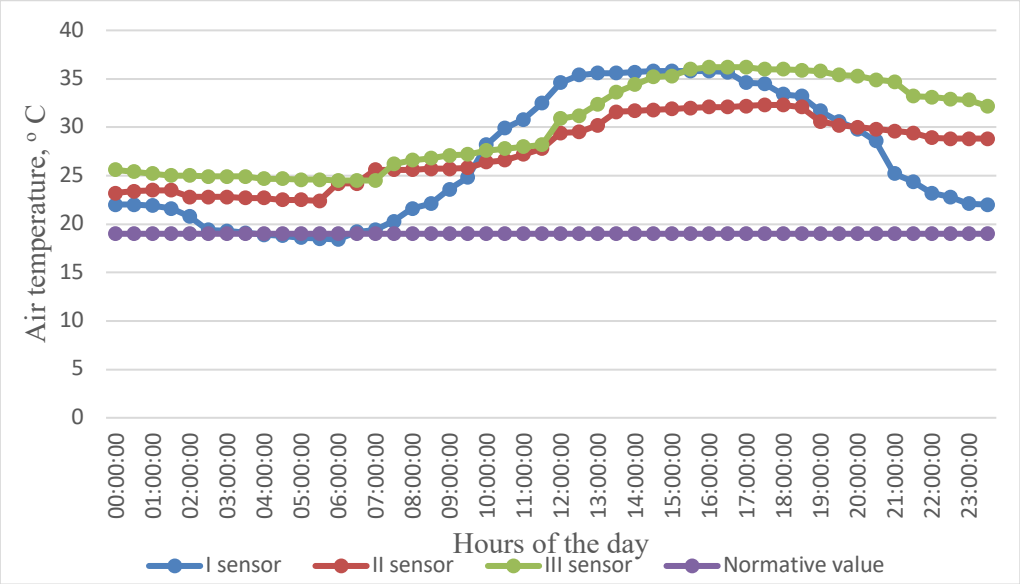

Figure 5. Oscillation of the air temperature of three sensors by transverse ventilation system in a boars housing in August (Notes: I sensor - air temperature outside; II sensor – air temperature area where boars lie at a level of 25-30 cm from the floor; III sensor - air temperature standing area boars at a level of 60-70 cm from the floor. Source: author's measurement data).

Observations show that from midnight to 6.00 am, the outside temperature steadily decreased from 22 °C to 18.4 °C, which is an obvious process at this time of year. Starting from 6.30 am, the outside temperature steadily increased from 19.2 °C to 35.8 °C by 2.30 pm. Then, the temperature remained unchanged at 35.8 °C until 4.00 pm. From 4.30 pm, the air temperature began to gradually and slowly decrease and by midnight its value stabilized.

However, the temperature parameters in the boar lying area at 25-30 cm from the floor and in the animal standing area at 60-70 cm from the floor differed depending on the type of climate control system in the boar housing.

August's average ambient air temperature was 26.7 °C at the time of measurement. With the transverse ventilation system, the average temperature in the boars' laying region was 27.5 °C, which is considerably ($P < 0.05$) 21.45% higher than the same parameter with the geothermal ventilation system. This area is located 25–30 cm from the floor.

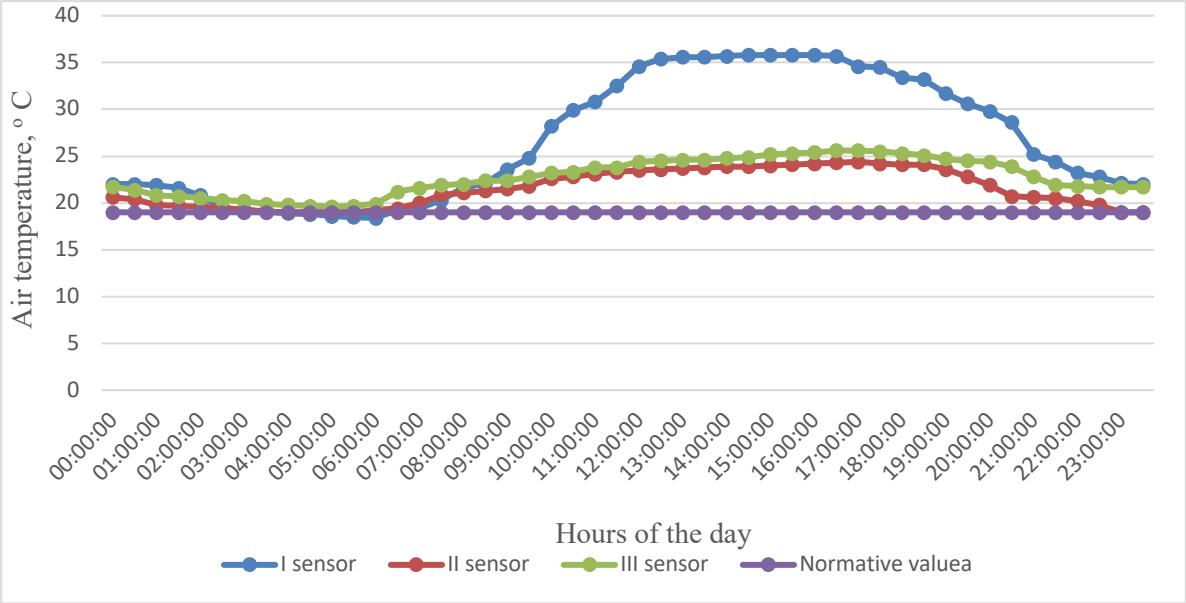

Figure 6. Oscillation of the air temperature of three sensors by a geothermal ventilation system in a boar housing in August (Notes: I sensor - air temperature outside; II sensor – air temperature area where boars lie at a level of 25-30 cm from the floor; III sensor - air temperature standing area boars at a level of 60-70 cm from the floor. Source: author's measurement data).

The temperature difference in terms of air cooling was in favor of geothermal ventilation in the boars' lying down area, as the air, having passed through the air ducts of the underground tunnel, lost 5.9°C of heat, which indicates the operation of the «basement effect» (Figure 7), which means that the underground cooling system copes with its main task.

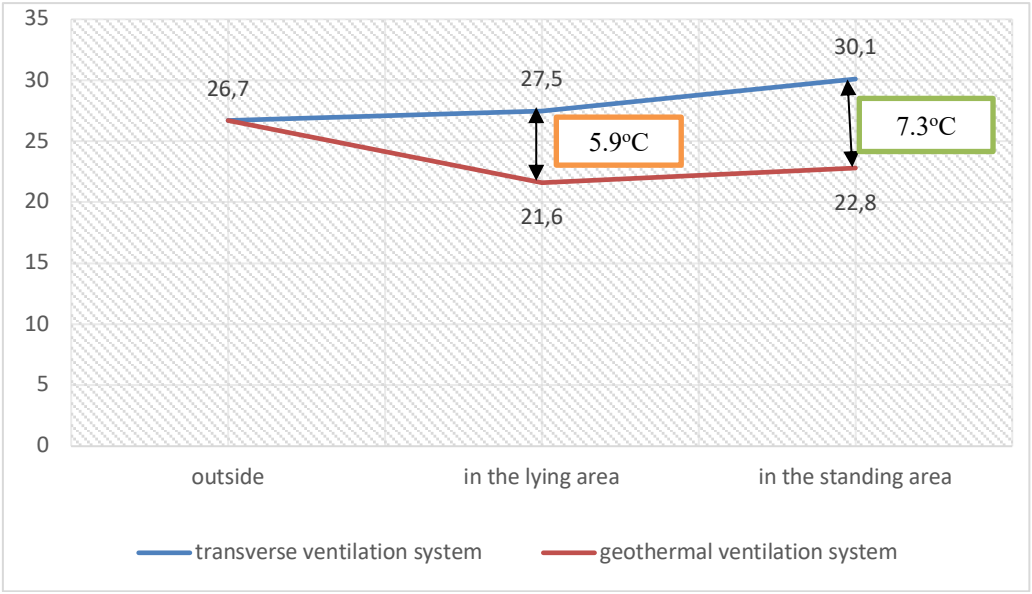

Figure 7. Temperature control in the boar housing (Notes: -the presence of the figure indicates the «basement effect»; - air heat loss. Source: author's development).

In the area of boars standing at the level of 60-70 cm from the floor, the air temperature decreased by 7.3°C, or 24.25% (P <0.05) under geothermal ventilation, which again indicates the operation of the «basement effect». Instead, with the transverse air ventilation system, the average temperature at the level of boars' standing was recorded at 30.1 °C.

August's average ambient air temperature at the time of measurement was 26.7 °C. When using the transversal ventilation system, the average temperature in the boars' lying region, which is 25–30

cm from the floor, was 27.5 °C. This is considerably ($P < 0.05$) 21.45% higher than the same parameter when using the geothermal ventilation system.

The temperature factor influenced the physiological parameters of boars with different types of indoor ventilation systems (Table 3). As Ross et al. (2015) showed, the response of animals to heat stress begins with an increase in respiration rate, followed by a decrease in feed intake, leading to an increase in rectal temperature, an indicator of reduced productivity in pigs. Experimental results showed that under the influence of increased temperature, the respiratory rate of boars housing with transverse ventilation systems significantly increased the respiratory rate by 68.3% ($P < 0.001$) of boar pens with cooled air supply. Oliveira et. al (2024) reported that heat stress increased pigs' respiratory rate by 112%. It is worth noting that increased respiratory rate is the primary mechanism of heat dissipation in the pig body an effective physiological system for maintaining heat generation and transfer. On the other hand, pigs' typical respiratory rates range from 10.0 to 32.7 bpm, according to Lykhach et al. (2022). If the temperature difference between the boars' skin and the outside air decreases, heat transfer by convection becomes impossible. Then the mechanism of increasing the frequency of respiratory movements is activated to stabilize the body temperature, which becomes dangerous for the animals (Scriba-Janulis and Wechsler, 2021).

Table 3. Boars' clinical triad parameters based on the housing's ventilation system, X ± SD

Parameter	Type of ventilation		Normative value
	transverse	geothermal	
Heartbeats rate, bpm	134.9±5.62***	89.2±4.25	80.0-100.0
Respiratory rate, bpm	74.5±4.72***	23.6±3.29	10.0-35.0
Rectal temperature, °C	39.2±2.44	38.6±1.78	38.0-39.0

Note: significant: ***$P < 0.001$ (in comparison with same parameter geothermal ventilation system);
Source: author's measurement data and calculations.

In terms of the heart rate parameter, boars housed in housing with transverse air ventilation as opposed to the geothermal system showed an increase in this signal by 45.7 bpm, or 33.88% ($P < 0.001$). The reason for this response is the direct activation of the hypothalamic thermal center, which triggers the cardiorespiratory system to try and evaporatively release heat through elevated heart rate and respiration (Rodrigo et al., 2018; Scriba-Janulis and Wechsler, 2021).

Measurements of rectal temperature in boars kept under different types of indoor ventilation systems did not reveal significant differences in the value of this indicator and were within the normative values, varying in the range of 38.0-39.2 °C. Thus, no increase in body temperature with an increase in ambient temperature was detected.

The results obtained from the experiment can be used to show schematically the effect of temperature under different ventilation systems used to maintain the metabolism of the boars (Figure 8).

The temperature zone in which the boars' metabolism is maintained at a constant level is called the thermoneutral zone, or comfort zone with warm and cool zones, in which animals maintain a constant body temperature through vasocontraction and vasodilation (Scriba-Janulis and Wechsler, 2021; Lykhach et al., 2023).

In the experiment conducted for boars, the thermoneutral zone was +17...+19 °C, which is consistent with the research Dekker, 2015. If the temperature reaches the lowest critical point (+5...0 °C), showed a drop in the body temperature of boars (hypothermia). Boars are more tolerant of low temperatures (Gourdine et al., 2021).

At ambient temperatures above +26 °C, boars reduce their movement, rest more time, and increase their body area to release heat from their skin. If the air temperature is above +30 °C, hyperthermia is observed, breathing becomes frequent, and appetite decreases up to the point of refusal to feed, end up leading to death (Lykhach et al., 2022).

The lower and upper temperatures of the thermoneutral zone are called critical temperatures. Therefore, when the air temperature is lower than the lower critical temperature, it is no longer possible to retain heat in the body by reducing heat transfer, muscle tremors are observed and animals are forced to increase the intensity of metabolism to produce heat. At air temperatures above the upper critical limit, there is an increase in heat transfer due to sweating and increased respiration (Dekker, 2015).

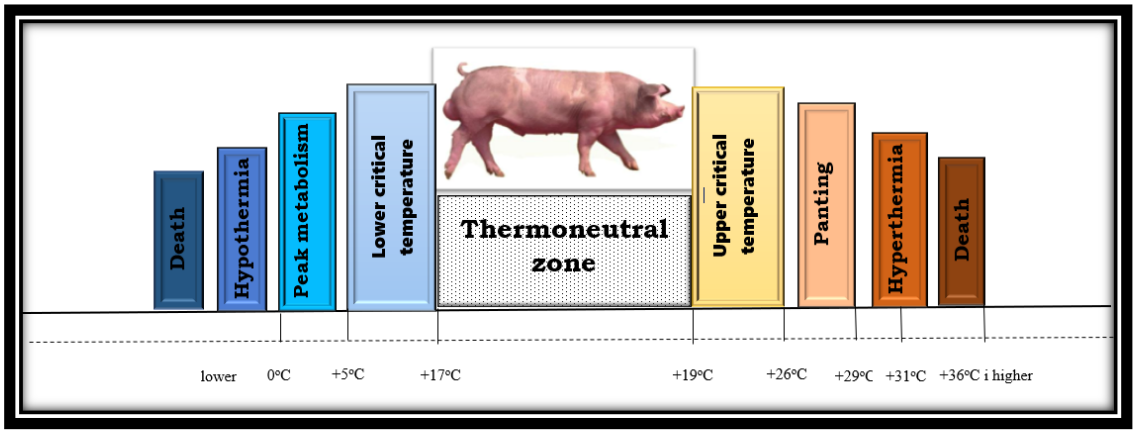

Figure 8. The effect of temperature under different ventilation systems used to maintain the metabolism of the boars (Notes: the lower critical temperature is the temperature of the environment with the lowest level of metabolism in boars at the lowest tension of heat transfer mechanisms, and the upper critical temperature is the temperature of the environment with a high level of metabolism in boars with reduced convection and increased heat transfer through the respiratory and circulatory systems with increased energy metabolism).

With energy prices on the rise both in Ukraine and globally, low-energy ventilation systems are becoming more profitable than ever. The economic efficiency of using different ventilation systems for boar housing is shown in Table 4.

Table 4. The economic analysis of using different ventilation systems for boar housing

Parameter	Type of ventilation		+/- geothermal/ transverse
	transverse	geothermal	
Average cost of a boar, UAH*	87300.00	87300.00	
EUR	2000.00	2000.00	
Average cost per day boar for 365 days of operation, excluding the cost of feeding, management and care, UAH*	239.20	239.20	
EUR	5.38	5.38	
Electricity consumption, kWh	10.00	2.50	
Electricity consumption for the day, kWh	240.00	60.00	-180.00
Electricity consumption for 365 days, kWh	87600.00	21900.00	-65700.00
Price of electricity consumption per 1 kWh, UAH*	6.00	6.00	
EUR	0.14	0.14	
Cost of electricity consumption per year, UAH*	525600.00	131400.00	-394200.00
EUR	11827.18	2956.80	-8870.38

Note: * - at average prices in the Ukraine in the first half of 2024.
Source: author's measurement data and calculations.

Based on the economic analysis, it was found that pig farms with a geothermal ventilation system will be able to save 394200.00 UAH (8870.38 EUR) in electricity consumption per year. Such a cost-saving technology in pig husbandry is extremely important for countries with hot climatic conditions and will reduce the heat load on animals through a geothermal cooling system and, as a result, improve boar welfare.

Conclusion

Monitoring of the microclimate in boar housing with the studied types of ventilation systems makes it possible to obtain significant information on the oscillation of air temperature and relative air humidity conditions during all seasons of the year. Based on the measurements and calculations, it was found that both the transverse ventilation and geothermal ventilation systems provide boars with microclimate parameters that meet biosecurity standards. The transverse ventilation system removes contaminated air from the room better, minimizing the negative impact of harmful gases on the physiological parameters of boars. However, the use of a transverse ventilation system did not ensure a comfortable temperature in the boar's housing and led to its exceeding 9.9°C in Summer. Therefore, the underground air supply ensures uniform air exchange, normalizing the temperature and air movement, providing standard humidity values at different times of the year, and copes well with the task of creating a satisfactory indoor microclimate for this technological group. In addition, the geothermal ventilation system provides a comfortable temperature for boars in Summer, which is particularly hot, thanks to the 'basement effect', which is confirmed by the normative values of the clinical triad parameters, and will save pork producers UAH 394200.00 (EUR 8870.38) in electricity consumption per year. Therefore, the study on the impact of ventilation systems on the microclimate of boar's housing and their physiological parameters is useful for pork farmers in developing strategies for sustainable practices that open up ways to ensure the comfort of boars and their welfare.

Ethical Statement

Ethical approval for this study was obtained from the Bioethics Commission of the National University of Life and Environmental Science of Ukraine (007/2021).

Conflict of Interest

The Authors declares that there are no conflicts of interest.

Funding Statement

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Author Contributions

A. Deschenko, A. Lykhach, L. Lenkov participated in the design of study, performed the experiments and writing original manuscript. V. Lykhach, Y. Barkar, M. Shpetny assisted in performing the study, data arranging, and calculation. V. Lykhach and A. Lykhach critically revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Research Article

Growth and Laying Performance of Local Guinea Fowl on Different Dietary Protein and Energy Levels

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Abstract: Constraints to Guinea fowl (*Numida meleagris*) production include poor growth and low laying performance. However, the lack of standard nutritional requirements significantly hinders commercial indigenous Guinea fowl production in Nigeria. This study aimed to determine the proper levels of crude protein (14, 16, and 18%) and metabolizable energy (2.65, 2.75, and 2.85 Mcal/kg) in the diets of native Guinea fowl in Nigeria. The dietary protein (PL) and energy (EL) levels for the fowl were evaluated in a completely randomized 3 (PL) × 3 (EL) factorial design with three replicates of 10 birds each. Thus, 270 birds with 20 weeks of age were allocated randomly to nine dietary treatments (18P:2.65E, 18P:2.75E, 18P:2.85E, 16P:2.65E, 16P:2.75E, 16P:2.85E, 14P:2.65E, 14P:2.75E and 14P:2.85E). The PL × EL interaction affected Guinea fowl's DFI, DWG, and WWG ($p<0.05$), while WFI and FCR remained unaffected. The 16:2.85E diet increased the DFI of the birds compared to other diets ($p<0.05$). The DFI of the 18:2.65, 18:2.75, and 16:2.75E Guinea fowls was higher than those of 18:2.85E, 14:2.65E, 14:2.75E, and 14:2.85 birds ($p<0.05$). The DWG of fowls improved by the 16:2.85E diet compared to other diets, except for the 18P:2.65E and 16P:2.75E diets ($p<0.05$). The interaction had a significant impact on the EN, EYH, and EM of the Guinea fowl egg while FCR remained unaffected. The 18P:2.85E diet improved the EN and EM of the birds compared to other diets ($p<0.05$), The 18P:2.85E also improved the FCR for laying except for 14P:2.85E and 18P:2.75E. The 18P:2.85E diet influenced the YW of the birds compared to other diets ($p<0.05$), whereas the EW of fowls improved by the 16:2.85E diet compared to other diets except for the 16P:2.65E diet ($p<0.05$). In conclusion, feeding guinea fowls with a diet comprising 18% protein and 2.85 Mcal/kg metabolizable energy significantly improved egg production and quality.

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1. Introduction

As a major source of protein, the poultry sector is vital to the world's food production (Oghenero et al., 2021). The native guinea fowl (*Numida meleagris*) is a valuable alternative for poultry farming (Baruwa and Sofoluwe, 2016; Shoyombo et al., 2021; Alabi et al., 2023) due to their gamey nature, meat and egg production. However, increasing their productivity demands understanding the connection between dietary nutrients and vital economic indicators like growth, egg production, and egg quality (Ebegbulem, 2018; Yakubu et al., 2022). Thorough management of diet is crucial for successful poultry production. Growth, reproductive efficiency, and product quality can be improved by tweaking the energy and dietary protein levels of poultry feeds based on specific requirements. However, there is no nutritional requirement standard for the native Guinea fowl in Nigeria (Mohammed and Dei, 2017; Rafiu et al., 2021).

Nutritional requirement studies have advanced greatly in relevance, therefore, the current study carefully deployed three energy and three dietary protein levels in a factorial design. The objective of the approach is to examine the effects of these factors, independently and in combination, on growth, egg production, and egg quality. Growth performance impacts meat production, while production and quality of eggs serve as essential aspects of poultry products (Ismoyowati et al., 2022). However, the local guinea fowl hens low laying on the range, and their ability to lay for long periods is dependent on the weather conditions; in harsh weather, they tend not to lay but only occasionally (Alli et al., 2016; Yakubu et al., 2019). Hence, by evaluating the effect of dietary protein and energy on the growth and egg-laying performance of the indigenous guinea fowl, this study's findings could contribute to setting a framework for developing nutritional standards and feed formulations that can improve the indigenous guinea fowl laying capability.

2. Material and Methods

2.1. Experimental design and bird management

The Landmark University Ethics Committee approved the research protocols and experimental birds in this work vide number LUAC/2021/0018A. The research was carried out at the Guinea Fowl Improvement Centre of the Teaching and Research Farm of Landmark University. Two hundred and seventy native Guinea fowls were selected from the grower flock at 20 weeks of age and then were allocated in a completely randomized 3 (protein levels, PL) × 3 (energy levels, EL) factorial design to nine dietary treatments (18P:2.65E, 18P:2.75E, 18P:2.85E, 16P:2.65E, 16P:2.75E, 16P:2.85E, 14P:2.65E, 14P:2.75E and 14P:2.85E) with three replicates of 10 birds each. Birds in each of the replicate pens were homogeneous in terms of live weight (LW) and egg production.

In this study, nine experimental diets (Table 1) were formulated using locally purchased ingredients to contain varying PL and EL. Three PLs (18, 16, and 14% crude protein, CP) were formulated, and three ELs (2.65, 2.75, and 2.85 Mcal/kg) in metabolizable energy (ME) were altered in the high, medium, and low CP diets. Thus, three levels of the energy content of each 18, 16, and 14% CP diet were created by replacing energy sources with protein sources in the layer diet. The Guinea fowls were fed with one of these diets in mash form (20–52 weeks) with ad libitum access to feed and fresh water. The fowls were reared in floor pens (1 × 2.5 m^2) with wood shavings and a lighting program with a minimum light intensity of 1-2 Lux for at least 12 hours, depending on the daily photoperiod, under controlled environmental conditions. The indoor temperature was set at 28°C with a relative humidity of 55% throughout the experiment. In addition, each of the indoor pens was equipped with a perch, individual nests (30 × 45 × 60 cm, 1 nest/5 hens), a drinker, and a red circular poultry feeder plate.

2.3. Data collection

The proximate composition (the percentages of dry matter [DM], ash, ether extract [EE], and crude fiber [CF]) of all diets was determined according to the standard method (AOAC, 2012). These analyses were performed at the Project Research Laboratory I, the College of Agricultural Sciences, Landmark University.

The LW of Guinea fowls was measured using the Ohaus (model PA512) digital sensitive weighing balance at placement and weekly during the experimental period. The daily (DWG) and weekly (WWG) weight gain for each treatment were calculated from these measurements. Feed intake (FI) and egg yield and weights were recorded daily per replicate pen using the Ohaus (model PA512) digital sensitive balance to two decimal places. Egg-laying number (EN) and weight (EW), egg yield per hen (EYH, mm) and week (EYW, mm), egg mass (laying rate × egg weight), daily (DFI) and weakly (WFI) FIs (total FI/number of days or weeks of the trial period) and feed conversion ratio (FCR, g feed: g egg mass) were calculated. Of the eggs produced during the last three days of each 7-day interval, 64 randomly selected eggs (4 from each replicate) were used to determine some egg quality parameters (reference should be given), such as egg weight (EW), egg height (EH), egg width (EWd), yolk height (YH), yolk diameter (YD), yolk weight (YW), shell thickness (ST), egg index (EI, [(egg width/egg height) × 100]): yolk index (YI, [(yolk width/yolk height) × 100]) (Musundire et al., 2017).

2.2. Statistical analysis

All data were subject to two-way analysis of variance using GenStat (2013) statistical package to analyze the effect of dietary PL and EL and their interaction (PL × EL) on growth performance and egg production indices. Differences in mean values were determined using Duncan's multiple comparison test, with a difference level of $p<0.05$ considered. All data in this study are expressed as mean ± SEM.

3. Results

Table 1 presents the composition of the diet formulated for the experiment using the 3 levels of energy and protein. The calculated nutrients are given for ME, CP, Ca, and Available P for all the diets. The analyzed nutrient composition of the diets was not significantly different ($p<0.05$). However, the ash content in interaction 16P:2.65E (5.07) was highest followed by 14P:2.65E (5.00). Interaction 18P:2.75E has the highest moisture content of 10.50% while interaction 16P:2.85E.

Table 1. Ingredients and nutrient composition of the experimental diets (as fed, %)

	Diet								
Ingredient	**18P:2.65E**	**18P:2.75E**	**18P:2.85E**	**16P:2.65E**	**16P:2.75E**	**16P:2.85E**	**14P:2.65E**	**14P:2.75E**	**14P:2.85E**
Maize	50.00	55.00	61.50	52.00	55.00	65.50	49.00	56.00	65.50
SBM, 44 CP %	18.00	18.00	28.50	12.00	13.00	14.50	10.00	11.00	17.00
Groundnut cake	11.00	12.00	0.00	9.00	11.00	10.00	8.00	8.00	0.50
Corn bran	7.00	5.00	0.00	9.00	11.00	0.00	19.00	14.00	7.00
Wheat offal	4.00	0.00	0.00	6.00	0.00	0.00	4.00	1.00	0.00
Bone meal	3.05	3.05	3.05	3.05	3.05	3.05	3.05	3.05	3.05
Limestone	5.80	5.80	5.80	5.80	5.80	5.80	5.80	5.80	5.80
Salt	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
L-Lysine	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Methionine DL	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Layer premix[1]	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Choline chloride	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Nutrizyme[2]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin E	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Calculated nutrient composition									
ME (Mcal kg^{-1})	2.65	2.75	2.85	2.65	2.75	2.85	2.65	2.75	2.85
CP (%)	18.00	18.00	18.00	16.00	16.00	16.00	14.00	14.00	14.00
Ca	3.163	3.155	3.14	3.16	3.159	3.133	3.173	3.159	3.138
Available P	0.933	0.900	0.895	0.933	0.882	0.882	0.901	0.876	0.861
Analyzed nutrient composition (%)									
Moisture	9.83	10.50	10.33	10.17	9.00	8.83	9.00	8.83	10.00
Ash	4.67	4.67	4.87	5.07	4.13	4.00	5.00	4.87	4.93
Crude protein	17.89	17.94	17.89	15.95	15.98	15.96	13.97	13.93	13.95
Ether extract	13.00	13.67	14.00	14.33	14.17	13.33	13.17	13.83	14.50
Crude fiber	4.37	3.10	3.39	4.22	4.00	4.17	4.06	4.41	4.38
N-free extract	50.24	50.13	49.55	50.26	52.72	53.70	50.24	50.13	52.24

[1]Vitamins [A, D3, E, K, pantothenate (B5), pyridoxine (B6), B12, niacin, biotin, and choline], Minerals [calcium, arsenic, magnesium, potassium, copper, iodine, iron, manganese, molybdenum, selenium, and zinc].
[2]Nutrizyme enzyme cocktail: Cellulase, Xylanase, β-glucanase, Mannanase, Protease, Alpha-amylase.

Table 2 presents the impact of dietary PL, EL, and the interaction between them on the growth performance of native Guinea fowls. The DFI, DWG, and WWG of Guinea fowl were affected by the interaction between these factors, while WFI and FCR remained unaffected. The 16P:2.85E diet increased the DFI of the birds compared to other diets ($p<0.05$). The DFI of Guinea fowls fed on the 18P:2.65E, 18P:2.75E, and 16P:2.75E diets was higher than those of birds fed on the 18P:2.85E, 14P:2.65E, 14P:2.75E, and 14P:2.85 diets ($p<0.05$). The DWG of fowls improved by the 16:2.85E diet compared to other diets, except for the 18P:2.65E and 16P:2.75E diets ($p<0.05$). The 18P:2.85E and 16P:2.65E birds had a lower DWG compared with other Guinea fowls, except for 14P:2.85E ($p<0.05$).

Table 2. Growth performance of local Guinea fowl fed diets with different protein and energy levels

Factor	Parameter				
	DFI (g)	WFI (g)	DWG (g)	WWG (g)	FCR (g feed: g live weight)
Protein level (PL, %CP)					
18	11.36	79.54	1.88	13.17	6.12
16	11.78	74.98	1.98	13.83	6.18
14	10.46	73.24	1.91	13.35	5.54
Energy level (EL, Mcal ME kg^{-1})					
2.85	11.28	72.95	1.94	13.55	5.97
2.75	11.11	77.75	1.96	13.69	5.70
2.65	11.21	77.05	1.87	13.11	6.17
PL × EL interaction					
18:2.65E	11.65	81.57	2.08	14.52	5.62
18:2.75E	11.70	81.87	1.97	13.78	5.97
18:2.85E	10.74	75.18	1.60	11.19	6.77
16:2.65E	11.18	73.89	1.58	11.04	7.32
16:2.75E	11.41	79.85	2.00	14.01	5.70
16:2.85E	12.75	71.19	2.35	16.43	5.51
14:2.65E	10.81	75.70	1.97	13.75	5.56
14:2.75E	10.22	71.53	1.90	13.26	5.42
14:2.85E	10.36	72.49	1.86	13.04	5.64
SEM	0.62	1.79	0.11	0.78	0.60
Main effect of					
PL	*	Ns	**	Ns	Ns
EL	*	Ns	*	Ns	Ns
PL × ML	**	Ns	***	**	Ns

DFI: average daily feed intake, WFI: average weekly feed intake, DWG: average daily weight gain, WWG: average weekly weight gain, FCR: feed conversion ratio, SEM: standard error of the mean.
[a,b,c] Means within a row with different superscripts are significantly different at $p<0.05$. ns: non-significant ($p>0.05$), *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Table 3 presents the effect of dietary PL, EL, and their interaction on the laying performance of native Guinea fowls. The EN, EYH, and EM of Guinea fowl were affected by the interaction between these factors, while FCR remained unaffected. The 18P:2.85E diet increased the EN of the birds compared to other diets ($p<0.05$). The EN of Guinea fowls fed on the 18P:2.65E and 18P:2.75E diets was higher than those of birds fed on the 16P6:2.85E, 16P:2.65E, 1P:2.75E, and 14P:2.65, 14P:2.75E, diets ($p<0.05$). The EM of fowls improved by the 18:2.85E diet compared to other diets, except for the 18P:2.75E, 18P:2.65E and 14P:2.75E diets ($p<0.05$). The birds on 14P:2.75E had a lower EM than other Guinea fowls ($p<0.05$). The FCR for laying was better in diet 18P:2.85E compared to other diets except for 14P:2.85E and 18P:2.75E, however, it was much in diet 14P:2.75E ($p<0.05$).

Table 4 shows the impact of dietary PL, EL, and the interaction between them on some egg quality traits of native Guinea fowls. The YD and YW of Guinea fowl eggs were affected by the interaction between these factors, while EI and YI remained unaffected. The 18P:2.85E diet influenced the YW of the birds compared to other diets ($p<0.05$). The YD of Guinea fowls fed on the 16P:2.65E, 16:2.85E, and 16P:2.75E diets was higher than those of birds fed on other diets ($p<0.05$). The EW of fowls improved by the 16:2.85E diet compared to other diets, except for the 16P:2.65E diet ($p<0.05$).

Table 3. Laying performance of local guinea fowl fed diets with different protein and energy levels

Factor	Parameter				
	EN	EYH	EM (g)	FCR (g feed: g EM)	LR (%)
Protein level (PL, %CP)					
18	165.70	26.10	831	3.31	54.60
16	65.90	11.10	603	3.97	45.20
14	61.40	9.60	562	3.94	41.10
Energy level (EL, Mcal ME kg^{-1})					
2.85	114.60	17.30	767	3.00	78.90
2.75	92.10	15.40	600	4.25	31.40
2.65	86.30	14.10	629	3.98	30.60
PL × ML interaction					
18:2.65E	138.70^{b}	23.10^{ab}	701^{ab}	3.64	66.00
18:2.75E	163.00^{ab}	27.20^{a}	829^{a}	2.98	77.60
18:2.85E	195.30^{a}	27.90^{a}	964^{a}	2.37	93.00
16:2.65E	72.00^{bc}	12.10^{b}	597^{b}	4.26	34.30
16:2.75E	77.00^{bc}	12.30^{b}	643^{ab}	3.76	36.70
16:2.85E	48.70^{c}	9.00^{bc}	569^{b}	4.73	23.20
14:2.65E	48.30^{c}	7.20^{c}	590^{b}	3.93	23.00
14:2.75E	36.30^{c}	6.70^{c}	326^{c}	5.18	21.30
14:2.85E	99.70^{bc}	15.00^{b}	769^{ab}	2.84	47.50
SEM	17.58	3.07	91.30	0.47	8.16
Main effect of					
PL	*	**	*	Ns	*
EL	*	*	*	Ns	*
PL × ML	**	***	**	**	***

EN: egg number, EYH: average egg yield per hen, TEW: total egg weight, EM: egg mass, LR: laying rate, SEM: standard error of the mean.
^{a,b,c} Means within a row with different superscripts are significantly different at p<0.05. *: p<0.05, **: p<0.01, ***: p<0.001.

Table 4. Some egg quality traits of local guinea fowl fed diets with different protein and energy levels

Factor	Egg quality trait								
	EW	EH	EWd	YH	YD	YW	ST	EI	YI
Protein level (PL, %CP)									
18	36.77	46.83	36.33	1.39	34.06	16.72	0.30	0.78	0.04
16	41.16	51.03	41.56	1.17	41.85	16.24	0.31	0.82	0.03
14	34.80	46.46	37.60	1.21	36.72	13.10	0.29	0.81	0.03
Energy level (EL, Mcal ME kg^{-1})									
2.85	38.30	49.80	41.62	1.26	38.25	14.58	0.29	0.84	0.03
2.75	36.05	46.16	36.55	1.27	38.37	14.75	0.37	0.79	0.03
2.65	38.38	48.36	37.32	1.25	36.01	16.73	0.24	0.77	0.04
PL × ML interaction									
18:2.65E	36.28^{b}	46.44^{bc}	35.93^{c}	1.28^{bc}	27.30^{c}	18.19^{a}	0.29^{b}	0.77^{c}	0.05^{a}
18:2.75E	34.20^{c}	46.03^{bc}	36.03^{c}	1.53^{a}	37.44^{bc}	15.89^{b}	0.34^{ab}	0.78^{b}	0.04^{b}
18:2.85E	39.83^{ab}	48.02^{b}	37.04^{bc}	1.37^{b}	37.45^{bc}	16.07^{b}	0.28^{b}	0.77^{c}	0.04^{b}
16:2.65E	45.38^{a}	52.12^{ab}	39.82^{b}	1.37^{b}	43.53^{a}	19.11^{a}	0.23^{c}	0.77^{c}	0.03^{a}
16:2.75E	37.92^{b}	46.58^{bc}	37.26^{bc}	0.95^{c}	40.88^{b}	14.36^{bc}	0.33^{bc}	0.80^{ab}	0.02^{b}
16:2.85E	40.18^{ab}	54.39^{a}	47.60^{a}	1.20^{bc}	41.14^{b}	15.26^{b}	0.36^{bc}	0.88^{a}	0.03^{a}
14:2.65E	33.47^{c}	46.52^{bc}	36.22^{c}	1.10^{c}	37.21^{bc}	12.89^{c}	0.21^{c}	0.78^{b}	0.03^{a}
14:2.75E	36.04^{b}	45.88^{c}	36.37^{c}	1.33^{b}	36.79^{bc}	14.00^{bc}	0.45^{a}	0.79^{b}	0.04^{b}
14:2.85E	34.89^{c}	46.99^{bc}	40.23^{ab}	1.20^{bc}	36.17^{bc}	12.40^{c}	0.23^{c}	0.86^{ab}	0.03^{a}
SEM	2.07	1.82	1.44	0.08	1.57	1.40	0.09	0.04	0.003
Main effect of									
PL	*	**	**	Ns	*	**	Ns	*	Ns
EL	*	*	*	Ns	**	**	Ns	*	Ns
PL × ML	**	***	**	Ns	**	**	Ns	*	Ns

EW: egg weight, EH: egg height, EWd: egg width, YH: yolk height, YD: yolk diameter, YW: yolk weight), ST: shell thickness, EI: egg index
YI: yolk index, SEM: standard error of the mean.
^{a,b,c} Means within a row with different superscripts are significantly different at p<0.05. *: p<0.05, **: p<0.01, ***: p<0.001.

4. Discussion

Guinea fowl though a domesticated game bird (Alabi et al., 2023), also experience significant dietary changes as they move into the egg-laying phase. A greater requirement marks this change for dietary protein to sustain the development of egg-laying cells and improve egg production and quality. Heo et al. (2023) noted that a sufficient provision of dietary protein is crucial to attaining better growth rates and egg production in poultry. As in the present study, when PL decreased from 18% to 14%, the DFI and DWG reduced without affecting FCR. However, FCR improves as protein levels decline due to more effective feed utilization at lower PLs. This case shows that not only PL but also EL of the diet plays an important role. Indeed, in the current study, the PL × EL interaction effect on the parameters examined was significant. Based on our results, providing the optimum energy levels in Guinea Fowl diets is vital because low dietary energy may result in the utilization of dietary protein for energy rather than protein synthesis as noted by Musigwa et al. (2021).

Our results indicate that dietary manipulations critically affected egg yield and quality traits during the early stages of the egg production cycle. Oke et al. (2020) reported that increasing dietary protein from 16% to 18% considerably enhanced egg production, as found herein. However, the results on the laying performance concluded that the PL or EL of the diet at the onset of egg production is not the only factor determining laying hen performance (Bryden et al., 2021). On the other hand, if the EL of the diet was a limiting factor for optimizing egg weight, then one should expect to observe a beneficial effect on egg weight due to increasing the EL of the diet (Mikulski et al., 2020). As known, laying hens have retained the ability to adjust feed intake to dietary energy, and a decrease in dietary energy content leads to increased feed intake. This acceptance may alter the differences in environmental conditions, diet composition, energy-to-protein ratio, egg production, hens' genotype, and age (Mikulski et al., 2020; Bryden et al., 2021). This information may explain the effect of the PL × EL interaction on egg yield and quality.

Our study showed that FCR was better at EL of 2.65 Mcal kg^{-1}, indicating a reduced efficient feed conversion as energy increased. The interaction effects of the PL × EL highlight their collective effect on growth parameters. The highest ADG was observed at 18% PL in combination with EL of 2.85 Mcal kg^{-1} and 2.75 Mcal kg^{-1}, showing weight gains of 81.87 g and 81.57 g respectively. However, at 2.65 Mcal kg^{-1} energy level at the same 18% protein inclusion showed a significantly reduced growth rate. At 16% inclusion, the responses were mixed but in particular, the interaction with 2.85 Mcal kg^{-1} energy showed a significant rise in growth rates. The growth rates observed across different ELs at 14% protein content were generally low. The FCR plays a pivotal role in laying performance, however, the best FCR recorded was 5.42 evidence that nutrient requirements during egg-laying, heat generation, and biological processes significantly impact FCR, further emphasizing the intricacy of dietary administration for the native guinea fowl in the egg-laying phase. A study by Kleyn et al., (2022) found that diets with higher CP content may require more energy consumption, depending on the energy system employed for formulation. Interestingly, the study also revealed that reducing energy levels per dietary protein composition may not provide any significant benefits.

In this study, the impact of varying PL and ELs, in the diet of the native guinea fowl was examined on some egg quality parameters. For the egg weight (EW) being the basis of a good egg, the PLs at 18% and 16% showed positive effects resulting in heavier eggs compared to the 14% level. This finding agrees with the submission of Heo et al. (2023) that higher protein diets enhance egg weight in poultry species. The EW in our study was higher than those of other studies on native guinea fowls (Veckic et al.,2018; Mohsenpour et al., 2020; Zeleke et al., 2020). However, it differed from the reported 53.63 g by (Gwaza and Elkana, 2017). The difference may be due to the interplay of genetic factors as they worked on the improved French-dual purpose breed and environmental factors that impact egg weight. These reports suggest that dietary intervention could directly impact egg weight and the prevailing ecological factor in the region. Yolk Quality (YH, YD, YW): Remarkably, the medial PL (16%) appears to give a balance, producing optimum yolk height, diameter, and weight. It could suggest an optimal protein threshold where further increases might not significantly enhance these yolk parameters (Kazemi et al., 2022). The interaction effect was significant in yolk height at 18: 26E, however, the results were to some extent lower than those reported by Mohsenpour (2020) and Vekic et al. (2018). This may imply that even though there is an apparent effect, it possibly may not be as obvious as observed in the aforementioned studies. The yolk height is a vital variable determining the nutritive

content of the egg (Da Nóbrega et al., 2022) and the observed values highlight the complex interaction between dietary effects and environmental factors. Yolk weight as the source of essential nutrients was impacted by the dietary interaction, with the 16:2.6E (19.11 g) and 18E:2.6E (18.19 g) interactions showing higher values than the 12.74 g and 11.00 g recorded by Mohsenpour et al. (2020) and Zeleke et al. (2020), This underlines the implication of energy content in the diet in enhancing yolk weight (Kazemi et al., 2022) and, in addition, the nutritional value of the egg. The 16: 26E interaction showed a higher yolk diameter of 43.53 mm, which was significant to other interactions. It could be linked with the higher protein level, signifying a possible path for definite dietary interventions to stimulate specific egg features (Karakolev et al., 2022). The eggshell thickness regulates the quality of the egg in terms of breakage, storage, and protection against bacterial infection (Kocetkovs et al., 2022), and in this study, at a higher energy level (2.85 Mcal kg^{-1}), the shell appears to be thicker, emphasizing the importance of energy intake in shell formation. This finding points to the potential role of energy supplementation in bolstering shell strength, and consequently, egg quality. However, the interaction effect on shell thickness was significant across all diets, with the highest value of 0.45mm recorded in 14: 27E. It was, however, lower than the 0.49mm reported by Veckic et al. (2018). It demonstrates that the level of inclusion did not show an apparent effect on shell thickness. Therefore, understanding the intricacies of shell thickness is central to enhancing egg value and can form a basis for future approaches in native guinea fowl nutrition. The protein and energy levels interaction is such that it affected both egg and yolk indices, indicative of a likelihood for more even and better eggs. This intricate relationship emphasizes the complexity of factors affecting egg shape. The Indigenous Guinea fowl egg-laying cycle may have been significantly impacted by factors such as growth rate, weather conditions, and human activities that affect wild animals, as reported by Soara et al. (2020) and Portillo-Salgado et al. (2022). These findings align with the current study observation that a correlation exists between egg count and diet given. Increased egg production metrics were observed at a higher protein level of 18% compared to 14% CP. with egg number, average egg/hen, total egg weight, and average egg/week all showing a pattern of improvement. Similarly, an energy level of 2.85 Mcal kg^{-1} slightly correlates with increased egg production indices compared to 2.65 Mcal kg^{-1}. It validates other research that demonstrates the effect of energy in supporting reproductive performance in poultry (Kazemi et al., 2022). The interaction effects reveal mixed effects on egg production metrics, with some showing synergistic effects that improve egg production indices against projections from individual effects. For example, the combination of 18% CP with medium energy at 2.75 Mcal kg^{-1} shows better egg production indices when compared to other interactions. It indicates the importance of understanding the interplay between protein and energy to optimize egg production in the indigenous guinea fowl. More importantly, it is worthy of note that, in the current study, the birds entered into lay at the peak of the dry season in January to buttress the fact that nutrition and management systems were factors that can affect the bird's performance and egg production, this is consonant with the report of Wasti and Mishra (2020), who observed that environmental conditions could seriously influence egg production. Consequently, careful consideration of the nutritional requirements of the birds is vital, coupled with good management practices to enhance egg production in different seasons.

Conclusion

The outcomes of this study highlight the importance of species-specific dietary considerations for indigenous Guinea fowl, mainly in enhancing egg production and quality. Based on the results obtained, a feed comprising 18% crude protein (CP) and 2.85 Mcal kg^{-1} is recommended, as it was found to significantly improve laying capacity. However, it is important to stress the need for more research to corroborate these findings across seasons (rainy and dry seasons) and to address limitations in the current study.

Ethical Statement

Ethical approval for this study was obtained from the Landmark University Ethics Committee vide number LUAC/2021/0018A.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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Author Contributions

Olajide Olasunkanmi Peter: Data curation, Writing, original draft preparation, review and editing, Project administration, Formal analysis.

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Oyawoye Enoch Olayiwola: Conceptualization, Project administration, Supervision.

Cyril Abang: Investigation, Formal analysis, Data curation;

Arije Olaniyi Damilare: Investigation, Methodology, Formal analysis, Data gathering

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Research Article

Micropropagation of *Vaccinium corymbosum* L.'Bluecrop' in Rocker Temporary Immersion System (TIS) Bioreactor

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Liquid medium,
Hyperhydricity shoots

Abstract: Blueberries are high-value fruits. The traditional method of propagation by cuttings cannot supply the modern market with large quantities of seedlings. The method of micropropagation of plants in vitro makes it possible to bring the production of blueberry seedlings to the highest level. Blueberries have not been sufficiently studied in in vitro culture, so the search for the simplest and most cost-effective methods of micropropagation remains relevant. The problem of accelerated micropropagation of blueberries can be solved using rocker-type bioreactors, which differ from other models in terms of simplicity of design and low cost. A study was carried out to evaluate the effectiveness of micropropagation of *Vaccinium corymbosum* 'Bluecrop' in rocker bioreactors. Two types of bioreactors were compared: the bioreactor of the Platform system and the TIS rocker bioreactor modified by the author. As a control, blueberries were grown on a semi-solid medium. The effectiveness of blueberry micropropagation was evaluated by the following indicators: multiplication coefficient, shoot length, and proportion of vitrified shoots. Experiments were conducted on WPM medium, with zeatin supplementation at a concentration of 1.0 mg/l, resulting in optimal results. It is shown that the rocker bioreactor is slightly inferior to the plantform bioreactor in micropropagation but outperforms the method of micropropagation on semisolid media. The rocker bioreactor can be fully utilized for production purposes. In order to reduce costs and increase technical reliability, the working principle of the mechanical drive of the author's model of a rocker-type bioreactor was changed.

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1. Introduction

Blueberries are highly nutritious, containing significant amounts of polyphenols and biologically active substances (Wang et al., 2019; Mengist et al., 2020). In recent years, blueberry planting areas and yields have rapidly increased worldwide. In 2016, the global blueberry planting area increased by 34.14% compared to 2012, reaching 110 928 hectares, and producing a total of 552 505 tons (Gallegos-Cedillo et al., 2018). Meanwhile, China's blueberry acreage reached 66 400 hectares in 2020, with a total harvest of 347 200 tons, making it the leading producer of blueberries in the Asia-Pacific region (Yang et al., 2022).

Traditionally, the propagation of blueberry varieties has been based on rooting cuttings. The formation of adventitious roots on blueberry shoots typically requires the exogenous application of auxins. Successful rooting occurs during a specific period of plant development (Litwinczuk et al., 2005; Debnath, 2007; Marino et al., 2014). The issue of obtaining large volumes of blueberry planting material can be resolved through the method of clonal propagation of plants under aseptic conditions. In vitro micropropagation protocols have been developed for numerous blueberry varieties. Initially, blueberry micropropagation was conducted on solid media. Subsequently, research emerged on the cultivation of blueberries in bioreactors (Fan et al., 2017; Wang et al., 2023). Nevertheless, reducing the expenses of blueberry micropropagation remains pertinent to this day.

Temporary immersion bioreactors (TIBs) are typically used for the micropropagation of blueberries. This method involves immersing explants in a liquid medium for limited time intervals rather than constant immersion (Georgiev, 2014; Welander et al., 2016). The process of periodic flooding of the culture is achieved in various ways, as described in relevant sources (Murthy et al., 2023). Temporary immersion systems (TIS) typically comprise multiple compartments or separate vessels within a container. The medium is transferred from the reservoir compartment/vessel to the compartment/vessel where the plants are cultivated using air pressure. Bioreactors such as RITA, PLANTFORM, and SETIS operate on this principle. However, these models may not always be accessible to blueberry producers. In addition, maintaining sterility in bioreactors of the aforementioned models can be challenging due to the interconnection of several containers. A simpler alternative is the Rocker system, which employs a mechanical platform to tilt individual plant containers at a fixed angle, facilitating the movement of the media. This system is easier to implement than the previous models. The BioMINT™ model (Patent No. PA/a/2004/003837, Centro de Investigación Científica de Yucatán (CICY), Yucatán, Mexico) is the most well-known. It reduces the risk of culture contamination by moving media within an individual container but does not effectively aerate the vessels.

The aim of the study was to evaluate the feasibility of blueberry micropropagation in a rocker TIS bioreactor, intending to achieve maximum efficiency at minimal cost. The study results suggest that the rocker TIS bioreactor is a viable option for blueberry micropropagation. In this study, the working efficiency of PLANTFORM and the rocker type bioreactor developed for this study were compared. The control was blueberry culture on semi-solid agar medium.The approach discovered for more economical micropropagation of this variety is expected to be applied to other varieties of *Vaccinium corymbosum*.

2. Material and Methods

The research was done in 2022-2023 at the laboratory of the Botanical Garden of the Southern Federal University and Don State Technical University, Rostov-on-Don, Russia. The original *Vaccinium corymbosum* 'Bluecrop' mother specimen was acquired from a private collection in Rostov-on-Don. For all stages of in vitro micropropagation, McCown's Woody Plant (WPM) basal medium (McCown and Lloyd, 1981) was used. The stimulation of shoot formation was performed with zeatin (Sigma, USA).

2.1. Plant material and culture establishment

Shoots of *Vaccinium corymbosum* L. 'Bluecrop' the current year, no longer than 10 cm, were obtained from the mother specimen and washed in running water for 20 minutes. Subsequently, they were washed for an additional 15 minutes in running water with a drop of hand soap, followed by another 20-minute wash in running water. Further processing was conducted under sterile conditions in a laminar flow box. Surface sterilization was carried out in several processing stages. The cuttings were sectioned into 5 cm explants and then submerged in a 70% ethyl alcohol solution for 30 seconds using a 300 ml glass beaker. Subsequently, they were immediately transferred to a solution of 1% sodium hypochlorite (NaOCl) for 10 minutes in a beaker of the same volume. After the treatment, they were washed three times in sterile water for 15 minutes with 200 ml portions of water in the same glass. The explants were then divided into single-node segments of 1 cm and planted in 20 ml tubes with 7 ml of nutrient medium. To initiate shoots, WPM medium was used with the addition of 30 g/l sucrose, 6 g/l agar, and 0.5 mg/l zeatin. The zeatin solution was filtered using a 0.22 μm membrane filter before being added to the medium after autoclaving. The pH of the medium was adjusted to 5.0 using a 1 M HCl solution (Borsai et al., 2019). The medium was then autoclaved at 121°C for 20 minutes. The explant tubes were placed

in a growth room. Growth room conditions were maintained at 60% humidity, 25 ± 2 °C, 16 h photoperiod with PPFD 50 µmol m^{-2} s^{-1} using cold white fluorescent lamps (6 500 K).

2.2. Multiplication and elongation stages

After six weeks, sterile axillary shoots were selected from the culture and transferred to the medium for multiplication. For this, the WPM medium was used with the same composition as in the initiation step, but the zeatin concentration was increased to 1 mg/L. After 6 weeks, axillary and adventitious shoots obtained by propagation were transferred to a hormone-free WPM medium for shoot elongation. After 4 weeks of cultivation in the elongation step, the shoots were used in bioreactor experiments and also transferred to a semi-solid medium as a control of the experiment.

2.3. Bioreactor culture

Two types of TIS bioreactors were tested: the Plantform (Adelberg et al., 2004; Welander et al., 2014) and a rocker-type bioreactor in the author's modification. The Plantform bioreactors were purchased from the Plant Form Company (Ireland) and used without modification.

The rocker bioreactor was manufactured according to the principle diagram given in the work of Georgiev et al. (2014), with improvements. The culture box was constructed from a transparent polyethylene container measuring 180 mm × 160 mm × 90 mm. This was divided into two equal parts, with one containing explants and the other a liquid nutrient medium. The bioreactor lid was equipped with ventilation holes protected by foam filters. The culture box was fixed on a plastic plane, with the help of which the angle of inclination could be changed. This ensured that the explants were periodically flooded. To change the slope of the platform, a simple and reliable pneumatic device was developed, controlled by a timer.

The bioreactors were filled with 2-3 node segments cut from shoots that had undergone the elongation stage in vitro. The composition of the medium for the cultivation of blueberries in bioreactors was taken from the protocol of Clapa et al. (2022) and included the following components: WPM salts, 100 mg/l myo-inositol, 2 mg/l thiamine, 1 mg/l pyridoxine, 1 mg/l nicotinic acid, 1 mg/l zeatin, 30 g/l sucrose. The pH of the medium was adjusted to 5.0. Autoclaving mode: 121 degrees for 20 minutes. 510 ml of medium was poured into Plantform bioreactor culture boxes and 60 explants were placed. 170 ml of medium was poured into rocker bioreactor culture boxes and 20 explants were transferred. Growth room conditions were maintained at 60% humidity, 25 ± 2 °C, 16 h photoperiod with PPFD 100 µmol m^{-2} s^{-1} using cold white fluorescent lamps (6 500 K).

After 8 weeks of cultivation, the results of plant growth and development were assessed, in particular shoot length, number of nodes per shoot, and multiplication rate. The multiplication rate was calculated by dividing the number of shoots produced by the number of explants inoculated.

In order to obtain the maximum effect from the operation of the bioreactors, it was necessary to find the optimum immersion mode for each model. To this end, three immersion modes were tested: 2, 4, and 6 times a day for 2 minutes. The efficiency of the bioreactors was evaluated after 8 weeks of cultivation using the following indicators: number of shoots per explant, and degree of their hyperhydricity. The degree of vitrification was determined visually. Both versions of the experiment were carried out on a medium with the same composition as above. In the control version of both experiments, the node segments of blueberry shoots were cultivated on a medium with 6 g/l agar of the same composition.

2.4. Ex vitro rooting and acclimatization

Shoots obtained on a semi-solid medium after the elongation stage and in both types of bioreactors were rooted ex vitro. In this way, the rooting phase was combined with the phase of acclimatization. The shoots were planted in 100 ml containers filled with a mixture of peat and perlite in a ratio of 5:1. The cultures were maintained in a climatic greenhouse at 100% humidity, 25 ± 2 °C, 16 h photoperiod with PPFD 250 µmol m^{-2} s^{-1} using cold white fluorescent lamps (6 500 K). After 4 weeks of rooting, the humidity in the greenhouse was gradually reduced until the plants had adapted to room conditions. After 8 weeks of rooting combined with acclimatization, the percentage of rooted acclimatized shoots out of the total number of shoots planted was determined.

2.5. Statistical analysis

All data obtained during this study were statistically analyzed using the Past 3.16 software package (Hammer et al., 2001) and an online software resource. Website www.socscistatistics.com [accessed 20 December 2023].

Each experiment was performed in triplicate as an independent experiment. 20 explants or shoots were used for each treatment. Normality was tested using the Shapiro-Wilk test. For multiple group comparisons, an ANOVA test was performed and significant differences between means were calculated using Tukey's HSD test.

3. Results

Optimal bioreactor operation mode: In an experiment to determine optimal bioreactor operation, flooding blueberry shoots for 2 minutes every 6 hours was found to be best. The multiplication rate was 8.9 and the vitrification rate was 2.4% on average. Flooding the shoots every 4 hours gave a multiplication rate of 7.5 and a vitrification rate of 3.5%. A further increase in the frequency of flooding (every 2 hours) resulted in an increase in vitrification (6.1%) and a slight decrease in the multiplication factor (7.3). The differences in reproduction coefficients in the last two versions of the experiment are not statistically significant at $p > 0.05$ (Figure 1).

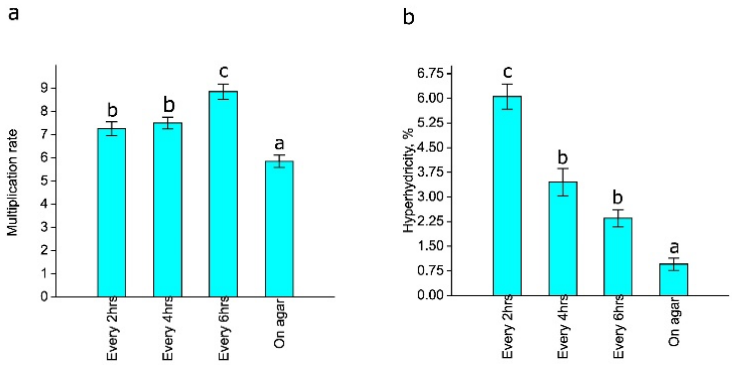

Figure 1. Comparison of rocker bioreactor operating modes for multiplication rate (a) and hyperhydricity (b). The control is a shoot culture on agar. All data are presented as mean ± standard error of the mean (SEM). Different letters indicate significant differences by Tukey's test at $p < 0.05$.

Comparison of bioreactor efficiency: After 8 weeks of blueberry cultivation in two types of bioreactors, the highest number of nodes was formed in the Plantform bioreactor (19.8). In the rocker bioreactor, an average of 16.1 nodes were formed per shoot, which was significantly lower ($p < 0.01$). In the control variant of the experiment on a semi-solid medium, an average of 9.9 nodes were formed per shoot, which is significantly lower than in the previous two variants ($p < 0.001$) (Figure 2a). The average shoot length in the Plantform bioreactor (6.5) was higher than in the rocker bioreactor (5.4) at $p < 0.05$. Compared to the control (3.7), the average shoot length was significantly higher in the Plantform system ($p < 0.001$), while in the rocker bioreactor, this indicator was not significantly different from the control ($p > 0.05$) (Figure 2b). The highest multiplication rate was observed in the Plantform bioreactor (12.5), while it was significantly lower (8.8) in the rocker bioreactor ($p < 0.001$). On the agar medium, this indicator was significantly lower (5.9) than in both types of bioreactors ($p < 0.001$) (Figure 2c).

Rooting together with acclimatization for 8 weeks under ex vitro conditions showed excellent results. After all in vitro cultivation methods, about 90% of the shoots were rooted and acclimatized with $p > 0.05$ (Figure 2d).

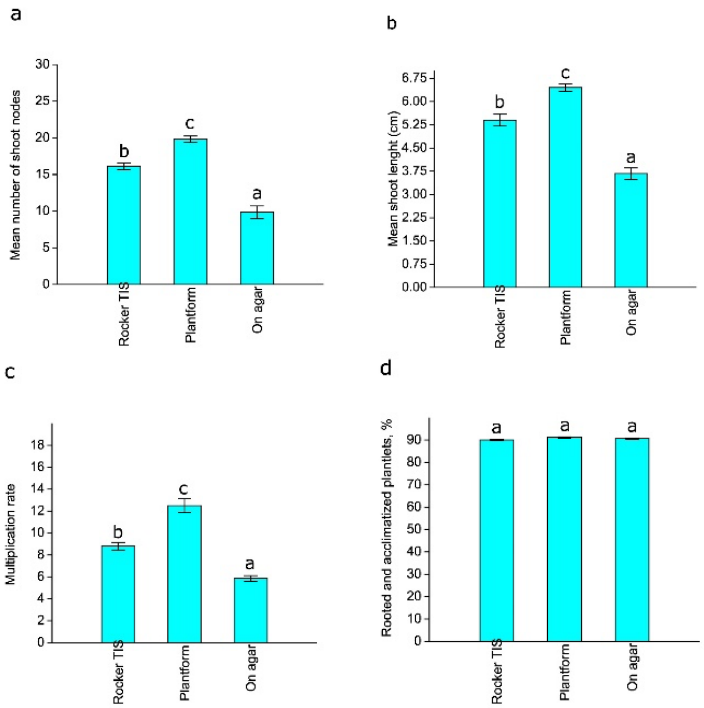

Figure 2. Comparison of the operation of a rocker bioreactor and a platform bioreactor according to the following indicators: the number of nodes of the shoots formed (a), the length of the shoots (b), the multiplication rate (c), the proportion of rooted and acclimatized regenerants obtained in different ways (d). All data are presented as mean ± standard error of the mean (SEM). Different letters indicate significant differences by Tukey's test at $p < 0.05$.

The blueberry culture obtained in different bioreactors and on semi-solid medium is shown in Figure 3.

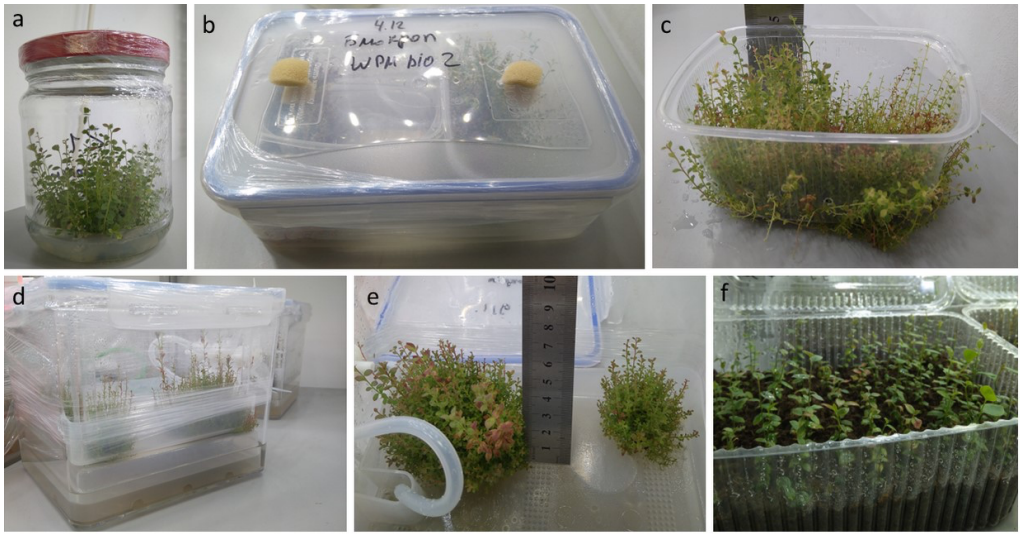

Figure 3. Culture of *Vaccinium corymbosum* 'Bluecrop' after 8 weeks of cultivation: on agar medium (a), in a rocker bioreactor (b, c), in a Plantform bioreactor (d, e), during rooting and acclimatization (f).

4. Discussion

The high efficiency of in vitro plant cultivation using temporary immersion bioreactors has been demonstrated by many researchers (Ahmadian et al., 2017; Jing et al., 2024; Sereda et al., 2024). Mass

micropropagation of berry crops was significantly faster in liquid media in bioreactors than in semi-solid media (Debnath, 2017; Bošnjak et al., 2021; Le et al., 2023). In studies with cranberries, the authors showed that after 8 weeks of cultivation, shoot reproduction was 2-3 times higher in liquid medium than in semi-solid medium (Arigundam et al., 2020). Clapa et al. (2022) reported that the highest reproduction rates of Duke blueberries were recorded in the TIS bioreactor compared to cultivation in semi-solid media. A wide variety of bioreactor models are available today. The choice of model depends on the biological crop and the end goal of cultivation (Murthy et al, 2023).

Obviously, to achieve the maximum effect of blueberry propagation in bioreactors, it is necessary to use the most economical TIS with the minimum risk of loss of the resulting shoots. A reduction in shoot quality can usually be caused by contamination of bioreactor parts and components or by vitrification of the shoots. The risk of shoot contamination increases with the complexity of the bioreactor design. A complex aeration system consisting of filters and air supply pipes, as well as the presence of additional media reservoirs, can lead to infection. This ultimately increases the cost of the plant material. On the other hand, simplification of the design due to the aeration system can lead to an increase in the number of vitrified shoots, as poor aeration of the culture vessels often leads to vitrification of the shoots (Sanyürek et al., 2021; Polivanova and Bedarev, 2022).

Most TIS bioreactors have a complex aeration and nutrient delivery system. Combining dozens of culture boxes in one system also increases the risk of contamination. One such model is the Plantform bioreactor. Undoubtedly, the TIS Plantform is a highly effective system for in vitro plant micropropagation, which has been proven by many works (Almusawi et al., 2017; Aka Kaçar et al., 2020). However, it was decided to test a rocker bioreactor, which is a simple and reliable system, in the modification to reduce the economic cost and the risk of contamination. Analysis of literature data shows that rocker bioreactor systems, such as BioMint, are used much less frequently than other systems. However, the application of this TIS has yielded good results (Etienne and Berthouly, 2002; Robert et al., 2006; Belo-Belo, 2010; Peña-Ramírez et al., 2010). It is believed that the diffusion of this type of bioreactor is hindered by a complex system of inclined platforms driven by an electric motor. In this study, the electric drive system was replaced by a simple pneumatic system that varied the inclination of the platform and was programmed with a household timer.

An experiment with different operating modes of a rocker-type bioreactor showed that with a flooding frequency of every 6 hours for 2 minutes, the maximum number of shoots is formed at a low level of vitrification. In this case, the value of the multiplication coefficient is higher than in the control with a semi-solid medium and decreases with increasing frequency of flooding. In addition, the degree of shoot vitrification directly depends on the frequency of flooding. The water content of the shoots is associated with insufficient aeration of the culture boxes, which leads to an excessive increase in humidity and the accumulation of ethylene and CO_2. As a result, a number of physiological parameters decrease (photosynthesis, amino acid synthesis, etc.), which has been repeatedly reported in the relevant literature (Kevers et al., 2004; Gao et al., 2018; Polivanova and Bedarev, 2022). The frequency and duration of flooding also depend on the type of digester and crop. In studies with blueberries grown in Planform TIS, the authors chose an optimal frequency of flooding every 4 hours for 1 minute, with forced aeration every hour for 4 minutes. At the same time, the authors did not note the vitrification of the shoots and obtained high rates of reproduction (Clapa et al., 2022).

The number of nodes per shoot and the shoot length are crucial indicators in the micropropagation process of blueberries. Cloning occurs in segments consisting of several nodes, each about 2 cm in length (Wang et al., 2023). A long, multi-node shoot can be divided into many segments, increasing the productivity of the entire propagation cycle. The number of shoot nodes in Rocker bioreactors was lower than in Plantform bioreactors but higher than in semisolid media. This difference can be attributed to the inadequate aeration in rocker-type bioreactors, as with the multiplication rate.

Blueberry rooting is typically conducted as a separate in vitro stage using auxins and activated carbon (Ruži et al., 2012). The combination of rooting and acclimatization processes under ex vitro conditions maximizes the yield of rooted and acclimatized regenerants (Osrolucka et al., 2007; Vescan et al., 2012). The experiment results indicate that the method of propagation did not affect the degree of rooting and acclimatization of regenerated plants. Around 90% of shoots obtained in a rocker-type bioreactor were successfully adapted to ex-vitro conditions.

Conclusion

The micropropagation of blueberries using a rocker-type bioreactor represents a promising approach for the rapid production of high-quality planting material. This method has been demonstrated to be more productive than growing on a semi-solid medium, as evidenced by higher reproduction rates, average number of nodes per shoot, and average shoot length. By comparison of the operational characteristics of a rocker bioreactor and a Plantform, bioreactor was observed that the micropropagation efficiency of a rocker bioreactor is inferior to that of the Plantform system. This is due to insufficient aeration, which increases the degree of vitrification of shoots. However, the slower rate of reproduction in the rocker bioreactor is compensated by the significantly lower cost of this system. In addition, the study demonstrated that shoots from a rocking-type bioreactor are capable of taking root and acclimatizing ex vitro in a single operation, with up to 90% of seedlings ready for planting in open ground. Future research will be conducted to enhance the efficiency of blueberry micropropagation in rocker-type bioreactors to implement this technology in mass production.

Ethical Statement

Ethical approval is not required for this study because given that only plant specimens were involved.

Conflict of Interest

The author declare 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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Research Article

Determination of the Biochemical and Antioxidant Enzyme Activities of Rose Oil (*Rosa damascena* Mill.) Collected in Different Time Periods

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Abstract: The aim of this study was to investigate the changes in the biochemical and antioxidant enzyme activities of oil of rose petals collected at different time intervals during the day. The results of the present study revealed that significant changes occurred in the biochemical content of the oil of rose petals due to collecting at various intervals in a day. The total phenolic content exhibited a statistically significant increase over the day, reaching a 151.57% increment by 14.00 p.m. compared to the initial level. Similarly, total flavonoid content and total antioxidant activity progressively increased. Proline, known for its versatile roles, including antioxidant defense, increased from 7.43 mg g^{-1} in the morning to 24.96 mg g^{-1}. Significant temporal changes were observed in antioxidant enzyme activities as well. Catalase (CAT) activity, for instance, increased by 588.22% from 6.00 a.m. to 14.00 p.m., with similar alterations noted in superoxide dismutase (SOD), ascorbate peroxidase (APX), and peroxidase (POD) activities. The results highlight a correlation between flower collection time and biochemical activities, with a noticeable increase in antioxidant enzyme activities as the day progresses. The findings emphasize the importance of considering plant physiology and environmental factors when determining optimal flower collection times. In conclusion, it can be said that the collection time of flowers influences the quality of rose oil and early morning collection may be more suitable.

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1. Introduction

Economically valuable and also known as Damask Rose, *Rosa damascena* Mill. is considered a member of the Rosaceae family. This plant naturally grows in Syria, Morocco, Andalusia, Iran, and the Caucasus (Babaei et al., 2007; Hatipoğlu et al., 2022; Macit et al., 2023). It originates from the ancient city formerly known as Damascus and is therefore commonly referred to as Damask rose. However, in Turkey, it is also known by various names such as Pink oil rose, Oil rose, Distillation rose, Damask rose, and Isparta rose (Özçelik et al., 2013; Baydar, 2016). While Oil rose is primarily cultivated for industrial purposes in the production of rose oil, especially in Turkey and Bulgaria, its medicinal usage has been gradually increasing in recent years.

Plants undergo significant biochemical changes under stress, including accumulating reactive oxygen species (ROS), particularly O_2 and H_2O_2, in chloroplasts, mitochondria, and peroxisomes. These ROS are natural by-products of cell metabolism and play a crucial role in signaling mechanisms. However, the excessive accumulation of ROS triggers oxidative stress by initiating lipid peroxidation, protein reduction, and DNA fragmentation, ultimately leading to cell death. Simultaneously, plants have developed enzymatic (superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT)), and non-enzymatic (glutathione, β-carotene, ascorbic acid, α-tocopherol) antioxidant defense mechanisms to reduce oxidative damage. Consequently, the balance between ROS production and antioxidant enzyme activities determines whether oxidative damage will occur (Kuşvuran et al., 2011; Farajzadeh et al., 2017; Popović et al., 2017; Selmi et al., 2017; Demir and Başayiğit, 2021).

Medicinal and aromatic plants are cultivated for their bioactive substances. However, bioactive substances can vary significantly depending on the different organs of the plant, growth stages, and harvest times. Therefore, producers of medicinal and aromatic plants need to thoroughly understand the variation in bioactive substance content of source plants and harvest, cut, and collect them when the active substances are most abundant (Başyiğit and Baydar, 2017). For instance, the mutagenic substance methyl eugenol in rose oil is either undesired or within certain limits. In Isparta roses, this ratio is generally around 2%. However, in roses with delayed harvesting and distillation, this ratio exceeds 4%, which is undesirable (Baydar et al., 2007). Therefore, this research aims to determine the time-dependent changes in the biochemical contents and antioxidant enzyme activities of petals collected from commercially grown *R. Damascena* in Isparta province at different intervals (06.00 a.m., 08.00 a.m., 10.00 a.m., 12.00 p.m., 14.00 p.m.).

2. Material and Methods

2.1. Material

The plant materials (*R. damascena*) used in the study were collected from a commercial garden located in the village of Yakaören, Isparta province. Petals were collected homogeneously to represent the entire plant (from the top, middle, and bottom parts of the plant). The study was designed according to randomized block experimental design, and the analyses were conducted with 3 replications for each collection time, with each replication consisting of 12 trees. The collected petals were frozen with liquid nitrogen and stored at -80°C in the Agricultural Biotechnology Department of the Faculty of Agriculture at Isparta Applied Sciences University until the next collection time.

2.2. Method

2.2.1. Biochemical analyses

Determination of total phenolic and flavonoid contents: Two grams (2±0.01 g) of rose petals were weighed and homogenized in 10 ml of 80% methanol. The obtained homogenate was centrifuged at 4000 g for 10 minutes, and the liquid portion was collected. The remaining pellet was re-extracted with an additional 10 ml of 80% methanol, and the above steps were repeated. The Folin-Ciocalteau method, as specified by Singleton and Rossi (1965), was used to determine the total phenolic content. The results were calculated and expressed as mg gallic acid equivalent per gram (mg GAE g^{-1}). The total flavonoid content was determined according to the method described by Zhishen et al. (1999) and expressed as mg catechin equivalent per gram (mg CE g^{-1}).

DPPH free radical scavenging activity: The antioxidant activity was measured based on the ability to capture DPPH radicals, following the method of Kumaran et al. (2006). The antioxidant activity of appropriately diluted samples in a specific concentration range was compared with the Trolox standard in terms of hydrogen bonding capability. The absorbance of the resulting color at 517 nm was measured using a UV spectrophotometer.

Total proline content: Six-tenths of a gram (0.6 g) of rose petals were weighed, and 3 ml of 3% sulfosalicylic acid was added. After homogenization, the mixture was centrifuged at 12 000 g for 10 minutes at room temperature. The analysis was conducted according to the method of Bates et al. (1973), and the results were expressed as mg/g and calculated relative to the D-Proline standard.

Lipid peroxidation (MDA) content: Ten grams (10 g) of rose petals were weighed, and 25 ml of cold 100 mM sodium phosphate buffer containing 0.5 g polyvinyl polypyrrolidone (PVPP) was prepared. After homogenization, the samples were centrifuged at 27 000 g for 50 minutes at 4 °C. The analysis was performed according to the method reported by Jiang et al. (2010), and the MDA content was calculated in nmol g^{-1}.

2.2.2. Antioxidant enzyme analyses

Catalase (CAT) enzyme activity: Ten grams (10 g) of rose petals were weighed, and a cold 50 mM sodium phosphate buffer containing 0.5 g polyvinyl polypyrrolidone (PVPP) was prepared (pH: 7.0). After homogenization, the samples were centrifuged at 27 000 g for 50 minutes at 4 °C. The analysis was carried out according to the method described by Beers et al. (1952), and the specific activity was expressed as U mg $protein^{-1}$.

Superoxide dismutase (SOD) enzyme activity: Ten grams (10 g) of rose petals were weighed, and a cold 100 mM sodium phosphate buffer containing 0.5 g polyvinyl polypyrrolidone (PVPP) was prepared. After homogenization, the samples were centrifuged at 27 000 g for 50 minutes at 4 °C, and the supernatant was used for analysis. The analysis was performed according to the method reported by Constantine and Stanley (1977), and the results were expressed as U mg^{-1} protein.

Ascorbate peroxidase (APX) enzyme activity: Four grams (4 g) of rose petals were weighed, and 12 ml of 50 mM potassium phosphate buffer (pH: 7.3) containing 1 mM (EDTA), 2 mM DTT, and 1 mM ascorbic acid was added. After homogenization, the samples were centrifuged at 10 000 g for 15 minutes at 4 °C. The analysis was conducted according to the method described by Nakano et al. (1981), and the results were expressed as mol/min/g protein.

Peroxidase (POD) enzyme activity: Ten grams (10 g) of rose petals were weighed, and a cold 100 mM sodium phosphate buffer containing 0.5 g polyvinyl polypyrrolidone (PVPP) was prepared. After homogenization, the samples were centrifuged at 27 000 g for 50 minutes at 4 °C, and the supernatant was used for analysis. The analysis was performed according to the method reported by Jiang et al. (2010), and the results were expressed as ΔA_{460} min^{-1} mg $protein^{-1}$.

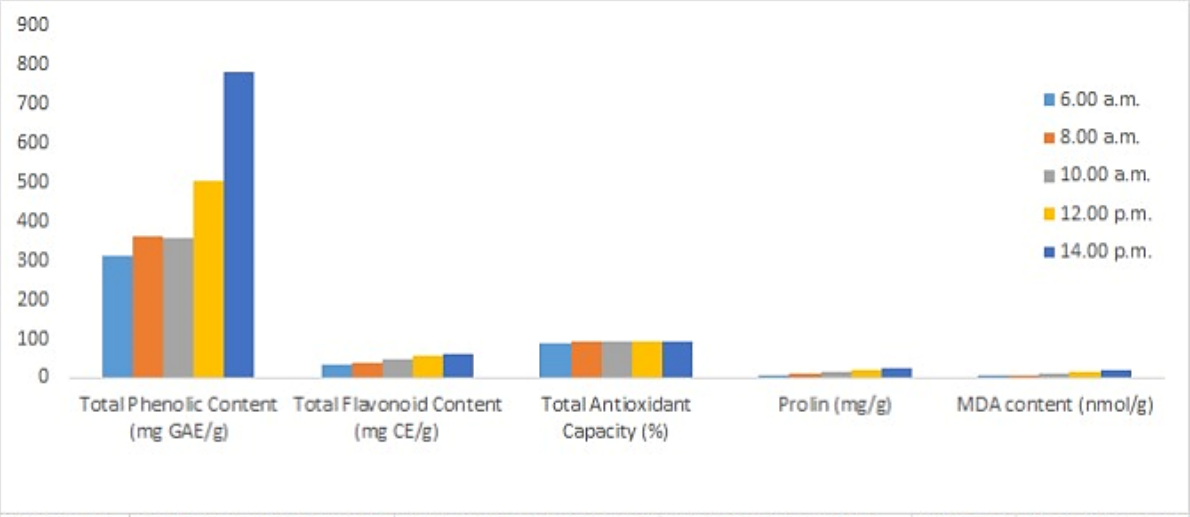
2.3. Statistical analysis

The experimental study was established in a factorial arrangement with 3 replications in a randomized block experimental design. The obtained data were subjected to one-way analysis of variance method using Minitab 17 statistical software. The differences that emerged were identified according to the Tukey multiple comparison test, and the differences between the means were indicated using different letters. Correlation matrix and biplot (Cos2) analyses between properties were made with the "FactoMineR, factoextra, pca3d, Methane" packages in the R package program. Principal component analysis was performed and (biplot) visuals were created for the first and second dimensions according to Cos2 values that show the importance of the variables in the components.

3. Results and Discussion

The results of the biochemical analyses conducted on rose petals collected at different time intervals are presented in Figure 1. Changes in the biochemical contents occurred over time, and this change was found to be statistically significant ($p<0.05$). Analysis of the rose petals collected at 6.00 a.m. in the morning (at dawn) revealed a total phenolic content of 310.32 mg GAE g^{-1}, with an increase in total phenolic content observed in samples collected at 08:00 a.m., 10:00 a.m., 12:00 p.m., and 14.00 p.m. hours due to the influence of sunlight and heat. The total phenolic content of rose petals collected at 14.00 p.m. was determined to be 780.70 mg GAE g^{-1}, indicating a 151.57% increase compared to the initial level. Similarly, an increase in total flavonoid content over time was observed. Analysis of rose petals collected at 6.00 a.m. in the morning showed a total flavonoid content of 33.90 mg CE g^{-1}, while in samples collected at 14.00 p.m., it was 62.33 mg CE g^{-1}, representing an 83.86% increase in flavonoid content. An increase in total antioxidant activity over time was also observed. Upon examination of Figure 1, it was determined that the DPPH radical scavenging activity increased by 8.87% between 6.00

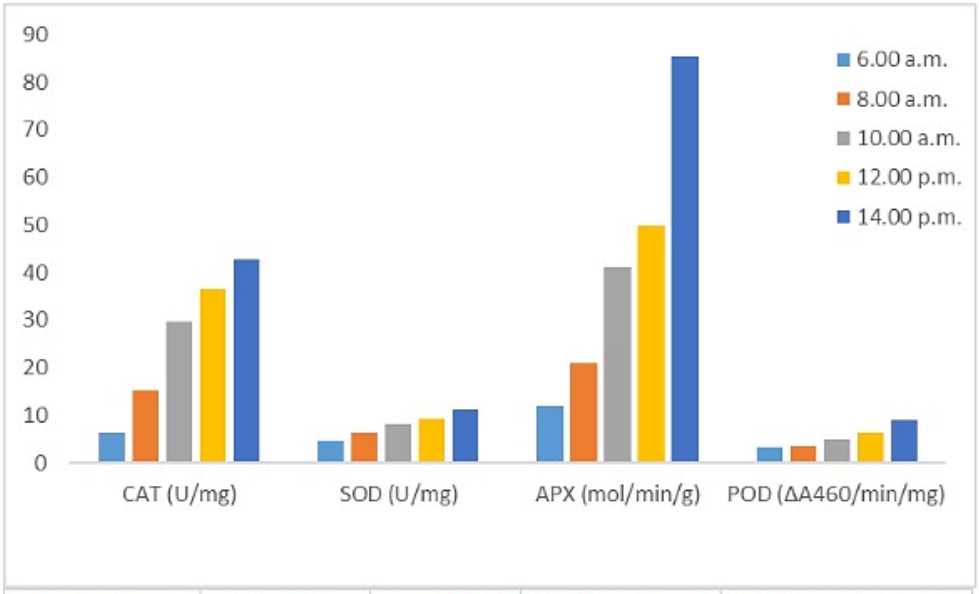
a.m. in the morning and 2:00 in the afternoon. Proline, despite acting as an osmolyte, is known as a potent antioxidant defense molecule, a metal chelator (separator), a protein stabilizer, a ROS scavenger, and an inhibitor of programmed cell death (Çelik, 2023). Research has shown a positive relationship between proline accumulation and increased stress tolerance in plants (Yildirim et al., 2021; Dindar et al., 2023; Yildirim et al., 2023). In the research, it was determined that while the proline content in the samples collected in the morning was 7.43 mg g^{-1}, it reached 24.96 mg g^{-1} at 14.00 p.m., an increase of 236%.



Sampling Time	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg CE/g)	Total Antioxidant Capacity (%)	Prolin (mg/g)	MDA content (nmol/g)
6.00 a.m.	310,32±5,02 c	33,90±2,69 c	87,50±0,57 d	7,43±1,66 c	5,00±1,38 c
8.00 a.m.	363,80±22,40 c	40,67±1,64 c	92,56±0,46 c	11,25±1,71 bc	8,42±1,56 bc
10.00 a.m.	356,30±18,30 c	48,73±0,89 b	93,85±0,12 b	16,37±2,74 b	13,18±0,48 b
12.00 p.m.	503,60±23,20 b	54,77±1,65 b	94,81±0,12 ab	22,11±1,42 a	14,05±0,85 ab

Figure 1. Changes in biochemical contents of oil rose petals over time. *a,b: The difference between the means indicated by different letters in the same column is significant at the P≤0.05 level.

The results of antioxidant enzyme analyses conducted on rose petals collected at different time intervals are presented in Figure 2. Changes in antioxidant enzyme activities over time were observed, and this change was found to be statistically significant ($p<0.05$). According to the findings, the lowest CAT activity was observed in oil roses collected at 6.00 a.m. in the morning, with 6.20 U mg^{-1}, while the highest CAT activity was found in roses collected at 14.00 p.m., with 42.67 U mg^{-1}. A significant increase was particularly observed from 8:00 a.m. onwards, reaching a 588.22% increase at 14.00 p.m. p.m. Analysis of rose petals collected at 6.00 a.m. revealed an increase in SOD activity over time, with the highest activity again observed in samples collected at 14.00 p.m. The SOD activity, which was 4.67 U mg^{-1} at 6.00 a.m., reached 11.33 U mg^{-1} with a 142.61% increase at 14.00 p.m.. An increase in APX activity was observed continuously between 6.00 a.m. and 14.00 p.m., reaching the highest APX activity at 14.00 p.m. The APX activity in oil rose petals collected at 6.00 a.m. was 12.11 mol min^{-1} g^{-1}, while it reached 85.55 mol min^{-1} g^{-1} with a 606.44% increase at 14.00 p.m. Similarly, an increase in POD activity was observed between 6.00 a.m. and 14.00 p.m. The POD activity in oil rose petals collected at 6.00 a.m. was 3.42 ΔA_{460} min^{-1} mg^{-1}, while it reached 8.91 ΔA_{460} min^{-1} mg^{-1} with a 160.52% increase at 14.00 p.m.



Sampling Time	CAT (U/mg)	SOD (U/mg)	APX (mol/min/g)	POD (ΔA460/min/mg)
6.00 a.m.	6,20±0,40 d	4,67±0,88 d	12,11±0,19 c	3,42±0,26 d
8.00 a.m.	15,20±1,77 c	6,33±0,12 cd	20,96±0,63 c	3,74±0,51 cd
10.00 a.m.	29,79±3,60 b	8,20±1,62 bc	41,11±1,92 b	5,02±0,29 bc
12.00 p.m.	36,62±1,15 ab	9,32±1,04 ab	49,81±6,24 b	6,22±0,78 b
14.00 p.m.	42,67±5,82 a	11,33±0,49 a	85,55±9,49 a	8,91±0,83 a

Figure 2. Changes in antioxidant activity of oil rose petals over time.*a,b: The difference between the means indicated by different letters in the same column is significant at the P≤0.05 level.

In the study, Pearson correlation coefficients for the collection times and biochemical as well as antioxidant enzyme activities, along with PCA variables, are presented in Figure 3. A strong positive correlation (ranging from 0.66 to 1.00) was observed between collection times and biochemical and antioxidant enzyme activities, indicating an increase in the examined features over time. It was revealed by the correlation matrix that there were statistically significant positive changes in biochemical and antioxidant enzyme activities over time. The first principal component explained 94.2% of the variance, and the second principal component explained 4.7%. The squares of the coordinates (cos2) were an indication of how successfully the relevant variable was expressed with the principal component. All features have high cos2 values. Cos2 values of the features in the same direction indicate that the correlation between features is positive.

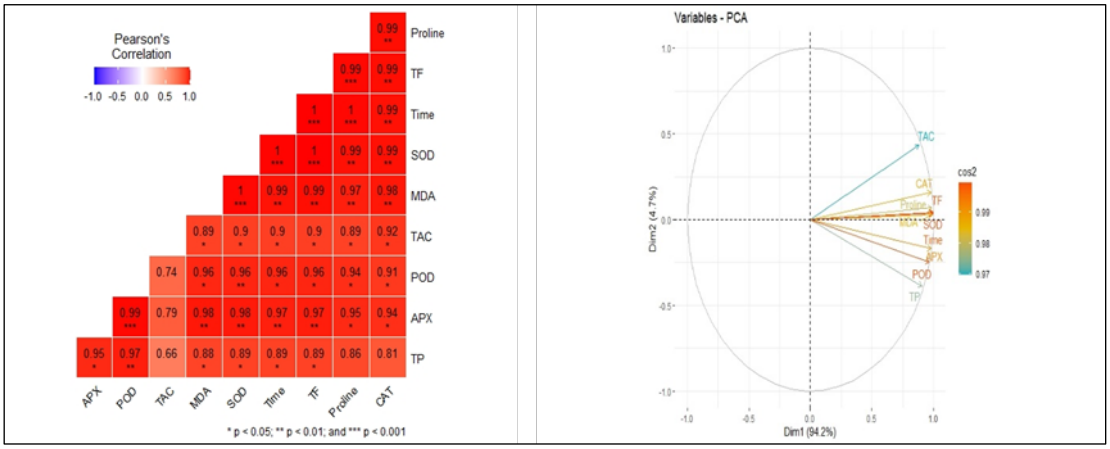


Figure 3. Pearson's correlation coefficients among biochemical and antioxidant enzyme properties used in the study. APX: Ascorbate peroxidase; POD: Peroxidase; TAC: Total antioxidant capacity; MDA: Malondialdehyde; SOD: Superoxide dismutase; TF: Total phenolic content; CAT: Catalase.

The cosmetic industry extensively utilizes plants' antioxidant properties and free radical scavenging abilities to regulate skin damage (Michalak, 2022). Therefore, plants abundant in antioxidants are commonly employed as a protective measure against oxidative degradation (Lee et al., 2015). Recent years have witnessed a growing interest in nutrition and food science, driven by diets that exhibit antioxidant activity (Fu and Mao, 2008). Generally, the health advantages of edible flowers are linked to their antioxidant activities, which exert notable inhibitory effects on free radicals. Recognized as natural antioxidants, the antioxidants in flowers serve as an alternative means to prevent the oxidative deterioration of food, thereby minimizing the harm caused by these oxidative compounds in humans (Franzen et al., 2018). In the Lakes region, a flowering season lasting approximately two months begins in the first week of May and continues until the first week of July, depending on the altitude. In the rose gardens situated at elevations between 800 and 1500 meters in the region, flowering is delayed by 2-3 days for every 100-meter increase in altitude. Throughout the flowering season, rose flowers are manually collected starting from the early hours of the day, broken individually from beneath the ovaries, and transported to rose oil factories in sacks for distillation (Baydar et al., 2013). The productivity and quality of rose products vary according to the climate and soil characteristics of the region where oil roses grow, the direction and altitude of the area, cultivation techniques, harvest timing and duration of storage, distillation, and extraction processes, as well as preservation and drying methods (Babu et al., 2002; Misra et al., 2002; Safari et al., 2004; Kazaz et al., 2009; Kazaz et al., 2010). Baydar et al. (2013) reported a decrease in the ratio of essential oil components of oil roses as the morning progressed towards the evening in their volatile oil component analysis conducted at different times of the day. Therefore, a positive relationship between the collection time and the quality of oil roses is evident. In our study, it was observed that as the collection time was delayed, there was a rapid increase in the biochemical and antioxidant enzyme activities in oil roses. It was determined that the petals, which determine the quality of rose oil, were exposed to stress throughout the day. Indeed, Çelik (2023) has reported a relationship between antioxidant enzymes, biochemical contents, and stress conditions. Upon reviewing previous studies, it is noteworthy that the biochemical and antioxidant enzyme responses of oil roses under salinity and drought stress conditions, as well as the effects of different collection times with stress-reducing applications, have not been investigated concerning antioxidant enzyme activities and some biochemical contents (Kashefi et al., 2012; Zahedi-Amiri et al., 2019; Alizadeh et al., 2021; Hamza et al., 2022; Hessini et al., 2022; Omidi et al., 2022; Demir and Başayiğit, 2022; Tiryaki et al., 2023). Therefore, our study was the first study on the subject. It is known that there are changes in plants' biochemical and enzymatic activities during the flowering development stages, and the production of reactive oxygen species (ROS) occurs in plant cells (Jajic et al., 2015). Ezhilmathi et al. (2007) reported an increase in antioxidant enzyme activities in plants at the beginning of the flowering stages. However, they noted a decrease in enzyme activities towards the end of flowering. The reason for this is associated with the accumulation of reactive oxygen species (ROS). Therefore, comparing the results obtained in our study with both Baydar et al. (2013) and Ezhilmathi et al. (2007) studies, it can be stated that as the collection time is delayed, there is an increase in ROS accumulation in rose petals, accompanied by an increase in enzyme activities.

Conclusion

This study examined the changes in biochemical contents and antioxidant enzyme activities in rose petals collected at different time intervals. The results of the research show that there are significant changes in biochemical contents and antioxidant enzyme activities over time, and these changes are statistically significant ($p<0.05$). The total phenolic content in rose petals collected at 6.00 a.m. was 310.32 mg GAE g^{-1}. Due to the influence of sunlight and heat, the total phenolic content increased to 780.70 mg GAE g^{-1} at 14.00 p.m., indicating a 151.57% increase. The total flavonoid content in samples collected at 6.00 a.m. was 33.90 mg CE g^{-1}, while in samples collected at 14.00 p.m., it was 62.33 mg CE g^{-1}, representing an 83.86% increase. The proline content in samples collected in the morning was 7.43 mg g^{-1}, reaching 24.96 mg g^{-1} by 14.00 p.m., showing a 236% increase. The DPPH radical scavenging activity showed an 8.87% increase from 6.00 a.m. to 14.00 p.m. The lowest CAT activity was observed at 6.00 a.m. with 6.20 U mg^{-1}, while the highest CAT activity was observed at 14.00 p.m. with 42.67 U mg^{-1}, showing a 588.22% increase. The SOD activity at 6.00 a.m. was 4.67 U mg^{-1}, reaching 11.33 U mg^{-1} at 14.00 p.m., showing a 142.61% increase. The APX activity at 6.00 a.m. was

12.11 mol min^{-1} g^{-1}, reaching 85.55 mol min^{-1} g^{-1} at 14.00 p.m., showing a 606.44% increase. The POD activity at 6.00 a.m. was 3.42 ΔA_{460} min^{-1} mg^{-1}, reaching 8.91 ΔA_{460} min^{-1} mg^{-1} at 14.00 p.m., showing a 160.52% increase.

As a result, the quality of rose oil depends on a series of factors, which can influence various stages, from the cultivation of flowers to their harvest, distillation process, and storage. Different rose species have different chemical compositions, affecting the quality of the oil. Growing conditions such as soil quality, climate, humidity levels, and sunlight directly impact the growth of the rose plant and the quality of its flowers. Additionally, the time of day when flowers are harvested also affects the quality of rose oil. Generally, the early morning hours are considered when rose petals have the most concentrated and high-quality volatile oil content. Therefore, based on the data obtained in our study, using appropriate distillation and extraction methods on rose petals collected at 6.00 in the morning would result in higher-quality rose oil. As time progresses, an increase in cellular ROS accumulation due to the stress experienced by the flowers may increase biochemical and antioxidant enzyme activities, which could also affect the oil quality.

Ethical Statement

Ethical approval is not required for this study.

Conflict of Interest

The authors declares that there are no conflicts of interest.

Funding Statement

There is not funding sources in the study.

Author Contributions

CÇ and AVP contributed to the study conception and design. CÇ and AVP performed the data analysis. CÇ and AVP wrote the first draft of the manuscript. CÇ and AVP acquired funds and supervised the analyses. CÇ and AVP supplied the seed material. All authors commented on previous versions of the manuscript and read and approved the final manuscript.

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Research Article

Herbage Quality of Eight Native Hordeum Ecotypes Collected From Natural Grassland & Pasture Ecology of Southeastern Anatolia

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Abstract: In this study, plant samples of eight different ecotypes of three distinct species of the genus *Hordeum* were collected at the anthesis stage of the plants in the 2023 spring in Southeastern Anatolia's natural grassland and pasture ecology. The quality analyses of the herbage samples of *Hordeum bulbosum*, *H. murinum, and H. spontaneum* ecotypes collected from five different locations (Karacadag-I, Batman-1, Diyarbakır-6, Diyarbakır-8, and Diyarbakır-13) were determined by NIRS analyzer. Crude protein (CP), dry matter (DM), acid detergent insoluble fiber (ADF), neutral detergent insoluble fiber (NDF), acid detergent insoluble protein (ADP), Ca, K, Mg, P, Ca/P, K/(Ca+Mg), digestible dry matter (DDM), dry matter intake (DMI) and relative feed values (RFV) were determined. The values were determined between 8.2-23.4% for CP; 92.1-93.4% for DM; 19.3-36.2% for ADF; 26.2-71.9% for NDF; 0.16-0.71% for ADP; 60.7-73.9% for DDM; 1.67-4.58% for DMI; 78.6-262.8 for RFV; 0.30-0.42% for P; 1.72-2.84% for K; 0.12-1.62% for Ca; 0.17-0.30% for Mg; 0.33-3.84 for Ca/P; 2.76-4.77 for K/(Ca+Mg). In conclusion, the CP, ADF, NDF, DMI, RFV, and Ca/P values were found very variable in collected ecotypes and can be used for forage barley breeding purposes.

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1. Introduction

An environment that is both demanding and unpredictable poses a threat to global food security. An effective and sustainable approach to enhancing food productivity stability is through selecting crops to exhibit high resilience to abiotic stressors. The use of the gene pools of the agricultural plant's wild relatives is a commonly used strategy for improving crop genetics (Chen et al., 2008).

One of the most important cereals in the world and one of the earliest domesticated crops is barley (*Hordeum vulgare* subsp. *vulgare*). The wild progenitor of barley, *H. vulgare* subsp. *spontaneum*, is considered as a significant source for barley improvement in response to changing climatic conditions. Both species are interfertile, primarily self-pollinating, and diploid (2n = 14). There are various phenotypic differences between cultivated and wild barley, which are combined to form the domestication syndrome. From the coasts of the eastern Mediterranean to the semideserts of Afghanistan, wild barley grows naturally in Southwest Asia (Jakob et al., 2014). *H. spontaneum*, the

parent plant of cultivated barley, is a self-fertilizing annual grass that is primarily found in the Mediterranean and Irano-Turanian regions. It can also be found in desert regions, where it can sustain steady populations. There are colder climates where wild barley grows, like in Tibet. Rich genetic diversities for drought, salt, and cold resistances have been collected in wild barley as a result of its adaption to both cold and desert environments (Chen et al., 2008).

Since many of the taxa that make up the *Hordeum*, such as the majority of tetra- and hexaploid species, are of hybrid origin, it is not simple to determine the relationships between its members. Within the *Triticeae* family, *Hordeum* is one of the largest genera, containing over 30 species distributed worldwide. These two species do not appear to be related, based on the observed dramatic variations in the number and distribution of the repeat sequences that were analyzed in *H. vulgare* and *H. bulbosum*. Conversely, no distinctions were found between *H. vulgare*'s two subspecies (subsp. *vulgare* and subsp. *spontanum*). Concerning the *H. murinum* complex, the results support the idea that *H. glaucum* and two extinct species were respectively the diploid donors of the subgenomes Xu, Xv, and Xw present in polyploids (Jouve et al., 2018).

Particularly in colder climates where other feed grain crops like wheat, sorghum, and maize are difficult to grow, barley is an extremely important feed grain (Poulsen, 2020). One of the few essential crops that were domesticated during the beginning of agriculture in the Fertile Crescent was barley, and it is still widely used in farming today in the region. According to Russell et al. (2011), barley serves as a model species for research on the evolution, adaptability, and dissemination of major crops worldwide. In the Fertile Crescent, the taxon makes up a significant annual component of the open herbaceous and park-like vegetation. It is mostly limited to artificial (secondary) habitats outside of this area, and only widely dispersed populations are found there, especially in the east of its distribution area. Extensive serrated lemma awns and the ear's ability to break apart after ripening, releasing spikelets resembling arrows, offer superior adaptability to epizoochory and colonization. Uncertainty surrounds whether wild barley populations in Morocco, Ethiopia, and Tibet are naturally occurring, human-introduced, or wild variations of farmed barley (Molina-Cano et al., 2005). The spike structure separates the two-rowed and six-rowed barley. Hulless barley is defined as having neither the palea nor the lemma attached to the seed. Compared to hulled barley, naked barley yields less and has a higher protein content. While naked barley is an old food crop, most farmed varieties of barley are hulled. By manipulating inflorescence, there is a great potential to increase its yields (Li, 2020).

Barley, both wild and cultivated, has been collected across its range in the past century, and seed samples have been preserved and kept in ex-situ gene banks. Approximately 470 000 barley accessions are kept in more than 200 collections across the globe (Knüpffer, 2009). According to Russell et al. (2011), this stock of accessions serves as the primary source of plant materials used in investigations into many facets of genetic variation in barley.

Annual forages are well suited to semiarid climates. In dryland systems, annual cereal forages are more resilient than grain crops in terms of water use (WU), water use efficiency (WUE), and weed control. Many types of fodder can survive in semiarid conditions, including warm- and cool-season grasses as well as mixes of legumes and grasses. Dryland cropping systems can be effectively diversified by using any of these annual forages (Lenssen et al., 2015).

For livestock producers in ecologies similar to Montana (USA), barley cut for hay is a major source of winter feed. Barley is a widely accessible and reasonably priced feed ingredient. As it grows and is harvested similar to legume forages, forage barley yields well and is beneficial to producers. Feed value can be maximized by careful harvesting. Compared to other minor grains, barley has been found to have a lower fiber concentration and a higher nutritional value. Awns on some barley cultivars, nevertheless, are harsh when chopped for hay. Cereal grains with rough or barbed awns can irritate the mouth and reduce palatability (Todd et al., 2003). Despite the significance of fodder quality, forage barley breeding programs often choose new barley lines primarily on yield and awnless features (Surber et al., 2004). The barley feed's palatability may be impacted by the awns. The forage's ingestion potential is inversely connected with neutral detergent fiber. Acid detergent fiber is a measure of the portion of fiber that is less digestible and has a negative correlation with the animal's potential for digestion. Barley hay is usually fed in the winter and spring, when the animals' protein needs may be at their peak, hence its nitrogen concentration is crucial. Particular varieties of hay barley are more likely to accumulate nitrates, which can make them harmful to cattle that consume them. It would be preferable if the nitrate accumulation potential was low. Thus, it would be preferable to have higher CP and digestibility and

reduced accumulation of NDF, ADF, and NO3-N. Although measuring these traits is not difficult, breeding programs do not employ them as selection criteria (Surber et al., 2001).

According to Liancourt et al. (2013), an ecotype is a population (or subspecies, or race) that has adapted to the environmental conditions of its particular location and is distinguished by particular physiological and morphological traits. The response of species to the environment is "ecotype-specific". Climate change effects may be mitigated or obscured by prevailing sources of variance affecting plant performance which exist in ecotypes.

In this study, plant samples of eight different ecotypes of three distinct species of the genus *Hordeum* were collected at the anthesis stage of the plants in Southeastern Anatolia's natural grassland and pasture ecology. The quality analyses of the herbage samples of *Hordeum bulbosum*, *H. murinum,* and *H. spontaneum* ecotypes collected from five different locations (Karacadag, Batman, Diyarbakır-1, Diyarbakır-2, and Diyarbakır-3) were determined in the study.

2. Material and Methods

Plant samples of eight ecotypes from three distinct species of the genus *Hordeum* constitute the research material. The samples were obtained in 2023 from various sites (Karacadag, Batman, Diyarbakır-1, Diyarbakır-2, and Diyarbakır-3 locations) in the Southeastern Anatolia Region of Turkiye. Table 1 provides information on the locations, dates, and geographic coordinates of the collected plants.

Tablo 1. Species, locations, collection dates, and geographic coordinates of the plant samples

Species	Locations	Latitude	Longitude	Altitude (m)	Date
H. bulbosum	Karacadag	37.77º	39.78 º	1469	21.05.2023
H. bulbosum	Diyarbakır-1	37.91 º	40.27 º	652	15.05.2023
H. bulbosum	Diyarbakır-2	38.21 º	39.27 º	1113	10.05.2023
H. murinum	Diyarbakır-2	37.91 º	40.27 º	652	15.05.2023
H.murinum ssp. *leporinum*	Batman	37.95 º	41.36 º	572	07.05.2023
H.spontaneum	Batman	37.95 º	41.36 º	572	07.05.2023
H.spontaneum	Diyarbakır-1	37.91 º	40.27 º	652	15.05.2023
H.spontaneum	Diyarbakır-2	38.19 º	39.36 º	982	10.05.2023

Samples of plants and herbarium specimens from *Hordeum* species were collected at the anthesis stage of the plants. Determination of species was conducted by Prof. Dr. Selçuk ERTEKİN from Dicle University, Faculty of Science, Department of Biology (Diyarbakır, Türkiye). Approximately 200 g of green grass samples from each species were cut from the the plants at soil level. The samples were dried in a drying cabinet (Memmert ULM 800) at 70 °C for 48 hours (Anonymous, 2021). The dried samples were ground in a laboratory type mill (IKA, A11), then sieved in a 1 mm diameter sample sieve (Retsch, DIN-ISO 3310/2), and made ready for analysis.

Quality analyses of the grass samples were conducted with a NIRS analyzer (Near Infrared Spectroscopy-Foss Model 6500) in the laboratory of Dicle University, Science and Technology Application and Research Center (Diyarbakır, Türkiye). Crude protein (CP), acid detergent insoluble fiber (ADF), neutral detergent insoluble fiber (NDF), acid detergent insoluble protein (ADP), Ca, K, Mg, P, Ca/P, and K/(Ca+Mg) values were determined via analysis. Additionally, digestible dry matter (DDM), dry matter intake (DMI), and relative feed values (RFV) were calculated with the help of the determined ADF and NDF values. The following equations were used for calculations (Morrison, 2003).

$$DDM = 88.9 - (0.779 \times ADF) \quad (1)$$

$$DMI = 120 / NDF \quad (2)$$

$$RFV = (DDM \times DMI) / 1.29 \quad (3)$$

A comparison of the quality of the samples with reference standards was conducted according to the classification method of Lacefield (1988) given for legumes, grasses, and legume + wheatgrass mixtures (Table 2).

Table 2. Reference quality standards for legumes, grasses, and legume + wheat mixtures (Lacefield, 1988)

Quality standards	CP (%)	ADF (%)	NDF (%)	DDM (%)	DMI (%)	RFV
P	>19	<31	<40	>65	>3.0	>151
1	17-19	31-35	40-46	62-65	3.0-2.6	151-125
2	14-16	36-40	47-53	58-61	2.5-2.3	124-103
3	11-13	41-42	54-60	56-57	2.2-2.0	102-87
4	8-10	43-45	61-65	53-55	1.9-1.8	86-75
5	<8	>45	>65	<53	<1.8	<75

2.1. Evaluation of data

The produced data regarding the searched features were analyzed according to the one-way ANOVA test in the Jump-Pro13 statistical package program. Differences between means were compared with the Tukey HSD test. According to the scatter plot model, the principal components analysis was conducted in the Genstat (2009)12th (Copyright 2011, VSN International Ltd) statistical package program and the scatterplot matrix was made in the Jump-Pro13 statistical package program. The research results were presented in a table using the one-way ANOVA test.

3. Results

The results of one-way variance analysis of the examined quality characteristics of *Hordeum* species and the resulting groups are given in Tables 3, 4, and 5.

Table 3. Comparisons of species according to one-way ANOVA using Tukey HSD test

		Crude protein				
Species	**Location**	**Mean**	**Std Dev**	**Std Err Mean**	**Lower 95%**	**Upper 95%**
H. bulbosum	Karacadag	10.09 d	0.20	0.12	9.60	10.59
H. bulbosum	Diyarbakır-3	8.15 f	0.22	0.13	7.60	8.70
H. bulbosum	Diyarbakır-2	11.47 c	0.16	0.09	11.06	11.88
H. murinum	Diyarbakır-2	13.85 b	0.21	0.12	13.33	14.38
H.murinum ssp. *leporinum*	Batman	11.93 c	0.05	0.03	11.79	12.07
H.spontaneum	Batman	9.19 e	0.22	0.12	8.66	9.73
H.spontaneum	Diyarbakır-3	9.72 d	0.18	0.10	9.27	10.17
H.spontaneum	Diyarbakır-1	23.38 a	0.04	0.02	23.27	23.48
Significance	**					
Coefficient variance (%)	**1.40**					
		Dry matter				
H. bulbosum	Karacadag	93.14 bc	0.064	0.037	92.982	93.300
H. bulbosum	Diyarbakır-3	92.09 f	0.098	0.057	91.847	92.335
H. bulbosum	Diyarbakır-2	92.88 d	0.057	0.033	92.735	93.018
H. murinum	Diyarbakır-2	93.35 a	0.002	0.001	93.350	93.357
H.murinum ssp. *leporinum*	Batman	93.09 bc	0.066	0.038	92.927	93.256
H.spontaneum	Batman	92.98 cd	0.050	0.029	92.856	93.104
H.spontaneum	Diyarbakır-3	93.23 ab	0.046	0.027	93.119	93.349
H.spontaneum	Diyarbakır-2	92.31 e	0.085	0.049	92.099	92.519
Significance	**					
Coefficient variance (%)	0.06					
		ADF				
H. bulbosum	Karacadag	30.86 c	0.595	0.344	29.379	32.338
H. bulbosum	Diyarbakır-3	34.93 b	0.146	0.084	34.564	35.290
H. bulbosum	Diyarbakır-2	35.37 ab	0.528	0.305	34.061	36.686
H. murinum	Diyarbakır-2	31.31 c	0.400	0.231	30.316	32.303
H.murinum ssp. *leporinum*	Batman	31.65 c	0.116	0.067	31.360	31.935
H.spontaneum	Batman	36.20 a	0.495	0.286	34.974	37.433
H.spontaneum	Diyarbakır-3	34.81 b	0.108	0.062	34.539	35.075
H.spontaneum	Diyarbakır-1	19.30 d	0.165	0.095	18.887	19.705
Significance	**					
Coefficient variance (%)	1.17					

Table 3. Comparisons of species according to one-way ANOVA using Tukey HSD test (continued)

Species	Location	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
			NDF			
H. bulbosum	Karacadag	55.31 d	0.987	0.370	54.525	56.093
H. bulbosum	Diyarbakır-3	45.99 e	0.508	0.370	45.202	46.771
H. bulbosum	Diyarbakır-2	59.51 c	0.881	0.370	58.729	60.298
H. murinum	Diyarbakır-2	59.17 c	0.581	0.370	58.388	59.957
H.murinum ssp. *leporinum*	Batman	62.39 b	0.399	0.370	61.602	63.171
H.spontaneum	Batman	71.86 a	0.705	0.370	71.072	72.641
H.spontaneum	Diyarbakır-3	63.61 b	0.306	0.370	62.822	64.391
H.spontaneum	Diyarbakır-1	26.15 f	0.437	0.370	25.369	26.938
Significance	**					
Coefficient variance (%)	1.15					

**; P≤0.01, *; P≤0.05 significant levels. Levels not connected by the same letter are significantly different.

3.1. Crude protein

In the research, the CP rate in different *Hordeum* ecotypes varied between 8.15-23.38%. The highest CP ratio (23.38%) was at *H. spontaneum* in Diyarbakır-1. The lowest CP value (8.15%) was at *H. bulbosum* in Diyarbakır-3 (Table 3). High CP value is an important characteristic of the quality roughage. The findings regarding the CP ratio in this research were within the limits specified by Sirat and Bahar (2020) (12.73-15.6%), but were found to be higher than the results of Sarı and Alatürk (2023) (6.30-15.89%).

3.2. DM

DM ratios in different *Hordeum* ecotypes varied between 92.09-93.35%. The highest DM ratio (93.35%) among the ecotypes was at *H. murinum* in Diyarbakır-3. The lowest DM value (92.09%) was obtained from *H. bulbosum* collected from the same region (Table 3). The findings regarding dry matter in the study were within the limits of the findings (65.80-95.72%) of Sarı and Alatürk (2023).

3.3. ADF

The ADF value in roughage refers to the amount of cellulose, lignin, and insoluble protein in the structure of the plant cell wall (Kutlu, 2008), and is desired to be as low as possible (Schroeder, 1994; Başbağ et al., 2020). The ADF values of different *Hordeum* ecotypes were between 19.30-36.20%. The highest ADF value (36.20%) was detected in *H. spontaneum* collected from the Batman region. The lowest ADF value (19.30%) was obtained from *H. spontaneum* at Diyarbakır-1 (Table 3). Unlike this research, some studies have reported the ADF values between 1.43-8.65% (Alijosius et al., 2016; Erbaş Köse and Mut, 2019; Sirat and Bahar, 2020).

3.4. NDF

In forages, NDF refers to the amount of hemicellulose, cellulose, lignin, cutin, and insoluble protein found in the structure of the plant cell wall (Aşçı and Acar, 2018). For this reason, low NDF value is desired in roughage (Mut et al., 2017; Başbağ et al., 2020). The NDF value in different *Hordeum* ecotypes was between 26.15-71.86%. The highest NDF value (71.86%) was detected in *H. spontaneum* collected from the Batman region. The lowest NDF value (26.15%) was obtained from *H. spontaneum* in the Diyarbakır-1 location (Table 3). Fife et al. (2008) reported NDF contents in barley between 19.9% and 24.5%. Also, Sirat and Bahar (2020) reported NDF values between 20.42 and 25.03%. It is stated by different researchers that NDF values may vary depending on genotype/varieties (Barteczko et al., 2009; Can and Ayan, 2017).

Table 4. Comparisons of species according to one-way ANOVA using Tukey HSD test

Species	Location	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
		ADP				
H. bulbosum	Karacadag	0.17 f	0.074	0.043	-0.015	0.351
H. bulbosum	Diyarbakır-3	0.71 a	0.020	0.011	0.660	0.757
H. bulbosum	Diyarbakır-2	0.46 c	0.012	0.007	0.435	0.492
H. murinum	Diyarbakır-2	0.58 b	0.033	0.019	0.498	0.660
H.murinum ssp. *leporinum*	Batman	0.45 cd	0.015	0.008	0.411	0.484
H.spontaneum	Batman	0.37 d	0.000	0.000	0.370	0.370
H.spontaneum	Diyarbakır-3	0.43 cd	0.014	0.008	0.392	0.460
H.spontaneum	Diyarbakır-1	0.26 e	0.010	0.006	0.237	0.285
Significance	**					
Coefficient variance (%)	7.14					
		DDM				
H. bulbosum	Karacadag	64.86 b	0.464	0.268	63.709	66.014
H. bulbosum	Diyarbakır-3	61.69 c	0.114	0.066	61.409	61.975
H. bulbosum	Diyarbakır-2	61.34 cd	0.412	0.238	60.322	62.367
H. murinum	Diyarbakır-2	64.51 b	0.312	0.180	63.736	65.284
H.murinum ssp. *leporinum*	Batman	64.25 b	0.090	0.052	64.023	64.470
H.spontaneum	Batman	60.70 d	0.386	0.223	59.740	61.656
H.spontaneum	Diyarbakır-3	61.79 c	0.084	0.049	61.577	61.994
H.spontaneum	Diyarbakır-1	73.87 a	0.128	0.074	73.550	74.187
Significance	**					
Coefficient variance (%)	0.46					
		DMC				
H. bulbosum	Karacadag	2.17 c	0.039	0.022	2.074	2.266
H. bulbosum	Diyarbakır-3	2.61 b	0.029	0.017	2.538	2.681
H. bulbosum	Diyarbakır-2	2.02 de	0.030	0.017	1.942	2.091
H. murinum	Diyarbakır-2	2.03 d	0.020	0.012	1.979	2.078
H.murinum ssp. *leporinum*	Batman	1.92 ef	0.012	0.007	1.893	1.954
H.spontaneum	Batman	1.67 g	0.016	0.009	1.629	1.711
H.spontaneum	Diyarbakır-3	1.89 f	0.009	0.005	1.864	1.909
H.spontaneum	Diyarbakır-1	4.59 a	0.077	0.044	4.399	4.780
Significance	**					
Coefficient variance (%)	1.27					
		RFV				
H. bulbosum	Karacadag	109.12 c	2.728	1.575	102.350	115.900
H. bulbosum	Diyarbakır-3	124.80 b	1.609	0.929	120.810	128.800
H. bulbosum	Diyarbakır-2	95.91 de	2.070	1.195	90.760	101.050
H. murinum	Diyarbakır-2	101.42 d	1.486	0.858	97.730	105.110
H.murinum ssp. *leporinum*	Batman	95.80 de	0.746	0.431	93.950	97.650
H.spontaneum	Batman	78.59 f	1.270	0.733	75.430	81.740
H.spontaneum	Diyarbakır-3	90.36 e	0.536	0.310	89.030	91.690
H.spontaneum	Diyarbakır-1	262.79 a	4.846	2.798	250.750	274.830
Significance	**					
Coefficient variance (%)	1.91					
		Phosphorous				
H. bulbosum	Karacadag	0.36 c	0.007	0.004	0.346	0.379
H. bulbosum	Diyarbakır-3	0.30 e	0.002	0.001	0.297	0.307
H. bulbosum	Diyarbakır-2	0.40 b	0.001	0.001	0.400	0.400
H. murinum	Diyarbakır-2	0.41 b	0.004	0.003	0.395	0.417
H.murinum ssp. *leporinum*	Batman	0.35 d	0.001	0.001	0.346	0.350
H.spontaneum	Batman	0.35 d	0.006	0.003	0.332	0.361
H.spontaneum	Diyarbakır-3	0.36 c	0.002	0.001	0.360	0.370
H.spontaneum	Diyarbakır-1	0.42 a	0.002	0.001	0.417	0.428
Significance	**					
Coefficient variance (%)	**0.83**					

**; P≤0.01, *; P≤0.05 significant levels. Levels not connected by the same letter are significantly different.

3.5. ADP

The ADP ratios of different *Hordeum* ecotypes were between 0.171-0.71%. The highest ADP ratio (0.71%) was at *H. bulbosum* in Diyarbakır-3 location. The lowest ADP value (0.17%) was obtained from *H. bulbesum* from Karacadağ region (Table 4). Low ADP values are desirable as they reflect the amount of indigestible protein in roughage (Aşçı and Acar, 2018).

3.6. DDM

The DDM values in *Hordeum* ecotypes varied between 60.70 and 73.87%. The highest DDM value (73.87%) was detected at *H. spontaneum* in Diyarbakır-1 location. The lowest DDM value

(60.70%) was obtained from *H. spontaneum* from the Batman region (Table 4). The DDM value varies depending on the species, and different results have been reported by different researchers (Kaplan, 2021; Tutar and Kökten, 2022; Arıkan et al., 2023) regarding the DDM value.

3.7. DMI

The DMI values in *Hordeum* ecotypes varied between 1.67 and 4.59%. The highest DMI value (4.59%) among the ecotypes was determined at *H. spontaneum*in in the Diyarbakır-1 location. The lowest DMI value was obtained from *H. spontaneum* (1.67%) in the Batman region (Table 4). Arıkan et al., (2023) reported the DMI value in barley as 2.02%.

3.8. RFV

RFV values in *Hordeum* ecotypes varied between 78.59 and 262.79. The highest RFV value (262.79) was determined at *H. spontaneum* in the Diyarbakır-1 location. The lowest RFV value (78.59) was obtained from *H. spontaneum* in the Batman region (Table 4). Canbolat (2012) and Arıkan et al., (2023) reported higher RFV value in wheat compared to barley.

3.9. Phosphorous (P)

P values in *Hordeum* ecotypes varied between 0.30-0.42%. The highest phosphorus value (0.42%) among the species was determined at *H. spontaneum* in Diyarbakır-1 The lowest P value (0.30%) was at *H. bulbosum* in Diyarbakır-2, (Table 4). Sirat and Bahar (2020) reported the P rates between 0.42-0.44%. In another study, P values varied between 0.363 and 0.408% (Mut and Erbaş Köse, 2018). Some researchers reported that P values vary according to genotypes (Poutanen, 2012; Jakobsone et al., 2015).

Table 5. Comparisons of species according to one-way ANOVA using Tukey HSD test

		Potassium				
Species	**Location**	**Mean**	**Std Dev**	**Std Err Mean**	**Lower 95%**	**Upper 95%**
H. bulbosum	Karacadag	1.73 d	0.092	0.053	1.497	1.953
H. bulbosum	Diyarbakır-3	2.56 b	0.033	0.019	2.480	2.645
H. bulbosum	Diyarbakır-2	2.50 b	0.074	0.043	2.314	2.680
H. murinum	Diyarbakır-2	2.84 a	0.020	0.011	2.796	2.893
H.murinum ssp. *leporinum*	Batman	2.25 c	0.088	0.051	2.035	2.472
H.spontaneum	Batman	1.79 d	0.075	0.043	1.600	1.973
H.spontaneum	Diyarbakır-3	2.32 c	0.021	0.012	2.264	2.368
H.spontaneum	Diyarbakır-1	2.59 b	0.053	0.031	2.460	2.723
Significance	**					
Coefficient variance (%)	2.58					
		Calcium				
H. bulbosum	Karacadag	0.12 f	0.062	0.036	-0.034	0.274
H. bulbosum	Diyarbakır-3	1.12 b	0.009	0.005	1.099	1.146
H. bulbosum	Diyarbakır-2	0.54 c	0.023	0.013	0.486	0.601
H. murinum	Diyarbakır-2	0.41 d	0.024	0.014	0.345	0.466
H.murinum ssp. *leporinum*	Batman	0.58 c	0.004	0.002	0.572	0.592
H.spontaneum	Batman	0.31 e	0.010	0.006	0.285	0.335
H.spontaneum	Diyarbakır-3	0.32 e	0.001	0.000	0.318	0.321
H.spontaneum	Diyarbakır-1	1.62 a	0.004	0.003	1.613	1.635
Significance	**					
Coefficient variance (%)	3.22					
		Magnessium				
H. bulbosum	Karacadag	0.25 c	0.004	0.002	0.237	0.257
H. bulbosum	Diyarbakır-3	0.30 a	0.002	0.001	0.301	0.309
H. bulbosum	Diyarbakır-2	0.17 f	0.000	0.000	0.170	0.170
H. murinum	Diyarbakır-2	0.24 c	0.003	0.002	0.234	0.250
H.murinum ssp. *leporinum*	Batman	0.23 d	0.001	0.001	0.231	0.235
H.spontaneum	Batman	0.22 e	0.000	0.000	0.220	0.220
H.spontaneum	Diyarbakır-3	0.17 f	0.003	0.002	0.162	0.179
H.spontaneum	Diyarbakır-1	0.29 b	0.004	0.003	0.282	0.304
Significance	**					
Coefficient variance (%)	0.86					

Table 5. Comparisons of species according to one-way ANOVA using Tukey HSD test (continued)

Species	Location	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
			Ca/P			
H. bulbosum	Karacadag	0.33 e	0.177	0.102	-0.107	0.773
H. bulbosum	Diyarbakır-3	3.72 a	0.056	0.032	3.576	3.854
H. bulbosum	Diyarbakır-2	1.36 c	0.058	0.033	1.215	1.502
H. murinum	Diyarbakır-2	1.00 d	0.049	0.028	0.878	1.122
H.murinum ssp. *leporinum*	Batman	1.67 b	0.007	0.004	1.656	1.690
H.spontaneum	Batman	0.89 d	0.017	0.010	0.851	0.937
H.spontaneum	Diyarbakır-3	0.88 d	0.006	0.004	0.861	0.892
H.spontaneum	Diyarbakır-1	3.84 a	0.033	0.019	3.764	3.925
Significance	**					
Coefficient variance (%)	4.09					
			K/(Ca+Mg)			
H. bulbosum	Karacadag	4.77 a	0.618	0.357	3.235	6.307
H. bulbosum	Diyarbakır-3	1.80 d	0.010	0.005	1.772	1.819
H. bulbosum	Diyarbakır-2	3.50 b	0.025	0.014	3.439	3.562
H. murinum	Diyarbakır-2	4.40 a	0.217	0.125	3.858	4.934
H.murinum ssp. *leporinum*	Batman	2.77 c	0.125	0.072	2.456	3.075
H.spontaneum	Batman	3.37 bc	0.078	0.045	3.175	3.564
H.spontaneum	Diyarbakır-3	4.73 a	0.078	0.045	4.533	4.922
H.spontaneum	Diyarbakır-1	1.35 d	0.034	0.020	1.268	1.437
Significance	**					
Coefficient variance (%)	6.90					

**; P≤0.01, *; P≤0.05 significant levels. Levels not connected by the same letter are significantly different.

3.10. Potassium (K)

K, which has important roles in photosynthesis, enzyme activity, and regulation of the water content of plants, is very important for the sugar and protein contents of cereal grains (Güneş et al., 2000). K value in *Hordeum* ecotypes varied between 1.73-2.84%. The highest K value (2.84%) was at *H. murinum* in Diyarbakır-2 location. The lowest K values were at *H. bulbosum* in Karacadağ (1.73%) and from *H. spontaneum* (1.79%) from Batman region (Table 5). Sirat and Bahar (2020) reported a K value between 0.68-0.79%. The findings obtained in this study regarding the K value were higher than the findings of other researchers. This difference may be sourced from the performance of different genotypes under different ecological conditions. Similarly, Poutanen (2012) stated that K values vary according to varieties.

3.11. Calcium (Ca)

Due to Ca deficiency, rickets, osteomalacia, and urinary system stone disease may occur in animals. Ca, which is found in limited amounts, especially in the bones of newborn animals, is absolutely necessary for the development of bones (Anonymous, 2021). Ca value obtained in *Hordeum* ecotypes varied between 0.12-1.62%. The highest Ca value (1.62%) was determined at *H. spontaneum* in Diyarbakır-1 location. The lowest Ca value (0.12%) was obtained from *H. bulbosum* from the Karacadag region (Table 5). Gül et al., (2022) determined Ca as 0.45% in their research.

3.12. Magnesium (Mg)

Mg acts as an enzyme activator and its deficiency may result in meadow tetany disease in animals (Underwood, 1981). Magnesium is also known as the "antistress mineral" as it helps reduce the hypersensitivity of the animal nervous system. It plays a role in activating enzymes and converting sugar into energy in the blood. Mg deficiency in sheep causes meadow tetany, in the form of contraction of the legs and lifting of the head backward (Ensminger et al., 1990).

Mg value of studied *Hordeum* ecotypes varied between 0.17-0.30%. The highest Mg value (0.30%) was at *H. bulbosum* in Diyarbakır-3. The lowest Mg values were in Diyarbakır-2 both at *H. bulbosum* (0.17%) and at *H. spontaneum* (0.17%) (Table 5). Sirat and Bahar (2020) reported that the Mg values varied between 0.17-0.19%, which is similar to this study's Mg values.

3.13. Calcium/Phosphorus (Ca/P)

The Ca/P ratio in studied *Hordeum* ecotypes varied between 0.33-3.84. The highest Ca/P ratio (3.84) was at *H.spontaneum* Diyarbakır-1. The lowest Ca/P ratio value (0.33) was obtained from *H. bulbosum* collected from the Karacadag region (Table 5). Ayan et al., (2010) and Albu et al., (2012) reported that the Ca/P ratio in an ideal feed should be 1/1 or 2/1. In their research, Gül et al. (2022) found the Ca/P ratio in barley as 1.3. The findings we obtained regarding the Ca/P ratio were within the range of the findings reported by Gül et al. (2022).

3.14. Potassium/(Calcium+Magnesium) [K/(Ca+Mg)]

The K/(Ca+Mg) ratios in studied *Hordeum* ecotypes varied between 1.35 and 4.77. The highest K/(Ca+Mg) ratios were obtained from *H. bulbosum* in Karacadag (4.77), *H. murinum* in Diyarbakır-2 (4.39) and *H. spontaneum* in Diyarbakır-3 (4.73). The lowest K/(Ca+Mg) ratio value (1.35) was obtained from *H. spontaneum*collected from Diyarbakır-1 (Table 5). Gül et al. (2022) reported the K/(Ca+Mg) ratio in barley as 3.31. The findings obtained from this study were higher than the findings of Gül et al. (2022).

3.15. Interpretation of relationships between features with Scatterplot matrix and Biplot analysis

The relationships between features can be interpreted in the scatterplot matrix graphic obtained based on correlation coefficient values (Karaman, 2022). If the distribution representing the relationship between any two features does not show a regular accumulation on the regression curve, the relationship between these two features can be commented as weak or non-existent. However, if the distribution on the regression curve is regular, it can be concluded that there is a strong relationship between these two features. There were generally strong relationships between the examined traits in our research, especially between ADF and CP; DDM and CP; DMI and ADF; RFV and ADF; RFV and NDF; DMI and DDM; RFV and DDM; RFV and DMI; Ca/P and Ca, K values. The correlation coefficient between /(Ca+Mg) and Mg and Ca/P values were close to ±1 and the distribution was regular on the regression line which shows that there were very strong relationships between these characters (Figure 1).

The relationships between the examined features of *Hordeum* ecotypes were determined by using the scatterplot biplot technique obtained from the average data of the relevant examined features through polygons and sectors (Figure 2). In the biplot analysis, it was observed that the two-dimensional PCA score PC1 was 61.96%, PC2 was 21.36%, and the total variation (PC1+PC2) was 83.33%. In different studies, PC1, PC2, and PC1+PC2 scores were also found very diverse (Sayar et al., 2018; Başbağ et al., 2021).

In the Scatterplot biplot graph, the regression coefficient between some features, was close to ±1, which gave the same result in the biplot produced by using sectors, polygons, and mega environments. This showed that the mentioned features were strongly interrelated (Figure 2).

There appeared four sectors in the graph: K/(Ca+Mg) and DM in the first sector; P, DDM, CP, DMI, RFV, K, Mg, Ca, and Ca/P in the second sector; ADF and ADP in the third sector; and NDF in the fourth sector. The mentioned features represent the highest values for the ecotypes in the relevant sectors. If there were ecotypes and features in different sectors, it can be commented that there was no genotype standing out in terms of the relevant feature. Instead, if they are located in the same sector, this indicates a positive relationship (Figure 2).

If all of the features are located in the same sector, it can be commented that these features show a complex interaction (Chinipardaz et al., 2016). The ecotypes located on the diagonals of the polygon and indicated by a number produced the best performance in terms of the feature in the relevant sector (Yan and Tinker 2006; Ahmadi et al., 2012). From this perspective, numbers 1 (*Hordeum bulbosum* in Karacadag), number 2 (*Hordeum bulbosum* in Diyarbakır-3), number 6 (*Hordeum spontaneum* in Batman), and number 8 (*Hordeum spontaneum* in Diyarbakır-1) represent the best averages for the observed features in the sectors.

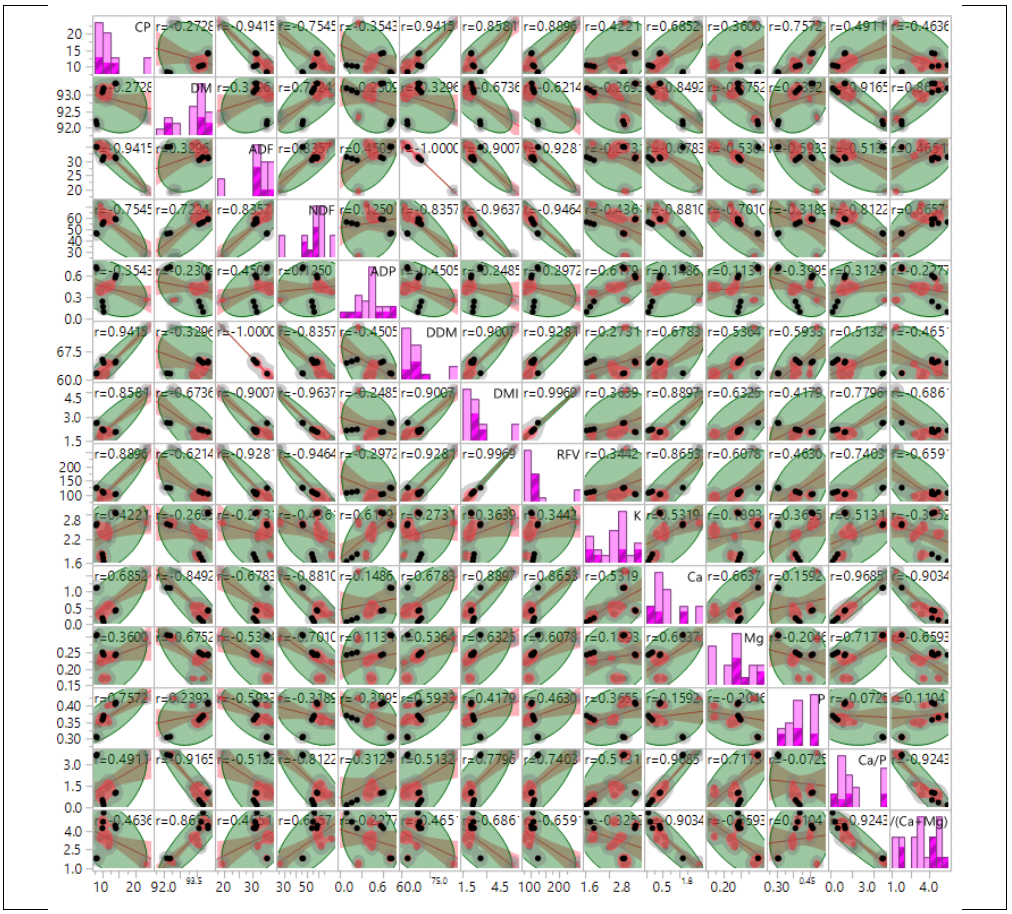

Figure 1. Representation of the relationships between features with a scatterplot matrix.

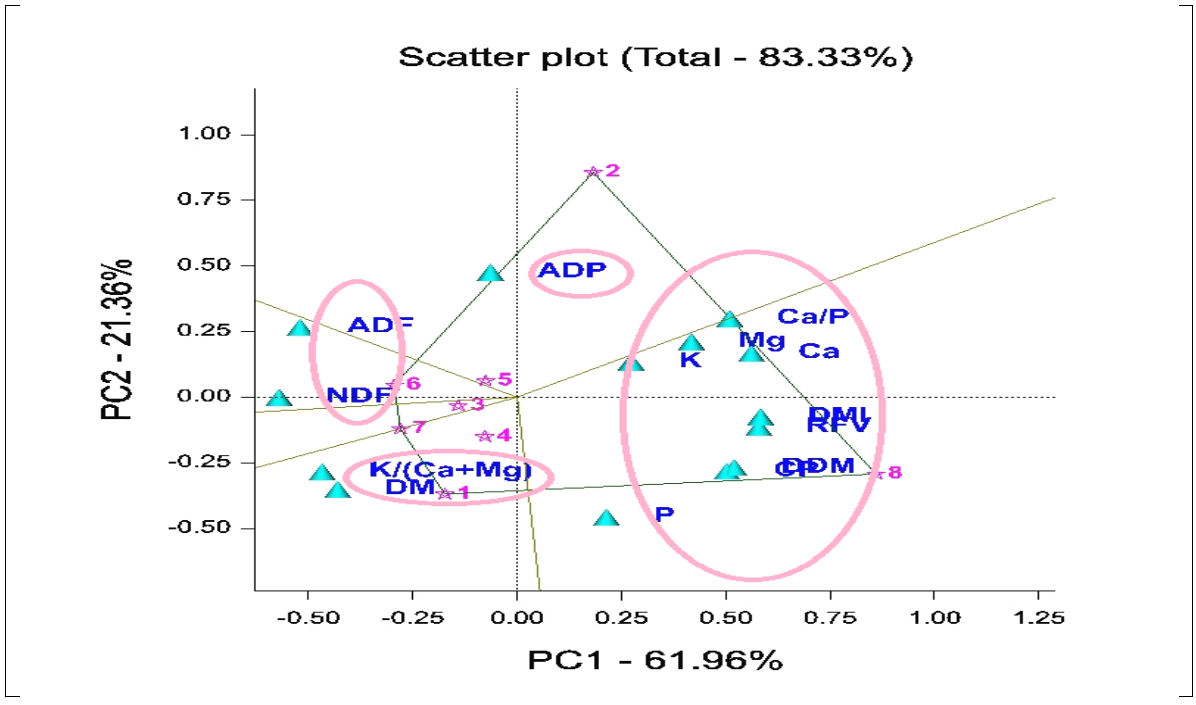

Figure 2. Representation of the relationship between features with scatterplot matrix. 1) *Hordeum bulbosum* in Karacadag, 2) *Hordeum bulbosum* in Diyarbakır-3, 3) *Hordeum bulbosum* in Diyarbakır-2, 4) *Hordeum murinum* in Diyarbakır-3, 5) *H. murinum* ssp. *leporinum* in Batman, 6) *Hordeum spontaneum* in Batman, 7) *Hordeum spontaneum* in Diyarbakır-3, 8) *Hordeum spontaneum* in Diyarbakır-1.

Conclusion

The values were determined between 8.2-23.4% for CP; 92.1-93.4% for DM; 19.3-36.2% for ADF; 26.2-71.9% for NDF; 0.16-0.71% for ADP; 60.7-73.9% for DDM; 1.67-4.58% for DMI; 78.6-262.8 for RFV; 0.30-0.42% for P; 1.72-2.84% for K; 0.12-1.62% for Ca; 0.17-0.30% for Mg; 0.33-3.84 for Ca/P; 2.76-4.77 for K/(Ca+Mg). As a conclusion, the CP, ADF, NDF, DMI, RFV, and Ca/P values were found very variable in collected ecotypes and can be used for forage barley breeding purposes.

Ethical Statement

Ethical approval is not required for this study because ethics is not required for plants.

Conflict of Interest

All authors declare that there is no conflict of interest related to this article.

Author Contributions

The authors declare that they have contributed equally to the article. All authors declare that they have seen/read and approved the final version of the article ready for publication.

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Research Article

Isolation and Immobilization of Biosurfactant-Producing Bacteria Capable of Degrading Carbofuran Pesticide

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Abstract: Pesticide residue has been detected not only on agricultural lands but also in bodies of water such as rivers, lakes, and the sea. This study was aimed at exploring the potency of local bacterial isolates to degrade carbofuran, an active pesticide compound. Two biosurfactant-producing bacteria were isolated from hydrocarbon-compound-contaminated seas (NF9) and agricultural land with a long-term history of pesticide application (AB2). Bacteria were selected according to their ability to grow on a mineral medium, Bushnell Haas Agar, with the addition of 41.86 ppm of carbofuran pesticide as the sole carbon source. Their growth was characterized morphologically, biochemically, and molecularly based on their 16S rRNA genes. All isolates were Gram+ and indicated as *Bacillus thuringiensis* KD168 for isolate NF9 and *Bacillus paranthracis* C9 for isolate AB2. Both of the isolates were immobilized in sodium alginate and polyurethane matrixes. Both *B. thuringiensis* NF9 and *B. paranthracis* AB2 were able to degrade carbofuran, as indicated by the presence of carbofuran residue that ranged from 1.03 to 1.89 ppm; however, the residue was undetected after 15 days of incubation. We also confirmed that bacterial cells were immobilized and retained in polyurethane as well as in the sodium alginate matrix. The immobilization of the bacterial cells showed the abilities of the cells to degrade pesticides and their potential to be developed as bioremediation agents in polluted areas.

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1. Introduction

In modern agriculture, the increasing use of various forms of pesticides is inevitable. Pesticides are used to limit the reproduction and development of pests and diseases; however, the uncontrolled application of pesticides has caused severe problems on agricultural land and in bodies of water. The accumulation of pesticides can threaten human existence, other organisms, and the environment. In the US, the usage of pesticides has increased more than twofold since 1962, and now it endangers groundwater quality in most parts of the country (Lima et al., 2009). Meanwhile, in developing countries such as African countries, people rely on pesticide use as the best way to protect their crops against

pests, as if pesticides can guarantee their crop yield. The annual global agriculture utilization of pesticides has been estimated to be in the order of five million tons, of which approximately seventy percent is utilized in farming and the rest by public health agencies and government departments for vector control and household goals (Sharip et al., 2017; Nabhan et al., 2018; Sarkar, 2021). Carbofuran, a carbamate pesticide, is a potent inhibitor of acetylcholine esterase (AChE) and butyrylcholinesterase, enzymes vital for the functioning of the central nervous system. In humans, carbofuran is associated with endocrine-disrupting activity and reproductive disorders by mimicking or inhibiting estrogen receptors. It is also believed to be responsible for a decrease in sperm count and a rise in testicular cancer in humans, as well as abnormal sexual development in some wildlife species and cytotoxic and genotoxic abnormalities (Islam et al., 2018; Mishra et al., 2020; Pathak et al., 2021). According to the World Health Organization (WHO), over half a million people are poisoned each year by pesticides, and five thousand of the victims die (Bertolote et al., 2006). It is clear that the amount of carbofuran residue in the environment should be controlled. Bioremediation is an emerging technology that has the potential to reduce environmental toxicity.

Carbofuran (2,3-dihydro-2,2dimethylbenzene-7-methylcarbamate) is one of the active ingredients in pesticides. Carbofuran is a broad-spectrum insecticide used extensively in agriculture to inhibit the digestion of insects and other pests. Carbofuran residue becomes a global issue due to its solubility in water and high mobility in soil, resulting in significant groundwater contamination and severe toxic effects on mammals due to cholinesterase inhibition. Numerous European nations have already prohibited the use of chlorpyrifos and carbofuran, and some Asian nations will follow suit by 2022 (PAN Asia Pacific, 2022). As an agricultural nation, Indonesia has a massive pesticide market worth approximately US$ 600 million, of which more than 90 percent is imported (ACECHEM, 2022). Utami et al. (2020) report that the pesticide use estimation was an average of 24.6 kg/ha/year. Mostly, pesticides are used in line with the recommended dosage, but about a quarter are used in larger amounts than the prescribed amount (Utami et al., 2020). Carbofuran is the most toxic carbamate pesticide among pesticides and is sold under various brand names, including Furadan, Jordan, Propinep, Methomyl, and others. Carbofuran is extensively used as an insecticide, acaricide, and nematicide in global agriculture, and its residues will remain in soil and water. Several studies have shown that pesticide behavior in soil is influenced by the absorption, motility, and degradation processes. The adsorption of pesticides in soil is very important because it can lead to environmental problems. Pesticides that do not bind to the soil particles will be degraded to produce less toxic metabolites, while in comparison, adsorbed pesticides will continue to be in the surroundings for a number of years and may build up into food chains many years after their use in soil (Cheah et al., 1997). As an example, it is estimated that carbofuran, which is widely utilized in paddy soil, remains in the water at about 54% and in the soil at about 46% (Arifin and Sukirah, 2020). Another way to improve soil fertility is the application of organic fertilizers including vermi compost, cattle and chicken manure (Alp and Sensoy, 2023; Rahimi et al., 2023).

Numerous bacterial species have been isolated through extensive research on the biodegradation of a variety of pesticides under varying conditions. Microorganisms that play major roles in degrading and utilizing carbamates as a sole source of carbon include *Achromobacter* spp. (Karns et al., 1986; Tomasek and Karns, 1989), *Arthrobacter* sp. (Racke and Coats, 1988), *Sphingomonas* spp. (Feng et al., 1997; and Xu et al., 2009), *Pseudomonas* spp. and *Chrysobacterium* spp. (Bano and Musarrat, 2004), *Bacillus brevis* (Kamboj et al., 2005), *Gliocladium* sp. (Slaoui et al., 2007), *Novosphingobium* sp. FND-3 (Yan et al., 2007), *Burkholderia cepacia* PCL3 (Plangklang and Reungsang, 2011), *Rhodococcus* sp., *Sphingobium* sp., *Bosea* sp., and *Microbacterium* sp. (Shin et al., 2012), and *Cupriavidus* sp. ISTL7 (Gupta et al., 2019). The removal of pesticide-active compounds is restricted by their toxicity. Biological degradation was devised to eliminate these toxic environmental pollutants. This cleaning method is regarded as eco-friendly and cost-effective in comparison to other methods (Mishra et al., 2020).

The immobilization of microorganisms is a multidisciplinary subject that bridges the pure and applied sciences. It has become an emerging method for wide applications in the areas of environmental bioremediation, bioprocessing, and biomedical technology (Nemati and Webb, 2019). Compared to conventional suspension systems, cell entrapment technology has numerous advantages, including high biomass, high enzyme activities, strong resistance to toxic compounds, possible reutilization of microorganisms, and being highly efficient in harsh environments (Partovinia and Rasekh, 2018). At 4 ^{o}C, a species of *Pseudomonas* degraded carbofuran substantially more efficiently in agar-immobilized cells than in free cell suspension (Fareed et al., 2019). Therefore, immobilized microbial technology has

been investigated as a potential wastewater treatment instrument in recent decades. The objective of this investigation was to isolate bacteria from hydrocarbon-contaminated sites and to compare the pesticide degradation efficiency of local isolates as immobilized cells versus their free cell suspensions.

2. Material and Methods

2.1. Chemicals

Analytical grade (99.9%) carbofuran and other chemicals (except as otherwise stated) have been purchased from Merck and Sigma Aldrich Singapore. Molecular identification was done using their 16S rRNA genes. All the samples were sequenced in Macrogen, Singapore.

2.2. Sampling and Screening of isolates

Water and soil samples were collected for the isolation of bacteria. A water sample was obtained from a polluted area by gasoline at the beach area at Belawan Port. Meanwhile, 100 g of topsoil was obtained from agricultural land that has been treated with carbofuran pesticide for a long time in Berastagi. Both are located in North Sumatra, Indonesia. One ml of water sample or 1 g of soil sample was diluted in 9 ml of phosphate buffer, repeated twice to obtain a dilution up to 10^{-2} times. As much as 0.1 ml was inoculated on Bushnell Haas Agar (BHA) medium that contained 41.86 ppm of carbofuran as the only carbon source. The composition of BHA medium per liter was: 0.2 g of magnesium sulfate, 0.02 g of calcium chloride, 1 g of monopotassium phosphate, 1 g of dipotassium phosphate, 1 g of ammonium nitrate, 0.05 g of ferric chloride, and 20 g of agar. pH was adjusted to 7.5 by the addition of NaOH. The obtained colonies were isolated and characterized to get the pure isolate.

2.2. Cell immobilization

Two different matrixes, sodium alginate, and polyurethane, were used to immobilize the isolates.

2.1.1. Cell immobilization using alginate

Cell suspension was prepared by measuring the absorbance of the cell culture using a spectrophotometer at 600 nm to achieve optical density (OD) = 1 which is equal to 10^9 cells ml^{-1}. The cell suspension was concentrated into 10^{13} cells ml^{-1} by centrifugation, and the pellet was suspended in a smaller volume of buffer. The bacterial cell suspension was mixed with 3% Na-alginate. Using a 3 ml syringe, the mixture was dropped into a 0.1–0.2 M $CaCl_2$ solution. Alginate polymerized spontaneously, forming alginate beads containing bacterial cells (Fravel et al., 1985). Alginate beads were rinsed with distilled water for 10 minutes to remove the $CaCl_2$. To evaluate the efficiency of cell entrapment by alginate beads, 10 alginate beads were soaked in 10 ml of 0.85% NaCl for 30 minutes, then transferred into a sodium citrate solution (60 g l^{-1}) for 30 minutes and shaken in a rotary shaker until the beads dissolved completely. Total bacteria were observed by standard plate counting (Schoebitz et al., 2013). The experiments were done in triplicate.

2.1.2. Cell entrapment using polyurethane

The equal volume (50 ml each) of polyurethane solutions A and B (obtained from the local supplier) were mixed and the mixture solidified at room temperature (28 ^oC) to form a hard foam of polyurethane. By using a cutter the foam was cut into pieces with a size of 2 cm square and 0.2 cm of thickness. As many as 0.5 g of polyurethane cubes were mixed with bacterial suspension with a population of 10^{13} cells ml^{-1}. The mixture was incubated for 30 minutes with a 125 rpm shaking incubator at 28 ^oC (Moon et al., 2024). Cell attachment efficiency was evaluated by counting the cells from polyurethane foam after the foam had been shaken vigorously using a vortex for 2 minutes. The cell present in the buffer was plated on Nutrient Agar in a Petri dish. The colonies were counted using the colony counter. The experiments were done in triplicate.

2.3. Determination of cell attachment on polyurethane using Scanning Electron Microscope (SEM) and Pesticide residue analysis

The preparation and observation of the sample using SEM were done at the National Research and Innovation Agency in Bogor, Indonesia. The types of SEM are JSM-5000, MAG-X-15,000, and ACVV-20kV. Meanwhile, the residue of the pesticide was analyzed using HPLC. Waters HPLC 2-2695 series with condition Column: C18, 250 mm x 4.6 mm, 5µ; Flow rate: 1.0 mL/min; Wavelength: 282 nm; Column temperature: 30 °C; Injection volume: 20 µL; Run time: 10 minutes; Diluent: Mobile phase; Elution: Isocratic; Needle wash: Water: Acetonitrile 90:10 (v/v).

2.4. Culture condition for determination of carbofuran degradation

One gram of sodium alginate beads or polyurethane cut into pieces was added to 99 ml of Bushnell-Haas Broth (BHB), whose composition is the same as BHA but without agar addition. As much as 41.86 ppm of carbofuran was added to the medium that served as the only carbon source. The isolates were incubated in a shaking incubator (Vison, Model: VS-8480SN) at 125 rpm and 28 °C for 15 days in a dark condition. The growth of cells was determined, and the residual pesticide was analyzed on days 5, 10, and 15. The same cultures with the addition of free cell suspension were conducted as a positive control. By using 2 ml of bacterial suspension, OD600 = 1 (equal to 10^9 cells ml^{-1}) was added to 98 ml of BHB with the same amount of carbofuran. The same medium with no bacteria was used as a negative control. There is no replication for HPLC analysis.

3. Results and Discussion

3.1. Bacterial isolation

From the screening of the ability of local isolates to grow on the medium with pesticide as the sole carbon source, 17 isolates were able to grow. They varied in morphology, colonies, some basic metabolism (biochemical tests), types of Gram staining, shape, and cell arrangement, as shown in Tables 1 and 2. The isolates with the NF code came from Belawan, the water sample, and the HS and AB codes came from agricultural land in Berastagi. Soils, mainly those with a history of pesticide application, are the main source of microorganisms. Pesticides showed shorter half-lives in soil with a history of pesticide application, mostly compared to soil that has no history of pesticide application (Cycon et al., 2017). The long application of pesticides has caused numerous microorganisms to expand their metabolic systems to break down toxic compounds through different mechanisms, approaches, and enzymatic pathways. The first bacterial isolate capable of degrading organophosphate was from a paddy field in the Philippines in 1973. Later on, numerous strains capable of metabolizing pesticides have been isolated by many researchers from different geographical regions (Das et al., 2005; Talwar et al., 2014; Wu et al., 2014).

Table 1. Characteristic of isolates

No.	Isolate	Colony Morphologies				Gram Staining	Shape and cell arrangement
		Shape	Edge	Elevation	Color		
1.	NF 1	Circular	Entire	Raised	White	-	Basil
2.	NF 3	Circular	Entire	Flat	Krem	+	Streptobasil
3.	NF 4	Irregular	Irregular	Flat	Milky white	+	Basil
4.	NF 5	Circular	Entire	Flat	Beige	+	Streptobasil
5.	NF 6	Circular	Entire	Flat	Beige	+	Streptobasil
6.	NF 7	Circular	Irregular	Flat	Beige	-	Basil
7.	NF 8	Irregular	Irregular	Flat	Beige	-	Basil
8.	NF 9	Circular	Entire	Raised	Yellow	+	Streptobacil
9.	AB 2	Circular	Undulate	Flat	Light yellow	+	Mono, diplobacil
10.	HS 1	Circular	Entire	Flat	Light brown	-	Mono, diplococcus
11.	HS 2	Circular	Undulate	Convex	Milky white	-	Mono, diplococcus
12.	HS 3	Irregular	Entire	Flat	Light brown	-	Mono, diplococcus
13.	HS 4	Irregular	Entire	Flat	Milky white	-	Mono, diplococcus
14.	HS 5	Circular	Entire	Flat	Milky white	-	Mono, diplococcus
15.	HS 6	Circular	Entire	Flat	Light yellow	-	Mono, diplococcus
16.	HS 7	Irregular	Entire	Flat	Light brown	+	Mono, diplococcus
17	HS 8	Circular	Undulate	Convex	Light orange	-	Mono, diplococcus

Soil ecosystems comprised of microorganisms in soil are able to metabolize carbamate pesticides and easily adapt themselves to various forms of that pesticide. Nevertheless, pesticides and their metabolism products play an important role in the soil's microflora and productivity (Gupta et al., 2016). Parekh et al. (1994) have gathered samples from five field locations with varying carbofuran treatment histories. The chemical was hydrolyzed more quickly in all soils treated with carbofuran earlier than in samples of identical soils that had not been treated. Sixty-eight bacteria, capable of degrading carbofuran as the sole source of carbon and nitrogen, were isolated from liquid cultures of treated soils. All carbofuran-degrading isolates were gram-negative aerobic rods that broke down the carbofuran to carbofuran phenol.

Table 2. Biochemical characteristics of isolates

No.	Isolate	Types of Biochemical test					
		Starch	Gelatine	Citric	Sulfide	Motility	Catalase
1.	NF 1	-	-	+	+	-	+
2.	NF 3	-	-	-	+	+	+
3.	NF 4	+	-	-	+	+	+
4.	NF 5	+	-	+	+	+	+
5.	NF 6	-	-	-	+	+	+
6.	NF 7	-	-	-	+	-	-
7.	NF 8	-	-	-	-	+	+
8.	NF 9	-	-	-	-	+	+
9.	AB 2	-	+	+	+	+	+
10.	HS 1	-	+	-	+	+	+
11.	HS 2	-	+	-	+	+	+
12.	HS 3	-	-	-	-	+	-
13.	HS 4	-	-	-	+	+	-
14.	HS 5	-	+	-	+	-	+
15.	HS 6	-	+	+	+	+	-
16.	HS 7	-	+	-	+	+	+
17.	HS 8	-	-	-	+	+	+

All isolates that were grown in BHB medium with carbofuran as the sole carbon source grew well, as shown in Table 3. Among all isolates, NF9 showed the best growth, steadily increasing weekly. In addition to the ability to break down carbofuran as their carbon source, the other important characteristic of hydrocarbon-degrading bacteria is their capability to secrete biosurfactants. Microbial biosurfactants are low-molecular-weight surface-active compounds that are stable under several environmental conditions. Biosurfactant functions to reduce the surface tension, thus facilitating the emulsification and solubilization of highly hydrophobic pollutants (Eras-Muñoz et al., 2022). It is assumed that bacteria secrete the biosurfactant into the medium. The biosurfactant was obtained by centrifugation of the culture to separate the cell pellet from the medium. The medium is considered a biosurfactant. Biosurfactant activity could be evaluated by measuring the emulsion volume produced by the mixture of biosurfactant and hydrophobic compound, n-hexane. Data in Table 3 showed that NF9 and AB2 had the highest and second highest biosurfactant activity, with an Emulsion Index (EI24) value of 43% and 38%, respectively.

Two isolates, NF9 and AB2, were selected for further testing based on their abilities to grow best among others and their highest biosurfactant activities. These isolates showed distinct colony characteristics, in which the NF9 isolate has a flat elevation and a white color, while the AB2 isolate has a raised elevation and a yellow color. They share a common form, margin, Gram+ staining, and streptobacillus arrangement. Based on molecular identification of 16S rRNA analysis, NF9 and AB2 isolates were closely related to *Bacillus thuringiensis* strain KH 168 with a percent identity of 99.73% and *Bacillus paranthracis* strain C9 with a percent identity of 99.54%, respectively. The phylogenetic structure of these strains is shown in the following figure. Subsequently, *Bacillus thuringiensis* NF9 and *Bacillus paranthracis* AB2, with accession numbers SUB14158312 NF9 PP152281 and SUB14158312 AB2 PP152282, respectively, are used as the strains of our isolates.

Table 3. Growth of isolates on carbofuran and the activity of their biosurfactant

No.	Isolate Code	Total Bacterial Cells (10^7 cells/ml)			Biosurfactant Activity (% Emulsion Index/EI_{24})
		Day 7th	Day 14th	Day 21st	
1.	NF 1	2	58	78	-
2.	NF 2	8	38	58	38
3.	NF 4	4	25	17	18
4.	NF 5	9	25	25	36
5.	NF 6	48	49	50	39
6.	NF 7	2	12	12	-
7.	NF 8	4	118	43	24
8.	NF 9	8	48	86	43
9.	AB 2	4.9	22	18	38
10.	HS 1	2	12	6	27
11.	HS 2	2.5	18.6	10.4	29
12.	HS 3	5	16	10.9	29
13.	HS 4	1.6	8.2	9.6	26
14.	HS 5	1.5	4	3.2	-
15.	HS 6	2.8	16	18.8	38
16.	HS 7	7.8	11.5	6.5	24
17.	HS 8	5.2	14.5	16	39

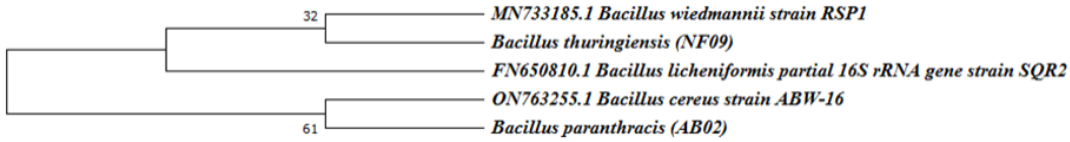

Figure 1. Phylogenetic tree of NF9 and AB2 isolates. The neighbor-joining tree is based on the 16S rRNA sequence, demonstrating the phylogenetic position of each strain.

Pesticide biodegradation by microorganisms is not new. Microorganisms simply supply all the required energy sources for simple chemical reactions to take place (Pandey et al., 2010). Many groups of microorganisms are characterized by the growth and degradation abilities of pesticides (Ishag et al., 2016). Isolation and characterization of microbes for pesticide degradation bring about new tools to restore environments polluted with pesticides. Several microbial species capable of degrading pesticides, such as *Pseudomonas*, *Flavobacterium*, *Achromobacterium* sp., *Sphingomonas* sp., *Arthrobacter*, and *Bacillus* species, have been isolated and characterized in an effort to know their mechanisms for removing pesticides. Various strains of *Bacillus* have been isolated from different types of contaminated soil as well as contaminated water. *B. amyloliquefaciens* FZB42, *Bacillus* sp. NH 217, and *B. subtilis* NH-100 showed high biosurfactant activities, and *B. atrophaeus* 176s lipopeptide biosurfactant, in addition to its very good activity, also showed antifungal activity (Sarwar et al., 2018). To improve further application of those isolates, the bacteria were encapsulated in sodium alginate and polyurethane.

3.2. The effectiveness of cell immobilization

The effectiveness of cell immobilization in alginate and polyurethane is shown in Table 4.

Table 4. The average number of cells immobilized in alginate and polyurethane

Bacterial Species	Number of cells (CFU) ml^{-1}		
	ml^{-1} cell suspension	g^{-1} alginate	g^{-1} Polyurethane
***B. thuringiensis* NF9**	2.08×10^{13}	6.60×10^{13}	1.25×10^{12}
***B. paranthracis* AB2**	4.07×10^{13}	1.94×10^{13}	6.60×10^{12}

The number of cells entrapped in alginate was statistically higher than that in the cell suspension. It is assumed that the bacterial cells were entrapped more easily in sodium alginate beads compared to the attachment of the cells to the polyurethane surface. Similarly, *B. thuringiensis* NF9 cells, which were

entrapped in alginate, were also significantly higher than *B. paranthracis* AB2. Conversely, there was no significant difference in the number of cells entrapped in polyurethane between the two strains. For almost thirty years, there has been interest in the immobilization of entire microbial cells and their applications to bioprocessing. In order to produce extracellular enzymes, whole cells can be immobilized. This has a number of advantages, including the ability to easily extract the cell mass from the bulk liquid for potential reuse, the ability to operate continuously for extended periods of time, increased reactor productivity, and increased catalysis efficiency (Kar and Ray, 2008).

A recent study reported by Jeon et al. (2019) stated that immobilization using a polyvinyl alcohol-sodium alginate matrix bead resulted in outstanding porosity, chemical stability, and mechanical strength. Moreover, based on the topology of the beads in the SEM image, Jeon et al. (2019) reported that the pores' coarse and irregular appearance will improve their specific surface area and interaction potential.

The number of bacterial cells that were released into the medium during incubation was observed and compared to the number of cells that remained in the matrix after incubation to evaluate the stabilization of cell immobilization. The result showed that the number of cells that were released into the medium remained stable (Table 5).

Table 5. The average number of bacterial cells encapsulated within sodium alginate which is released into the medium during incubation

Bacterial Species	**Number of cells which is released into medium (CFU) ml^{-1}**		
	Day 5th	Day 10th	Day 15th
***B. thuringiensis* NF9**	2.08×10^{13}	6.45×10^{12}	9.77×10^{12}
***B. paranthracis* AB2**	7.94×10^{12}	3.89×10^{12}	3.80×10^{11}

During 15 days of incubation, the number of cells that were released from the beads varied between the two isolates. *B. paranthracis* AB2 showed a continued decrease in the number of cells, while *B. thuringiensis* NF9 showed a slightly increasing cell population at the end of incubation. However, both strains showed that there was no statistically significant difference in cell numbers released into the medium on days 5, 10, and 15. As the incubation time proceeded, the beads absorbed some water and appeared to be a little bit swollen (*B. thuringiensis* NF9). This caused more bacteria to be released into the medium, but other beads of *B. paranthracis* AB2 remained intact. The stability of the interaction between bacterial cells and alginate beads is affected by the chemical characteristics and composition of the alginate beads and the bacterial cells. It was reported that the combination of alginate and other compounds such as active carbon, biochar, and cornstalk cubes improves the surface area and pore distribution of alginate beads (Fareed et al., 2019; Fravel et al., 1985; Li et al., 2020). The pores protect bacteria from harsh environments that could harm the cells. Furthermore, Jeon et al. (2019) noted that keeping an eye on the beads' structural integrity was necessary to monitor their ability to function properly and provide a sign of any deterioration. Other studies reported that after 102 days of use, alginate beads became transparent and their shape began to alter from spherical to round (Damayanti et al., 2021). It is thought that the bead's damage was caused by calcium ions, which dissolved over time and left the bead translucent, mushy, and partially fractured. The beads eventually break and become more brittle (Hu and Chen, 2007).

Table 6. The average number of bacterial cells entrapped in polyurethane which is released into the medium during incubation

Isolate	**Number of cells which is released into medium (CFU ml^{-1})**		
	Day 5th	Day 10th	Day 15th
***B. thuringiensis* NF9**	3.63×10^{13}	1.99×10^{12}	6.02×10^{9}
***B. paranthracis* AB2**	5.12×10^{12}	1.34×10^{13}	1.34×10^{13}

Unlike cell release from alginate beads, in polyurethane, cell release of *B. thuringiensis* NF9 decreased steadily, while *B. paranthracis* AB2 remained stable. Statistically, there was no significant difference in the number of released cells during incubation time, except for strain NF9 on day 5. During five days, the average number of cells showed the highest, 3.63×10^{13} CFU/ml.

Alginates are the polymers of choice in most systems of immobilization because they are easy to handle, nontoxic to humans, the environment, and entrapped microorganisms, legally safe for human use, available in large quantities, and inexpensive. From a physiological perspective, a major advantage of alginate is that immobilized cells do not suffer extreme changes in physicochemical conditions during the procedure of immobilization, and the gel is transparent and permeable (Buque et al., 2002). Organic carriers are such as modified celluloses, dextran, and chitosan agarose (Lu and Toy, 2009).

Despite providing high mechanical strength, cell immobilization on polyurethane showed some disadvantages, such as cell leakage and releasing cells into the medium. Moon et al. (2020) said that bacterial cell entrapment in polyurethane foam was not effective for thermal stabilization presumed to be due to the poor direct covalent linkage of the enzyme to the polyurethane matrix.

At the end of incubation, the number of cells retained in the beads was still high. Those results demonstrated that alginate beads served as an excellent matrix for cell immobilization. One advantage of applying cell immobilization is the possibility of using the beads several times without reducing the ability of cells to undergo pesticide degradation. A study by Soo et al. (2017) showed that increasing concentrations of alginate and $CaCl_2$ enhanced the stability of beads for up to 15 days in a vigorous shaking incubator. Furthermore, combining sodium alginate with chitosan extends the reusability of the beads up to 10 times in oil waste treatment (Jeon et al., 2019).

Table 7. The average number of bacterial cells retained in the sodium alginate beads and polyurethane after incubation

Isolates	**Number of cells (CFU ml^{-1})**			
	At the initial incubation		At the end of incubation	
	Alginate	Polyurethane	Alginate	Polyurethane
B. thuringiensis NF9	6.60 x 10^{13}	1.25 x 10^{12}	1.64 x 10^{11}	7.58 x 10^{10}
B. paranthracis AB2	1.94 x 10^{13}	6.60 x 10^{12}	9.50 x 10^{10}	2.88 x 10^{11}

At the end of the incubation, the number of bacterial cells in polyurethane was still relatively high. Statistically, there was no significant difference between the initial number of cells and the number of cells at the end of incubation, except for the number of *B. thuringiensis* NF9 cells in alginate beads. In this case, the average number of cells decreased significantly from 6.60×10^{13} to 1.64×10^{11}. It indicated that polyurethane foam provides excellent support for bacterial cells to attach. Due to its excellent mechanical characteristics, high porosity, and substantial adsorption surface, polyurethane foam has recently gained significant relevance as a carrier. Furthermore, it is cost-effective (De Ori et al., 2020). Compared to *B. thurungiensis* NF9, *B. paranthracis* AB2 showed a higher cell population. It was assumed that *B. paranthracis* AB2 had a better, stronger interaction between the bacterial cell and the polyurethane matrix. It was assumed that *B. parathracis* AB2 had a better, stronger interaction between the bacterial cell and polyurethane. Figure 2. shows how *B. parathracis* AB2 cells attached randomly to the polyurethane matrix.

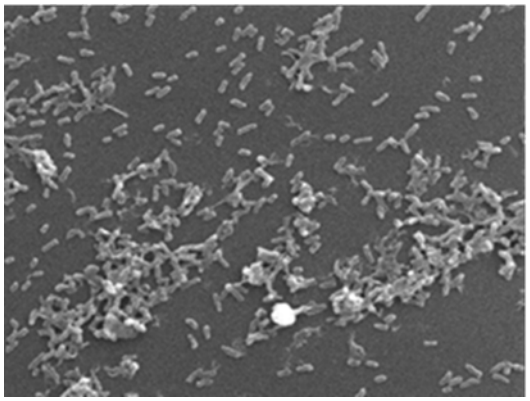

Figure 2. The attachment of *Bacillus parathracis* AB2 on polyurethane foam.

It is assumed that cell wall hydrophobicity enhanced the adhesion of bacterial cells to polyurethane foam. Jeon et al. (2019) reported that SEM imaging of *Acinetobacter* and *Paenibacillus* that were entrapped in sodium alginate showed that the shape of bacteria affected the interaction between bacteria and alginate. It was said that *Paenibacillus*, which is rod-shaped, is more easily entrapped or absorbed during immobilization compared to *Acinetobacter*, which is coccobacillus-shaped. Matshui and Tomohiko (2017) reported that *Brevibacterium ketoglutamicum*, which is immobilized in polyurethane, was able to degrade n-tetradecane in repeated batches up to 300 h with only a slight loss of activity.

3.3. Carbofuran degradation

Figure 3. shows the results of carbofuran degradation by two isolates in the form of immobilized cells as well as free cell suspensions. The result showed that all isolates were capable of degrading carbofuran completely after 15 days of incubation. Meanwhile, there was still 18.71 ppm (55.3%) of carbofuran in the control. No residue of carbofuran could be detected after 5 days of incubation when the carbofuran was treated with *B. parantraxis* AB2 as immobilized cells in alginate beads. Meanwhile, the residue of carbofuran was undetected after 15 days of incubation by those two isolates in all conditions. Based on the data of the number of cells retained within alginate beads, both strains of isolates showed almost the same population, which was $\pm 10^{10}$ cells ml^{-1}. It indicated that the *B. parantracis* AB2 strain had much better activity in carbofuran degradation. The result indicated that immobilized cells within alginate beads degraded carbofuran better than its free cell suspension. A study on carbofuran biodegradation by *Bacillus* sp. strain DT1, a soil bacterium, showed a similar result. When the isolate was immobilized in rice straw, it was capable of decreasing pesticide concentrations up to 97.5%, which was 19.8% higher than its free cell suspension (Duc, 2022).

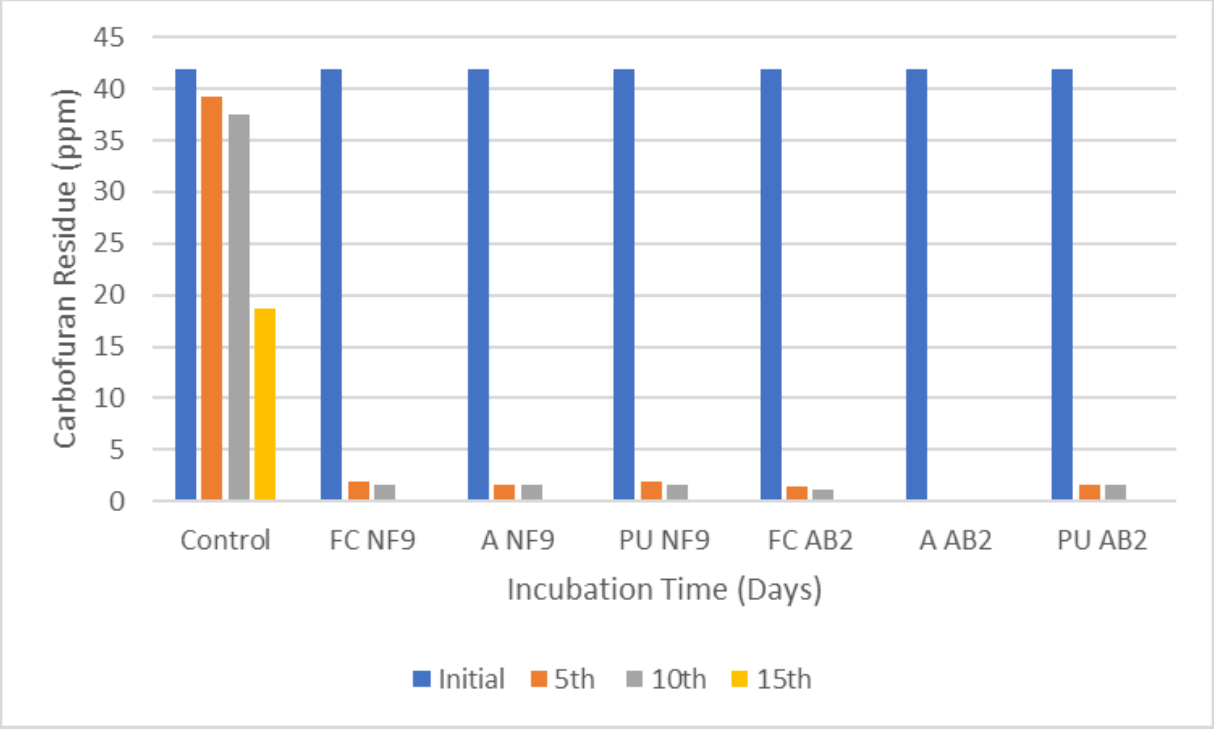

Figure 3. The residual of carbofuran after being incubated with bacterial cells in the form of free cells and immobilized in alginate beads and polyurethane. FC: Free Cells; A NF9, A AB2: Alginate NF9 and Alginate AB2; PU NF9, PU AB2: Polyurethane NF9 and Polyurethane AB2.

At the end of incubation, the residue of carbofuran in the negative control was 18.71 ppm. It meant that carbofuran underwent natural degradation up to 52.4%. When pesticides reach vegetation, soil, aquatic environments, or even the air, they can be degraded spontaneously by photooxidation. Herbicides like 2,4-d bromoxynil will be degraded completely in the presence of sunlight. Chemical

degradation through oxidation-reduction, hydrolysis, and ionization were other examples of natural degradation. Those processes might occur in aquatic environments and are mostly closely related to environmental pH. The strong acid or base environment inhibits the growth of microorganisms or inhibits the enzymes of microorganisms. Such conditions favor the chemical degradation of pesticides (Morel-Cheville et al., 1996).

In general, *B. paranthracis* AB2 degraded carbofuran higher than *B. thuringiensis* NF2 in all conditions, as a free cell suspension immobilized within alginate as well as in polyurethane. Comparing the matrix of immobilization, the result showed that alginate is a better carrier for both isolates and is better than their free cell suspension. On the contrary to immobilization by alginate, *B. paranthracis* AB2 immobilized on polyurethane showed lower degradation ability compared to its free cell suspension. It was assumed that the ability of *B. paranthracis* AB2 to immobilize in polyurethane was not as efficient as in alginate. With a total population of 9.50 x 10^{10} cells per g of alginate, it could degrade carbofuran completely on day 5 of incubation. Meanwhile, the bacterial cells entrapped in polyurethane were 10^{12} cells g^{-1}. Polyurethane at initial was much higher than that in alginate, which was $\pm 10^{10}$ cells g^{-1}. Likewise, the number of cells released into the medium ($\pm$ 10^{13} cells ml^{-1}) was significantly higher than in the alginate medium. Various species of bacteria have been reported to degrade carbofuran. Gupta et al. (2019) reported that *Cupriavidus* sp. ISTL7 degraded carbofuran efficiently, 98% in 96 hours. As in *Pseudomonas*, the bacteria break the chemical compound carbofuran into carbofuran-7 phenol and methyl amine by producing EPS such as glucose, xylose, sorbitol, and fructose.

Conclusion

Two local bacterial isolates capable of degrading carbofuran, *Bacillus thuringiensis* NF9 and *B. paranthracis* AB2, have been isolated from Belawan Port and Berastagi agricultural land. The isolates were immobilized in alginate beads and polyurethane. The immobilization processes were highly efficient, in which the number of cells reached $\pm$ 10^{13} cells g^{-1} alginate and 10^{12} cells g^{-1} polyurethane. The immobilized cells showed very good stability; the number of cells in the polyurethane matrix was about the same between the initial and after 15 days of incubation, allowing them to be applied on a larger scale. Both strains, in the form of free cells as well as immobilized cells, degraded carbofuran within 15 days of incubation. The strain *B. paranthracis* AB2, which was immobilized in alginate, was able to hydrolyze carbofuran completely, even much faster, within 5 days of incubation. This study suggests that both local strains have the potential to be further developed as agents of bioremediation, not only against carbofuran but also other hydrocarbon compounds. Furthermore, this study allows the possibility of using polyurethane as plastic waste to be utilized as a matrix of immobilization.

Ethical Statement

Ethical approval is not require for this study because no animal is used.

Conflict of Interest

There are no known conflicts of interest associated with this publication.

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Author Contributions

Nunuk Priyani: Material and equipment engagement, literature search, monitored search, data analysis and experimental development and wrote manuscript.

Edison Purba: Designed research methodology and data analyses.

Dwi Suryanto: Designed research methodology, conducted field sampling and data interpretation.

Erman Munir: Conceived the original idea, design the study and review and approval of manuscript.

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Research Article

An Ethnobotanical Survey, Pharmacognostic Profile and Phytochemical Analysis Investigation of *Chrysobalanus icaco* L.

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Chrysobalanusicaco, *Chrysobalanus icaco* seeds, Ethnobotanical survey, Pharmacognosy, Phytochemical investigation

Abstract: The spice known as *Chrysobalanus icaco* L. (Chrysobalanaceae) was found near the coast of the Niger Delta in Nigeria. A survey on the ethnobotany of *C. icaco* seeds was conducted in Warri, Abraka, Delta State, Ezetu village, and Onitsha. Standard techniques were used to determine the pharmacognostic profile, phytochemical screening, physiochemical parameters, and elemental analysis. The findings showed that the Ezetu village people utilized the seeds traditionally for stomachache, anti-diarrhea, and post-child delivery. Histochemical analysis revealed the presence of tannins and proteins in the seed. The seed powder's physicochemical parameters are as follows: pH (1 g/100 ml distilled water) (6.00±0.00) and pH (10 g/100 ml), water-soluble ash (1.5±0.00%), alcohol soluble ash (1.0±0.00%), acid insoluble acid (0.25±0.00%), acid soluble ash (1.0±0.00%), and sulfated ash (2.0±0.00%). The seed oil's physicochemical properties were: density (0.936±0.0%), refractive index (1.491±0.00%), iodine value (15.9±0.01%), peroxide value (25.31±0.01%), acid value (29.44±0.00%), and ester value (34.75±0.21%). The elements discovered in the seed included lead, copper, nickel, chromium, cadmium, potassium, sodium, calcium, phosphorus, magnesium, iron, and zinc. Phytochemicals found in the extract include reducing sugars, proteins, amino acids, fats, oils, alkaloids, tannins, phenolic compounds, flavonoids, cholesterol, steroids, terpenoids, triterpenoids, phytosterols, saponins, and cardiac glycosides. Quantitative phytochemical results include total phenolics (11.63±0.03), total flavonoids (2.35±0.06), total alkaloids (5.50±0.03), and total tannins (12.48±0.01). Consequently, it is possible to verify the authenticity of the seeds using these pharmacognostic features.

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1. Introduction

Ethnobotanical study together with phytochemical screening, pharmacognostic properties, and bioactive activities of plants is a convincing procedure for the identification of chemical constituents

from medicinal plants. New medications from medicinal plants can be identified rationally by combining phytochemical screening, pharmacognostic properties, and ethnobotanical documentation (Hammad et al., 2017; Ohemu et al., 2024). The survival of folk medicine relies majorly on the variety of medicinal plants and the related ethnobotanical documentation mode of preparation and use (Joseph et al., 2019)

Pharmacognostic profiles provide plant identification and authenticity, guard against adulteration, and guarantee repeatable quality, all of which improve the effectiveness and safety of herbal medicines (Sumitra, 2014).

Chrysobalanus icaco is a plant that belongs to the Chrysobalanaceae family. It is commonly called paradise plum. In Brazil, this species is called the Abajurú (De Aguiara et al., 2017). According to Silva et al. (2017), *Chrysobalanus icaco* is popular in the Caribbean, South Florida, Central America, Northwestern South America, and tropical West Africa. According to Erhenhi et al. (2016), the Ijaws in Bayelsa State, Nigeria, refer to it as "Ebulo," while Iteskiri in Delta State calls it "Omilo." *C. icaco* in Yoruba is locally known as Amukan, Awọnrinwán, Ikat, and Elewu (Janick et al., 2008; Ogunka-Nnoka, 2008; Onilude et al., 2020; CABI, 2023).

Chrysobalanus icaco is used in folk medicine to treat diabetes, malaria, chronic diarrhea, bleeding, infections, inflammation, dyslipidemia, and leucorrhoea. It is also used as a diuretic agent as well as possesses antioxidant activity and antiangiogenic effects (Vargas Simon et al., 1997; De Paulo et al., 2000; Presta et al., 2007; Silva et al., 2008; Ferreira-Machado et al., 2014; De Aguiara et al., 2017; Venancio et al., 2018).

Chrysobalanus icaco plant possesses a high level of phytochemicals such as polyphenols which include steroids, triterpenoids, alkaloids, saponins, tannins, phenolic acids such as flavones, and flavonoids**;** as reported by de Oliveira Barbosa et al. (2013), Silva et al., 2017, Onilude et al. (2020).

Literature search revealed that limited ethnobotanical studies have been conducted on the *C. icaco* plant. Also, comprehensive preliminary phytochemical screening and pharmacognostic profile have not been carried out on the seed *C. icaco*. Therefore, this study aimed to conduct an ethnobotanical survey on *C. icaco* seed and carry out phytochemical screening and pharmacognostic profile on the seed of *C. icaco*.

2. Material and Methods

2.1. Ethnobotanical survey on *C. icaco* seeds

A systematic questionnaire was used to perform an ethnobotanical survey on the traditional applications of *C. icaco* seeds among the local people in Ezetu village, Warri, Onitsha, and Abraka in the Southern region of Nigeria. The questionnaire gathered primary data on the seed, including its toxicity, preparation method, route of administration, and traditional medicinal uses. Pidgin English, Ijaw, Urhobo, and Itsekiri were the common languages used to interview respondents. All pertinent data was then translated into English language and recorded.

2.2. Ethical consideration

The World Medical Association's Declaration of Helsinki (Percie et al., 2020) was followed when conducting the study. Ethical permission was requested (using reference number REC/FBMS/DELSU/22/146) to the College of Health Sciences Ethical Approval Review Committee at Delta State University in Abraka, Nigeria. Enrollment in the research was entirely voluntary.

2.3. Collection and preparation of the seeds

The dry seeds of *C. icaco* were purchased from Relief Market, Onitsha, Anambra State, Main Market, Abraka, Delta State, Ogbe-Ijaw Market, Warri, Nigeria. The seed was authenticated in the Department of Botany, Delta State University, Abraka, with a voucher number (DELSUH-209), and a voucher specimen was deposited in the herbarium. The seeds were allowed to air dry for several days. The seeds were dehulled and crushed with a mortar and pestle to a coarse consistency. Finally, the coarse powder was ground into fine powder form using an electric blender that had been dried and sterilized. Using a 2.0 mm sieve, the dried powdered seeds were sieved and then kept in an airtight sterile container.

2.4. Organoleptic/macroscopic evaluation of *C. icaco* seeds

According to the African Pharmacopoeia (1986), the World Health Organization (1998), and Wallis (2005), the seeds were macroscopically evaluated for color, shape, size, surface characteristics, odor, appearance, and taste.

2.5. Histological study of *C. icaco* seeds

The histology of the seeds with hard seed coats was done according to the guidelines provided by Ribeiro (2014) and Yusuf (2015).

2.6. Histochemical analysis *C. icaco* powder

After boiling the seed powder for ten minutes with chloral hydrate, the mixture was stained with phloroglucinol, hydrochloric acid, safranin, glycerin, Million's reagent, Sudan IV, and iodine solution, respectively. The mixture was examined under a microscope to check for starch grains, calcium oxalate crystals, lignified cells, etc. (Kokate, 2003; Mali, 2017).

2.7. Physicochemical parameters of *C. icaco* seeds

As stated by (Vilash et al., 2016; Magbool et al., 2018), physicochemical parameters such as pH, moisture content, total ash, acid-insoluble ash, water-soluble ash, alcohol soluble ash, extractive value, crude lipid content, crude protein, crude carbohydrate, heavy metals, and minerals were carried out on the powdered sample. Peroxide value, acid value, saponification value, iodine value, and refractive index were among the physicochemical characteristics that were measured for the seed oil, as stated by (Warra et al., 2011; Aremu, 2014; Zahir et al., 2014).

2.8. Extraction of seed material

Five hundred (500 g) dried seed powder was extracted with ethanol (70%) using a Soxhlet extractor. The extract filtrate was concentrated using a rotary evaporator.

2.9. Phytochemical screening of *C. icaco* seeds

Qualitative and quantitative phytochemical screening of *C. icaco* seed powder was used to identify and quantify the presence of classes of phytochemicals in the seed using the methods described by Silva et al. (2017) and Singh (2017).

2.10. DPPH (α, α-diphenyl-β-picryl-hydrazyl) radical scavenging assay

α, α-diphenyl-β-picryl-hydrazyl was done as McCune (2002) and Chandha (2009) described.

2.11. Statistical analysis

Statistical analysis of the data was carried out using one-way analysis of variance (ANOVA) to assess the significance level in the mean concentrations of parameters. All statistical analyses were done using SPSS version 16.0 (IBM Corp., USA) software for Windows. P-value of < 0.05 (95% confidence interval) was statistically significant.

3. Results

3.1. Ethnobotanical survey

The study included fifty-one (51) respondents between the ages of 20 and 65, all female (100%). Figure 1 shows locations of the respondents; seventeen (33.33%) were from Ezetu village, Ekeremor LGA of Bayelsa State; twelve (12) (23.53%) were from Ogbe-Ijaw Market, Warri South LGA of Delta State; eight (8) (15.69%) were from Relief Market, Onitsha, Anambra South LGA of Anambra State; and fourteen (14) (27.45%) were from Abraka Main Market, Abraka town, Ethiope East LGA of Delta State. In Figure 2, the occupations of the respondents were presented. Twenty-one 21 (41.8%) worked as fishermen, and 30 (58.82%) were traders who sold spices. The survey also recorded the traditional

medicinal uses of the seed, as seen in Figure 3, which include stomachache: 32 (67.75%), anti-diarrhea: 9 (17.65%), and post-child delivery: 10 (19.60%). When consumed in excess, the fruit of the seeds can cause harmful side effects such as constipation and hard stool. Nonetheless, it has been noted that particular creatures, such as birds, eat the plant or the seed fruits. According to the survey shown in Figure 4, the following modes of usage were observed to include concoction ingredients (11 (21.57%), powdery (15 (29.41%), and entire seed (25(49.02%)). According to the survey, the seed was often used with other spices to prepare pepper soup, which Iteskiris, Ijawas, and Urhobos primarily consume. In April 2023, a trip to Ezetu village was undertaken to gather the *C. icaco* plant. Ezetu village is a village in Ekeremor LGA, Bayelsa State, Nigeria.

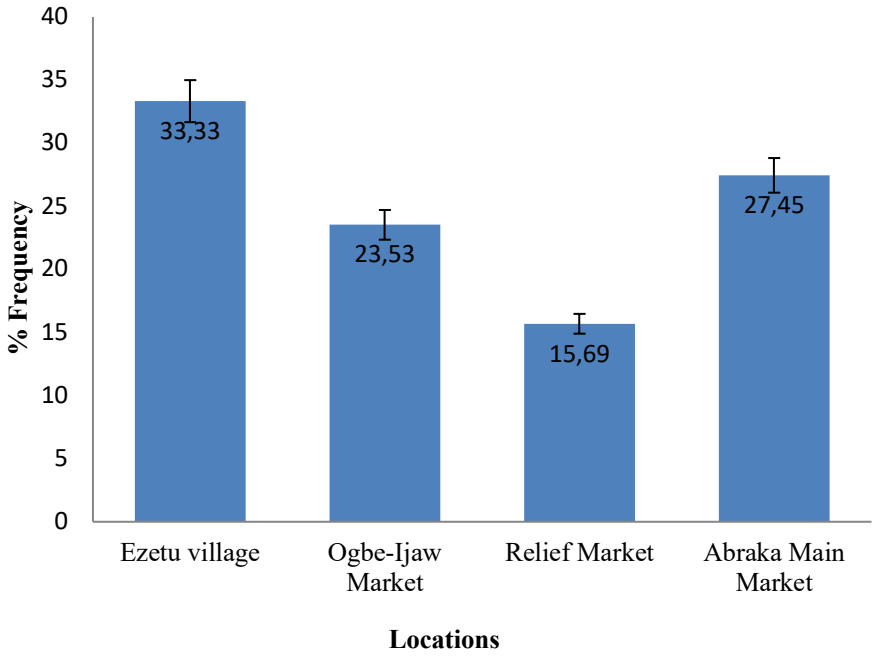

Figure 1. Showing places where the survey was carried out.

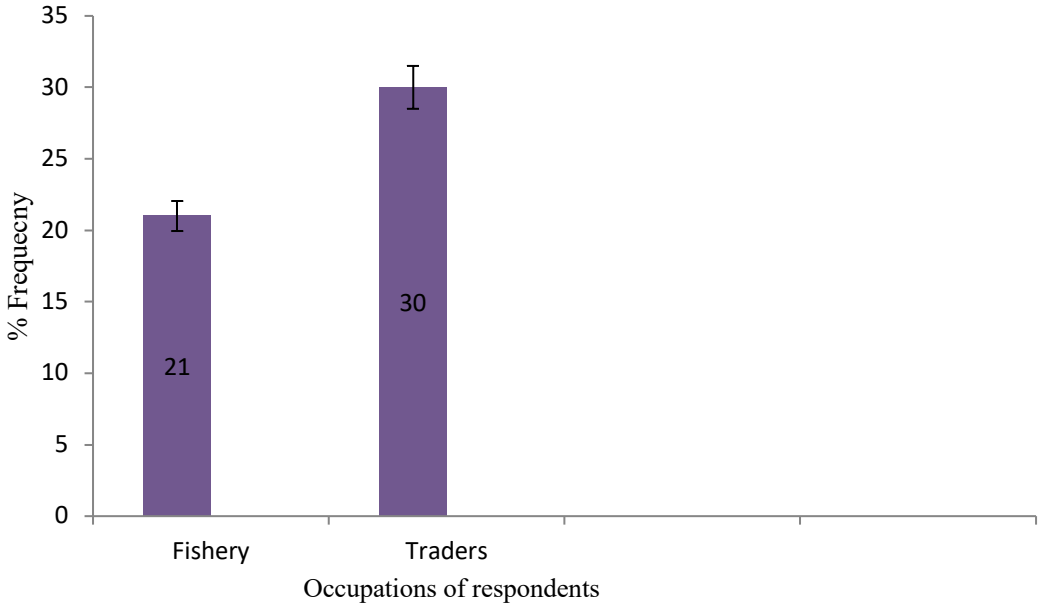

Figure 2. Showing occupations of the respondents.

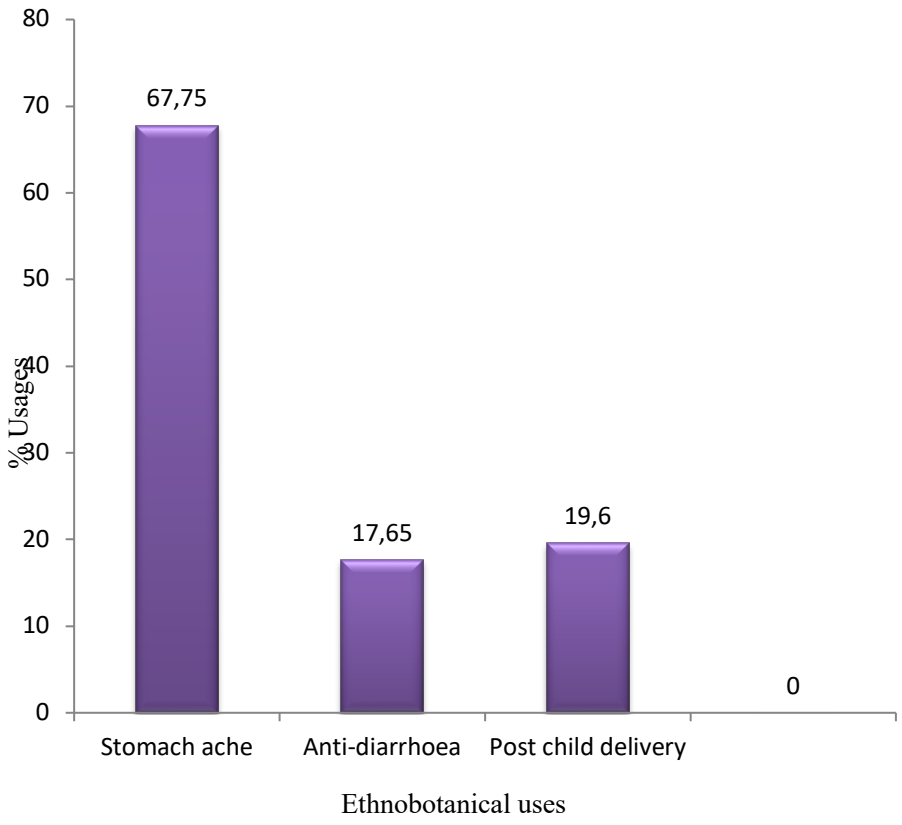

Figure 3. Showing ethnobotanical uses of *C. icaco*.

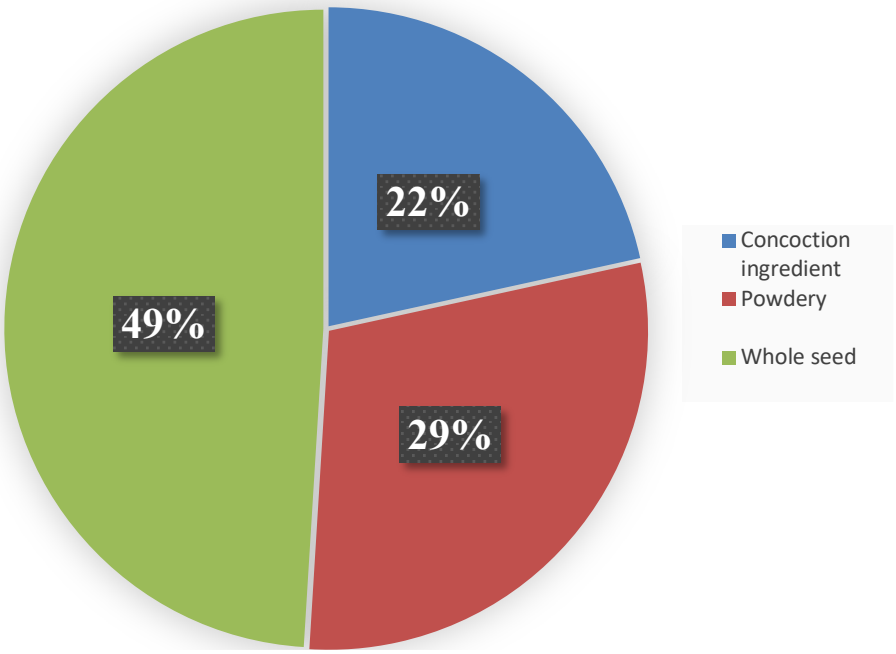

Figure 4. Showing the mode of use of *C. icaco* seeds in traditional medicine.

3.2. Macroscopic evaluation of *C. icaco* seeds

The macroscopic and organoleptic characteristics of *C. icaco* seeds and powder are shown in Table 1. The macroscopic and organoleptic characters revealed that the seed size was 7–10 mm, the shape was oblong or oval, and the fruit was pinkish when fresh, berry blue and brown when dried. The seeds appeared brown and slightly hard when the seed coat was removed. The seed coat was rough and cracked like a shell when dried. The number of seeds per fruit was one. The seed had a weakly aromatic odor and possessed a characteristic taste. The powder was greyish brown and had an aromatic odor and characteristic taste. The part of the *C. icaco* fruit is shown in Figure 4.2. The various parts of *the C. icaco* fruit include the exocarp, mesocarp, endocarp, seed coat, and seed.

Table 1. The macroscopic character of *C. icaco* seeds

Character	Observation
Seed test	
Size	7-10 mm
Shape	Oblong or oval
Color	Fruit is pinkish or blackish when fresh and black or brown when dry. The seeds appear brown when the seed coat is removed.
Odor	Weakly aromatic
Surface characteristics	The seed coat is complex, and the seed is slightly stiff when the seed coat is removed
Texture	The seed coat is rough and cracks like a shell when dry
Taste:	Characteristic
Number of seeds per fruit:	1
Powder study test	
Color	Grayish brow
Odor	Aromatic
Taste	characteristics
Filter paper test	+++

3.3. Histochemistry of *C. icaco* dry seed

After mounting the finely cut slides of the seed on the microscope, the slides were observed, and pictures of the observed features were taken and presented in Plates 1, 2, and 3 and Plate 4. Plate 1 shows the transverse section of *C. icaco* seed, showing the epidermis, endodermis, and epicarp stained with hematoxylin and eosin x 400. Ep-epidermis, en-endodermis, and ei-epicarp. Plate 2 shows a transverse section of *C. icaco* seed showing stomata seeds stained with hematoxylin and eosin x 400; plate 3 shows a transverse section of *C. icaco* seed showing schizogenous cavities; and plate four shows cork cells of a transverse section of *C. icaco* seed.

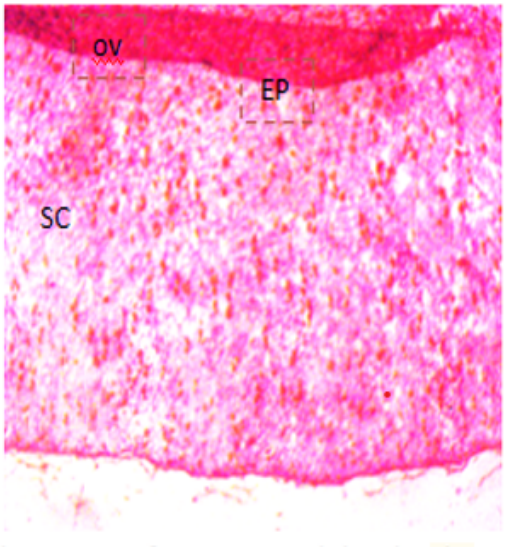

Plate 1: T. S of *c. icaco* seed showing the ovule stalk, secretory cavity, epidermis, and epicarp stained with H&E x 400. OV-Ovule stalk, SC-secretory cavity, EP-epicarp

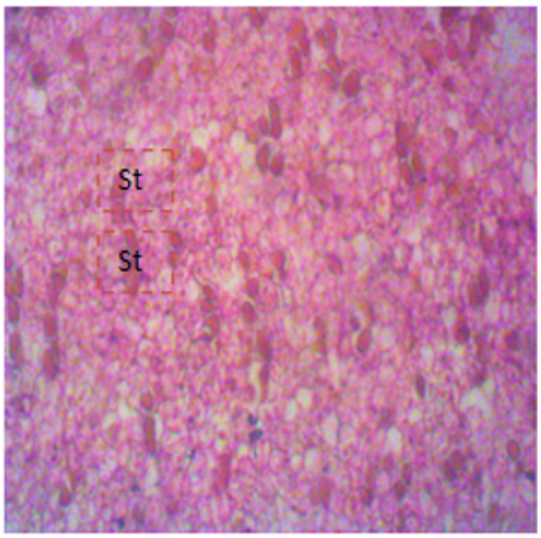

Plate 2: Transverse section of *C. icaco* seed showing stomata seeds

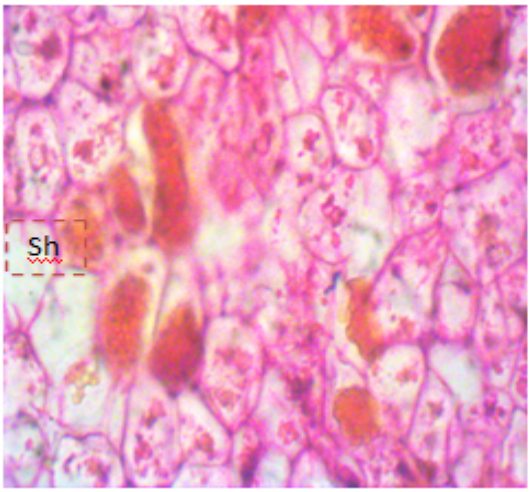

Plate 3:T.S of *C. icaco* seed showing schizogenous cavities stained with H & E x 400. Sh- Schizogenous cavities

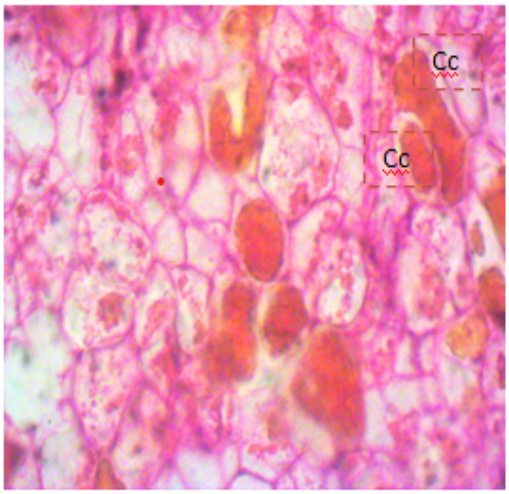

Plate 4: T.S of *C. icaco* seed showing cork cells, stained with H&E, Cc- Cork cells

Figure 5. After mounting the finely cut slides of the seed on the microscope.

3.4. Histochemical Characters of Dry Powder C. icaco Seed

The histochemical characteristics of dry powder *C. icaco* seeds are presented in Table 2. The yellow color was observed when a million reagents were added to the powder, confirming the protein's presence. A greenish color showed the presence of tannin when ferric chloride was added to the powder. No starch, blue, or black was observed when iodine and 66% H_2SO_4 were combined with iodine, which confirmed the absence of starch and cellulose, respectively. The absence of lignin was confirmed with no color change when phloroglucinol and conc. HCl was added to the seed powder.

Table 2. Histochemical characters of dry powder *C. icaco* seed

Reagent	Observation	Inference
Cell wall materials		
Sudan IV	Pinkish coloration	Oils present
66 % H_2 SO_4 + iodine	No blue-black	Cellulose absent
Phloroglucinol + conc.HCl	No red color	Lignin absent
Cell constituents		
80 % H_2SO_4	No crystals	Calcium oxalate absent
Glycerol + 5 % acetic acid	No evolution of gas	$CaCO_3$ absent
Million's reagent	Yellow colour	Protein present
Picric acid	Yellow colour	Protein present
Ferric chloride	Greenish color	Tannin present
N/50 iodine	No blue-black	Starch absent
Safranin	Black present	Nuclei present
Fast green	No purple or red stain	

3.5. Fluorescence analysis of dry powder of C. icaco seeds.

Table 3 presents the results of the fluorescence analysis observed in daylight and UV light. The fluorescence results for the *C. icaco* powder treated with different chemicals for daylight (UV-365 and UV-254 nm) are summarised as follows: HCl (green), (black), and (brown); HNO3 (green), (black), (brown); Acetic acid (yellow), (black), (yellow); Aqueous (prick red), (dark brown), (dark brown); NaOH (orange), (black), (black); Ammonia (brown), (dark brown), (yellow); FeCl3 (yellow), (dark brown), (green); Iodine (green), (black indigo), (brown); N-hexane (brown), (dark brown), (violet); Ethyl acetate (orange), (indigo), (brown); Butanol (orange), (indigo), (brown); Ethanol (orange), (indigo), (brown); Methanol (orange), (indigo), (brown); and Chloroform (colorless), (brown), (brown).

Table 3. Fluorescence analysis of dry powder of *C. icaco* seeds

Extract	Ordinary	UV-345 nm	UV-254 nm
HCl	Green	Black	Brown
HNO_3	Green	Black	Brown
Acetic acid	Yellow	Black	Yellow
Water	Prick red	Dark brown	Dark brown
NaOH	Orange	Black	Black
Ammonia	Brown	Dark brown	Yellow
$FeCl_3$	Yellow	Dark brown	Green
Iodine	Green	Black indigo	Brown
N-hexane	Brown	Dark brown	Violet
Ethyl acetate	Orange	Indigo	Brown
Butanol	Orange	Indigo	Brown
Ethanol	Orange	Indigo	Brown
Methanol	Orange	Indigo	Brown
Chloroform	Colourless	Brown	Brown

3.6. Physiochemical and proximate parameters of C. icaco seeds dried powder

Physiochemical and proximate parameters of *C. icaco* seeds dried powder in percentage are shown in Table 4. The results were moisture content (3.18±0.0%), crude protein (5.29±0.01), crude fat (9.53±0.01), crude fibers (2.54±0.01), crude carbohydrates (54.26±0.01), pH (1 g/100 ml distilled water) (6.00±0.00) and pH (10 g/100 ml distilled water) (5.57±0.007), ethyl acetate (10.0±5.29 %), *n*-hexane (10.0±2.00%), dichloromethane (14.67±1.16%), butanol (8.0±2.00%), water (4.0±0.00%), methanol (5.33±1.15%), ethanol (7.3±1.16%), water-soluble ash (1.5±0.00%), alcohol soluble ash (1.0±0.00%), acid insoluble acid (0.25±0.00%), acid soluble ash (1.0±0.00%) and sulfated ash (2.0±0.00%).

Table 4. Physiochemical and proximate parameters of *C. icaco* seeds dried powder

Physiochemical parameters	Composition (%)
Moisture content	3.18±0.00
Crude carbohydrates	54.26±0.01
Crude fat	9.53±0.01
Crude protein	5.29±0.01
Crude fiber	2.54±0.01
pH (1 g/100 mL distilled water)	6.00±0.00
pH (10 g/100 mL distilled water)	5.57±0.007
Extractive value	
Ethyl acetate	10.0±5.29
n-hexane	10.0±2.00
Dichloromethane	14.67±1.16
Butanol	8.0±2.00
Water	4.0±0.00
Methanol	5.33±1.15
Ethanol	7.3±1.16
Total ash	
Water soluble ash	1.5±0.00
Alcohol soluble ash	1.0±0.00
Acid insoluble acid	0.25±0.00
Acid soluble ash	1.0±0.00
Sulfated ash	2.0±0.00

*Mean value of three determinations. ($P < 0.05$).

3.7. Physiochemical analysis of ethanol of *C. icaco* seeds oil

The physicochemical parameters of the ethanol extract of *C. icaco* seed oil were determined using standard guidelines. The results were preset in Table 5 below as follows: refractive index (1.491±0.00%), density (0.936±0.0%), peroxide value (25.31±0.01%), iodine value (15.9±0.01%), acid value (29.44±0.00%), and ester value (34.75±0.21%).

Table 5. Physicochemical analysis of oils obtained from *C. icaco*

Physiochemical parameter	Composition (%)
Refractive index	1.491±0.00
Density	0.936±0.0
Peroxide value	25.31± 0.01
Iodine value	15.9±0.01
Acid value	29.44±0.00
Ester value	335.75±0.21
Saponification value	349.1±0.28

*Mean value of three determinations. ($P < 0.05$).

3.8. Elemental content composition of C. icaco seeds dried powder

The elemental analysis of the dry powder of *C. icaco* seeds showed the presence of potassium (1.0692 ppm), sodium (2.1095 ppm), calcium (1.5240 ppm), phosphorus (0.2018 ppm), magnesium (2.996 ppm), manganese (0.3163 ppm), iron (0.3159), and heavy metals such as zinc (1.1098 ppm), lead (0.0138 ppm), copper (1.4509 ppm), nickel (1.1040 ppm), chromium (1.2023 ppm), and cadmium (0.1788 ppm), as summarised in Table 6.

Table 6. Elemental content composition of *C. icaco* seeds dried powder

Metal	Conc. in sample (ppm)	FAO/WHO (1984) limit (ppm)

Potassium	1.0692	NA
Sodium	2.1095	NA
Calcium	1.5240	NA
Phosphorus	0.2018	NA
Magnesium	2.996	NA
Manganese	0.3163	NA
Iron	0.3159	NA
Zinc	1.1098	NA
Copper	1.4509	NA
Nickel	1.1040	NA
Chromium	1.2023	NA
Lead	0.0138	10.0
Cadmium	0.1788	0.30

For edible plants, ppm: Parts per million. NA: Not applicable (Obiajunwa et al., 2002; Abdulkadir et al., 2023; Karahan, 2023).

3.9. Qualitative phytochemical screening of ethanol extracts of *C. icaco* seeds

The qualitative phytochemical analysis of the ethanol extract of *Chrysobalanus icaco* seeds showed it contains alkaloid, tannin, phenolic compounds, flavonoid, cholesterol, steroid, terpenoid, triterpenoids, phytosterol, saponin, cardiac glycoside, carbohydrate, reducing sugars, proteins, amino acids, fats, and oil, as summarised in Table 7.

Table 7. Qualitative phytochemical screening of ethanol extracts of *C. icaco* seeds

Phytochemicals	**Extract**
Alkaloids	+++
Phlobatannins	++
Tannins	++
Phenolic compounds	++
Flavonoids	++
Cholesterol	+
Terpenoids	+
Triterpenoids	+
Phytosterols	++
Saponins	+++
Cardiac glycosides	++
Carbohydrates	++
Reducing sugars	+++
Proteins and amino acids	+
Detection of fats and oils	+++
Coumarins	++

Key: += low, ++ = moderate, +++ = high.

3.10. Quantitative phytochemicals screening of ethanol extract of C. icaco seeds

The results for the quantitative phytochemicals present in *C. icaco* were total phenolics (11.63±0.03), total flavonoids (2.35±0.06), total alkaloids (5.50±0.03), and total tannins (12.48±0.01), which are presented in Table 8.

Table 8. Quantitative phytochemicals screening of ethanol extract of *C. icaco* seeds

Bioactive compounds	**Standard (mg Gallic Acid Equivalents /g dry matter)**

Total phenolics	11.63±0.03
Total tannins	12.48±0.01
Bioactive compounds	**Standard (mg Quercetin Equivalents /g dry matter)**
Total flavonoids	2.35±0.06
Bioactive compounds	**Standard (AE/g dry matter)**
Total alkaloids	5.50±0.03

*Mean value of two determinations. ($P < 0.05$).

4. Discussion

Ethnobotany survey, phytochemical screening, and pharmacognostic profile of medicinal plants have been conducted by various researchers and the uses and potential of plants have been recorded (Mahajan et al., 2017, Fonseca-Kruel et al., 2020; Rakib-Uz-Zaman et al., 2020; Elhewehy et al., 2024). In this study, the seed of *C. icaco* used in traditional medicine was studied ethnobotanically, phytochemically, and phramacognostically to assess its folkloric uses, phytochemical constituents, and pharmacognostic properties

The ethnobotanical survey was conducted among communities' people who are into fishery along the coastal area in the southern region, such as Ezetu in Bayelsa State, Ogbe-ijoh in Delta State as well as local traders who deal in spices in Onitsha, Anambra State, Abraka, and Warri in Delta State. The ethnobotanical uses of the seed in the Southern region of Nigeria have revealed that the *C. icaco* plant was used for the treatment of stomachache, diarrhea, and post-child delivery. A similar ethnobotany study reported by Fonseca-Kruel et al. (2020) showed that *C. icaco* has the potential to treat diabetes. The study revealed that the seed of *C. icaco* was used in the form of whole seeds, concoction ingredients, and powdery by the local communities. The pharmacognostic properties of this plant highlighted in this review are crucial for accurate identification of the plant

The pharmacognostic profiles of this seed highlighted in this study are very vital for the proper identification of seeds. The results of the macroscopical characteristics of the seed showed that the fruits were pinkish and berry blue when fresh and brown when dried. The seed had an oval or oblong shape and measured 7–10 mm. The seeds appeared brown once the seed coat was removed. When the harsh seed coat was dried, it cracked like a shell. There is just one seed in the fruit, which is somewhat firm on the inside and has a hard seed coat. Hematoxylin staining of the *C.* icaco seed revealed stomata, cork cells, schizogenous cavities, epidermis, and epicarp in the transverse slice. The seed powder of *Chrysobalanus icaco* showed various colors after chemical treatment when exposed to UV-345 nm, UV-254 nm, and daylight.

Assessing the pH of a crude extract was a crucial first step in determining whether the powder seed could irritate the gastrointestinal tract when taken orally. The seed powder's pH values were 6.00±0.00 at 1 g/100 ml distilled water and 5.57±0.007 at 10 g/100 ml distilled water, respectively. The powder's comparatively low acidity lends credence to Franco's (1996) recommendation that it be classified as a low-acid food.

Several solvents were used to ascertain the extractive value of the powdered form of *C. icaco* seed. The highest extractive value was 14.67±1.16 for dichloromethane, followed by 10.2±5.29% for ethyl acetate, 10.0±2.00% for n-hexane, 8.0±2.00% for butanol, 7.3±1.16% for ethanol, 5.33±1.15% for methanol, and 4.0±0.00% for water.

The physiochemical analysis revealed that the powder of *C. icaco* seeds had a relatively low ash value. This implied that the *C. icaco* seeds used in the investigation were unadulterated and free of extraneous elements. The research conducted by Rajan et al. (2011), Sree et al. (2018), and Baidoo et al. (2019) are consistent with this study. *Chrysobalanus icaco* seed powder had a moisture value of 3.18 ± 0.0%. A low moisture content is evident from this. This is far less than the eight to fourteen percent (8 to 14%) maximum water content restriction for vegetable treatments (African Pharmacopoeia, 1985; Fatokun et al., 2017). Crude drugs with lower moisture content have a longer shelf life and are safe from bacterial and fungal contaminants (Anokwah et al., 2021; Pandiyan, 2022; Obia et al., 2022).

According to the outcome of the proximate analysis, the primary nutrients included in the powdered seed of *C. icaco* were crude protein (5.29±0.01), crude fiber (2.54±0.01), crude fat (9.53±0.01), and carbs (54.26±0.01).

The atomic absorption spectroscopy analysis showed that the powdered seed of *C. icaco* had thirteen (13) elemental components. Iron (0.3159 mg kg^{-1}), calcium (1.5240 mg kg^{-1}), phosphorus (0.2018 mg kg^{-1}), magnesium (2.996 mg kg^{-1}), manganese (0.3163 mg kg^{-1}), potassium (1.0692 mg kg^{-1}), and sodium (2.1095 mg kg^{-1}) were the macrominerals found. The following trace elements were discovered: arsenic (0.1020 mg kg^{-1}), chromium (1.2023 mg kg^{-1}), zinc (1.1098 mg kg^{-1}), copper (1.4509 mg kg^{-1}), nickel (1.1040 mg kg^{-1}), lead (0.0138 mg kg^{-1}), and mercury (0.1001 mg kg^{-1}). The elements were below the hazardous metals' minimal allowable limits (African Pharmacopoeia, 1986. The ethanol oil of *C. icaco* seeds was subjected to physiochemical analyses. The results obtained were refractive index (1.491±0.00), density (0.936±0.0%), iodine value (15.9±0.01), peroxide value (25.31±0.01), acid value (29.44±0.00), ester value (335.75±0.21), and saponification value (349.1±0.28) These findings were just slightly higher than the vegetable oil range of refractive indices given in the Codex Alimentarius Standard (Zhang et al., 2015). This research is similar to the findings reported by Owaba et al. (2017) and De Aguiara et al. (2017).

The oil extracted from *C. icaco* had an astonishingly high acid value of 29.44±0.00 mg KOH g^{-1} (Adegbe et al., 2016). Owaba et al.(2022) observations of lower acid levels in the dichloromethane and hexane fractions of *C. icaco* seeds contradict these findings. As per Bahl et al. (2005) report, there might be a significant hydrolysis of the glycerides indicated by the elevated acid levels. Therefore, due to the elevated acid value, direct consumption of *C.* icaco *oil* is not advisable. The oil exhibited a high degree of saponification (349.1±0.28 mg KOH). Its low impurity concentration might be the reason for this. According to Ezeuko et al. (2017), vegetables with high saponification are suitable for manufacturing paints, shampoos, soaps, and detergents. The peroxide value obtained from the physiochemical analysis of *C. icaco* seed oil was 25.31± 0.01 meq kg^{-1}. This is far higher than the permissible peroxide limits (10–15 meq/kg oil) (Olaniy et al., 1991). Olaniy et al. (1991) stated that vegetable oil with high peroxide value suggests a solid vulnerability to oxidative rancidity. The iodine value discovered in *C. icaco* seed oil was 15.9±0.01 g 100 g^{-1}. Compared to the FAO/WHO norm for edible oil (80 and 106 g 100 g^{-1}) (Orhevba et al., 2017), the iodine value of *C. icaco* seed oil is relatively low. Higher iodine values correspond to higher unsaturation and, hence, higher fluidity(Orhevba et al., 2017). The ester value was relatively high at 335.75±0.21. Belsare (2017) asserts that a high ester content indicates a high low molecular weight fatty acid content in the oil. This implies that *C. icaco* seed oil contains many low molecular weight fatty acids.

Numerous phytoconstituents were identified based on the preliminary qualitative phytochemical results. These included amino acids, proteins, carbohydrates, reducing sugar, alkaloids, saponins, tannins, terpenoids, steroids, phenolic compounds, phytosterol, cholesterol, triterpenoids, flavonoids, etc. Quantitative phytochemical research found that the *C. icaco* seed ethanol extracts had significant tannin and phenol levels but low alkaloids and flavonoids.

Conclusion

The study's findings showed that Chrysobalanus icaco has a variety of secondary metabolites, inorganic compounds, acids, crude fibers, proteins, carbohydrates, and mineral components. These pharmacognostic characteristics may be used to confirm the seed's authenticity, which is essential for foiling any adulteration attempts.

Ethical Statement

Ethical approval for this study was obtained from the College of Health Sciences Ethical Approval Review Committee at Delta State University in Abraka, Nigeria (REC/FBMS/DELSU/22/146).

Conflict of Interest

The authors declare that there are no conflicts of interest.

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There were no sources of funding.

Author Contributions

Jacinta E. Apitikori-Owumi: Conducted experiments, analyzed data, and wrote the manuscript.
Mubo A. Sonibare: Supervised the experiment and reviewed and edited the manuscript.
Marwa A. A. Fayed: Edited the manuscript.
Sayed M. Firdous: Edited the manuscript.

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Research

Effect of Transport Condition on the Structural Integrity of Ovarian Tissue and the Development of Sheep Embryos *In-Vitro*

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Abstract: The oocyte quality decreases during ovarian tissue transport to the laboratories of in vitro embryo production. To provide additional information on how the conditions of transporting sheep ovaries impact the ovarian tissue and oocytes' ability to develop into blastocyst stages, we have studied new transport media Ankara University Zootekni (AUZ1, AUZ2) supplemented with antioxidants (melatonin, Vit E, and Vit A), buffer solution, and energy substrates, and compared them with the traditional transport media: Phosphate-Buffered Saline (PBS), and Charles Rosenkrans 1 (CR1), Normal Saline (NS) at different temperatures (-6 to 30 °C). We also studied and compared how well different transport media preserve the ovarian tissue's structural integrity while transporting sheep ovaries at 4°C. Our findings indicated that various temperatures and transport media play critical roles in embryo development. The embryo development rates showed that when sheep ovaries are transported in AUZ1, they produce oocytes with a higher embryo development rate than other transport media at any temperature. In addition, histology examination revealed that the transport of sheep ovarian tissue in any medium at a temperature of 4 °C did not negatively impact the viability and histomorphology of the primordial, primary, and secondary follicles. In contrast to other transport media, the AUZ1 medium maintained the normal morphology of antral follicles, Graafian follicles, and the cumulus oophorus of sheep ovarian tissue. In conclusion, adding melatonin, buffer solution, and energy substrates to the transportation medium of ovarian tissues has a beneficial and positive role in maintaining ovarian tissue and increasing the rates of embryonic development.

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1. Introduction

The improvement of the efficiency of the in vitro embryo production (IVEP) protocols in sheep requires the use of ovaries from slaughtered animals because a significant number of oocyte samples must be obtained (García-Álvarez et al., 2011). Oocytes obtained from slaughtered animals have lower developmental potential than those retrieved from living animals (Jiao et al., 2016). Oocytes can be

harvested from live goats and sheep by ovum pick-up (OPU) methods (Wieczorek et al., 2014). However, this procedure needs technical competence and comes at a hefty price (Galli et al., 2014).

Since the quality of cumulus-oocyte complexes (COC) influences the developmental potential of embryos following in vitro fertilization (IVF), the maintenance of oocyte integrity from the time an animal is slaughtered and during the ovary transport from the slaughterhouse to the laboratory is crucial (Martín-Maestro et al., 2020). Medium composition, temperature, and storage period are all recognized to play a role in organ preservation (Combelles et al., 2009). Low oocyte in vitro maturation ratio and developmental competence could be caused by improper transportation and conservation circumstances (Wang and Sun, 2006). Often, slaughterhouses are located far from research centers requiring extended periods of preservation of the ovaries collected which may threaten the oocyte viability (Tellado et al., 2014).

The female reproductive organs (ovaries) have one of the highest blood flow rates per unit of tissue of any adult mammalian organ, as well as a fast metabolic rate (Reynolds et al., 2002). During the transportation to the laboratory, blood flow would be blocked, lowering oxygen delivery to the ovaries, and causing them to become ischemic (Wongsrikeao et al., 2005). Ischemia affects the viability of follicles in the ovaries, with oxygen-free radicals being pivotal contributors to ovary damage during preservation (Cruz et al., 2014). Low antioxidant enzymes and reactive oxygen species (ROS) generated in the follicular milieu due to ischemia may activate programmed cell death during transportation of the ovaries.

Several antioxidants have been used to reduce the harmful effects of ROS in the follicular milieu during ovary transportation (Sánchez-Ajofrín et al., 2020; Soto-Heras and Paramio, 2020). The protective properties of melatonin as a potent direct scavenger of free radicals have been well-documented (Tian et al., 2014). These therapeutic effects have been related to its ability to decrease pro-apoptotic and increase anti-apoptotic gene expression, as well as its ability to neutralize ROS. Under hypoxic conditions, glycolysis is the most common anaerobic pathway for ATP synthesis, resulting in lower pH and higher lactate levels (Guibert et al., 2011). Hypoxic and acidic environments adversely affect the net electric charge, cell membrane permeability, and chromosomal instability (Schomack and Gillies, 2003). As a result, the absence of buffer systems in the ovarian tissue transportation medium could significantly influence the oocyte's eventual developmental ability (Bohlooli et al., 2015). The removal of organs from the blood supply exposes them to induced hypothermia which protects them from damage (Guibert et al., 2011). At low temperatures, the metabolic activities of cells are reduced or entirely stopped, and the metabolic rate is bisected for every 10 °C reduction in temperature. As a result, the remaining metabolic rate at 4 °C is around 10 % of normal condition (Cantu and Zaas, 2011). A study has found that transporting ovaries at 38 °C for several hours reduces the blastocyst rate in several animals (Naoi et al., 2007).

With this background, this study hypothesized that the use of unconventional transport medium may have beneficial effects by maintaining the follicular quality of ovaries. Therefore, the first part of this study was designed to assess the impacts of five different ovaries transport media: Phosphate-Buffered Saline (PBS), Charles Rosenkrans 1 (CR1), Normal Saline (NS), Ankara University Zootekni 1 (AUZ1), and Ankara University Zootekni 2 (AUZ2) at four different temperatures (-6 to -4, 2 to 4, 8 to 12, and 24 to 30 °C), on the embryo development rate. The second part of this study was designed to compare the efficiency of NS, PBS, CR1, AUZ1, and AUZ2 transport media in maintaining the structural integrity of sheep ovarian tissue during transport at 4 °C.

2. Material and Methods

In this study, IVEP was performed at the Reproductive Biology and Animal Physiology Laboratory, Ankara University, Turkey. Histological analysis of the ovarian tissue was performed at the Department of Histology and Embryology, Faculty of Veterinary Medicine, Ankara University. Unless mentioned otherwise, all chemicals used in this study were purchased from Sigma-Aldrich Chemical Company. Materials procured from other companies are described in the relevant sections.

2.1. *In vitro* embryo production

Post-mortem ovaries (n = 825) were collected from adult sheep of various Turkish breeds from a local slaughterhouse from January 2022 to April 2022; the range of temperature on the days of the

collection was (-4–30 °C), and the days of ovaries collection was 28. The ovaries were randomly distributed into 100 ml tubes containing 60 ml of NS as the control medium (154 mM NaCl (S5886)), PBS (137 mM NaCl, 2.70 mM KCl (P5405), 8 mM Na_2HPO_4 (S5136), and 2 mM KH_2PO_4 (P5655)), CR1 (135 mM NaCl, and 10 mM KCl), AUZ1 (108 mM NaCl, 7 mM KCl, 3.6 mM $NaHCO_3$ (S5761), 16.9 mM L-Glutamine (G8540), 520 mM TRIS (252859), 1.11 mM Glucose (G7021), 1.5 µl/ml Na Pyruvate (P4562), and 1 µl/ml Melatonin (M5250)), or AUZ2 (51 mM NaCl, 2.7 mM KH_2PO_4 (P5655), 1.15 mM Sucrose (S0389), 1 µl/ml Vit E, and 1 µl/ml Vit A) at -6 to -4, 2 to 4, 8 to 12, or 24 to 30 °C. The temperature was continuously monitored with thermometers during transportation. Then the ovaries were transported to the laboratory in an ice chest 1 to 2 h post-mortem. The cumulus-oocyte complexes (COC) were collected by aspiration from visible follicles with a 3–5 mm diameter, and oocytes with two or more compact cumulus cell layers and homogeneous cytoplasm were chosen. Then, the oocytes were placed in groups of 40–60 into 500 µl of IVM medium based on TCM-199 supplemented with: 0.36 mmol/L Na Pyruvate (P4562), 5 µl/ml gentamycin (GEN-10B), 750 µM Glutamax (35050-06), 1 IU/ml FSH (Life Technologies),1 IU/ml LH (Bio98), and 1% FBS (16A) in a 4-well dish (Nunc) for 24 h at 38.5 °C in a humidified atmosphere with 5% CO_2. At the end of the IVM period, the oocytes were evaluated under an inverted microscope (LEICA DM IL LED; Wetzlar, Germany), and only those that had expanded cumulus cells were selected for in vitro fertilization (IVF) (Nikiforov et al., 2020). The oocytes were washed twice in BO (IVF Bioscience, 71001) medium 40-60 oocytes were placed in 500 µl of BO medium supplemented with 5 µl/ml gentamycin, 1.25 mM sodium pyruvate, 5 mg/ml BSA (A3311), 1 µl/ml heparin (P4562), 40 µl/ml D- penicillamine, Hypotaurine and epinephrine (B2794), 2 mM caffeine, and 10% FBS. For IVF, sperm cells were examined for quality and assessed according to individual/collective motility (Majeed et al., 2019) and viability. The COC was fertilized with sperm concentrations of 7 x 106/ml and co-incubated at 38.5 °C in 5% CO_2 and 90% humidity. After IVF, the oocytes were evaluated as fertilized when showing a 2nd PB or sperm heads in the cytoplasm (Arat et al., 2016). After 18 h of the IVF, cumulus cells were partially removed using 1 µl hyaluronidase (H3884) followed by pipetting. The resulting zygotes were cultured in 500 µl of SOF medium supplemented with 4 mg/ml BSA, 10 µl/ml glutamax, 10 µl/ml sodium citrate, 10 µl/ml Non-Essential AA (M7145), 20 µl/ml Essential AA (B6766), 10 µl/ml Myo-inositol (P4562), 0.5 µl/ml Na pyruvate, and 5 µl/ml gentamycin (GEN-10B), and incubated at 38.5 °C, 5% CO_2, and 90% humidity. After 48 h of IVF, the cleavage, morula, and blastocyst rates were evaluated at 24 h intervals using an inverted microscope (LEICA DM IL LED; Wetzlar, Germany) (Ferraz et al., 2018).

2.2. Histomorphology of ovaries

After slaughtering the animals, the ewes' abdomen was opened, the ovaries removed, collected (n = 120), and randomly assigned to the different transport media (PBS, CR1, NS, AUZ1, and AUZ2) at 4 °C. The samples were transported to the laboratory and fixed for 24 hours in a container with 10% neutral buffered formalin. The samples were rinsed in distilled water after fixing for 24 hours and dehydrated in a graded alcohol series (70%, 80%, 95%, 100% ×3), then were cleared in xylene (×3), embedded in paraffin, and sectioned into 5 µm thick slices. For each block, 8 serial sections were collected per slide with 100 µm (20 sections) discarded between slides. The slides were stained with Masson's trichrome (hematoxylin, acid fuchsin, and aniline blue) (MT, Sigma Aldrich) to determine the morphological integrity of ovarian tissue. They were examined under a light microscope (Leica DM2500, LEICA Microsystems GmbH, Wetzlar, Germany) and photographed with a digital microscope camera (Leica DFC450, Leica Microsystems GmbH, Wetzlar, Germany). Diameter and density measurements were not included in this assessment. The follicles were classified according to the method developed by Chaves et al. (2008) and Youm et al. (2014). The following morphological standards were used to assess the follicles: basement membrane integrity, granulosa cells (GC), oocyte, and cell density. The follicles were then categorized as normal (spherical oocyte, homogenous cytoplasm, well-organized GC) or degenerative (deformed and disordered GC, poor cell density). Sections selected for analysis were distributed throughout the tissue and all sections were assessed blindly.

2.3. Statistical analysis

The data were expressed as the mean (±S.E.M). Data analysis was done using two-way ANOVA in the SPSS version 23.0 statistical software. Duncan's Multiple Range test was carried out to compare the mean values, and p-values < 0.05 were considered significant.

3. Results

The AUZ1 transport medium at 2 - 4 ºC exhibited 72.5% ($p<0.05$) fertilization rate and embryo development with a significant superiority over the other treatments (Table 1). The AUZI medium also showed a significant superiority (60.83%, $p<0.05$) in IVF rate at 8 - 12 ºC when compared with the other transport media. At -6 - -4ºC, the fertilization rates showed no significant differences ($p> 0.05$) among transport media. Similarly, there were no significant differences in IVF rates ($p>0.05$) among the employed transport media at 24 - 30 ºC.

Table1. Impacts of different temperatures and transport media on the IVF rate and embryo development

Temperature	Media	No. of oocytes	No. of fertilized oocytes	IVF rate (%)	Cleavage rate (%)	Morula rate (%)	Blastocysts rate (%)
-6 - -4 ºC	NS	120	48	40±1.63 ^{a}	39.58±1.24 ^{a}	14.58±0.37 ^{ab}	3.77±0.24 ^{ab}
	PBS	120	40	35.33±1.78 ^{a}	37.5±0.54 ^{a}	12.5±0.60 ^{ab}	2.17±0.20 ^{c}
	CR1	120	39	32.5±1.01 ^{a}	28.20±0.66 ^{a}	5.12±0.44 ^{b}	-
	AUZ1	120	59	49.16±1.59 ^{a}	42.37±1.58 ^{a}	16.94±0.67 ^{ab}	6.77±0.20 ^{a}
	AUZ2	120	40	33.72±2.09 ^{a}	25±0.89 ^{a}	2.5±0.24 ^{b}	-
2-4 ºC	NS	120	60	50±1.78 ^{b}	58.33±0.22 ^{b}	30±0.50 ^{b}	10±0.37 ^{b}
	PBS	120	48	40±2.27 ^{b}	58.33±1.63 ^{b}	18.75±0.80 ^{bc}	6.25±0.24 ^{bc}
	CR1	120	51	42.5±1.77 ^{b}	39.21±1.18 ^{b}	5.88±0.40 ^{c}	1.9±0.20 ^{c}
	AUZ1	120	87	72.5±3.66 ^{a}	67.81±2.41 ^{a}	35.9±1.01 ^{a}	16.09±0.37 ^{a}
	AUZ2	120	54	45±1.39 ^{b}	40.74±1.28 ^{b}	7.40±0.37 ^{c}	1.85±0.20 ^{c}
8-12 ºC	NS	120	53	44.16±1.24 ^{b}	41.50±0.92 ^{b}	22.64±1.16 ^{ab}	5.66±0.40 ^{ab}
	PBS	120	44	36.66±1.39 ^{b}	25±0.73 ^{b}	15.90±0.50 ^{b}	2.27±0.20 ^{b}
	CR1	120	43	35.83±1.43 ^{b}	25.58±0.66 ^{b}	4.65±0.40 ^{b}	-
	AUZ1	120	73	60.83±2.11 ^{a}	50.68±1.07 ^{a}	26.02±0.37 ^{a}	10.95±0.67 ^{a}
	AUZ2	120	41	34.16±2.72 ^{b}	41.46±0.92 ^{b}	7.31±0.24 ^{b}	-
24-30 ºC	NS	120	50	41.66±1.70 ^{a}	40±0.70 ^{b}	22±0.66 ^{b}	4±0.24 ^{b}
	PBS	120	40	33.33±0.83 ^{a}	42.5±1.20 ^{b}	15±0.24 ^{bc}	2.5±0.20 ^{b}
	CR1	120	41	34.16±0.80 ^{a}	29.26±0.74 ^{b}	4.87±0.24 ^{c}	2.43±0.20 ^{b}
	AUZ1	120	62	51.66±1.69 ^{a}	54.83±0.73 ^{a}	29.03±0.50 ^{a}	11.29±0.24 ^{a}
	AUZ2	120	42	35±1.74 ^{a}	30.95±1.12 ^{b}	4.76±0.24 ^{c}	2.38±0.20 ^{b}

Data from 7 replicates; a, b Different superscripts indicate significant differences between transport media at the same temperature ($p< 0.05$). The total number of oocytes used was 2400; 120 were divided into each treatment.

As shown in Table 1, transport media had a significant impact on cleavage, morula, and blastocyst rates ($p>0.05$). At 2-4 ºC, the AUZ1 medium achieved higher cleavage and morula rates (67.81% and 35.9%, respectively) while CR1 and AUZ2 media achieved the least rates of morula (5.12% and 2.5%) and (4.87% and 4.76%) at -6 - -4 ºC and 24 - 30 ºC, respectively. In the AUZ1transport medium, more than 16% of fertilized oocytes reached the blastocyst stage at 2 - 4 ºC, which shows significant differences ($p<0.05$) when compared with other media.

Histology analysis revealed that the primordial follicles had normal histological characteristics (Figure 1). All treatments had the structure of the primary oocyte, which is enveloped by follicular cells and marks the boundary between the follicle and the surrounding stroma. The primary follicles in PBS, CR1, NS, AUZ1, and AUZ2 transport media had normal morphology (Figure 1) that consisted of oocytes surrounded by a layer (unilaminar primary follicle) or layers of cuboidal follicular cells (multilaminar primary or preantral follicle) in addition to the basal lamina, granulosa cells, and theca interna that showed no structural changes. In secondary follicles transported with PBS, CR1, NS, AUZ1, and AUZ2 media, the follicles had oocytes that maintained normal morphology and arranged granulosa cells; there were no defects in the formation of the zona pellucida. Furthermore, ovarian tissues that were transported without a medium (NON) showed follicles with normal histological characteristics and follicle viability.

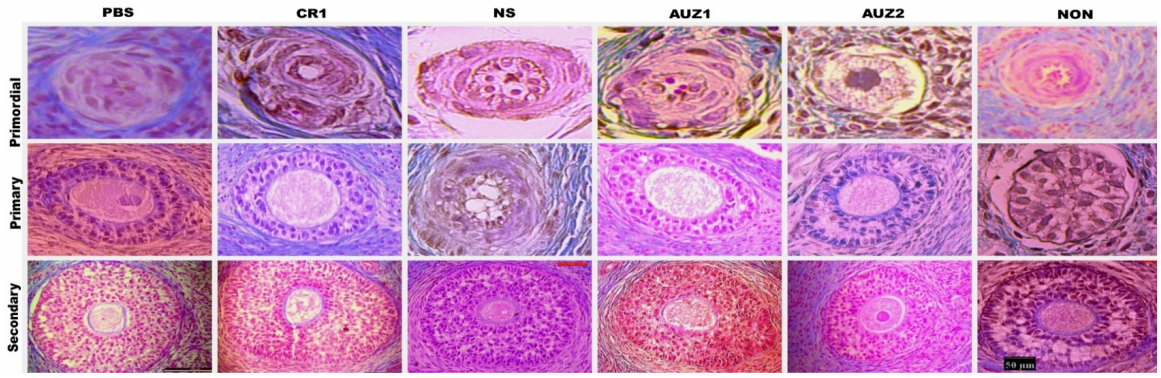

Figure 1. Histological image of primordial follicles, primary follicles, and secondary follicles of ewes' ovarian tissue transported in different media.

However, antral follicles transported in the AUZ1 medium showed normal histological profiles while those in CR1, AUZ2, or PBS exhibited disrupted granulosa cells and cumulus cells around the oocyte (Figure 2). The most significant difference was in treatments NS and NON where the oocytes were surrounded by several layers of granulosa cells separated from the antral follicles. For tissues transported in the AUZ1 medium, the Graafian follicle contained cumulus oophorus and was surrounded by theca externa and theca interna layers that maintained normal morphology. The Graafian follicles seemed to be affected by the transport conditions in the other media possibly due to the separation of the cumulus oophorus from the granulosa cells, in addition to the rupture of the corona radiata in follicles, thus exposing the oocyte to damage.

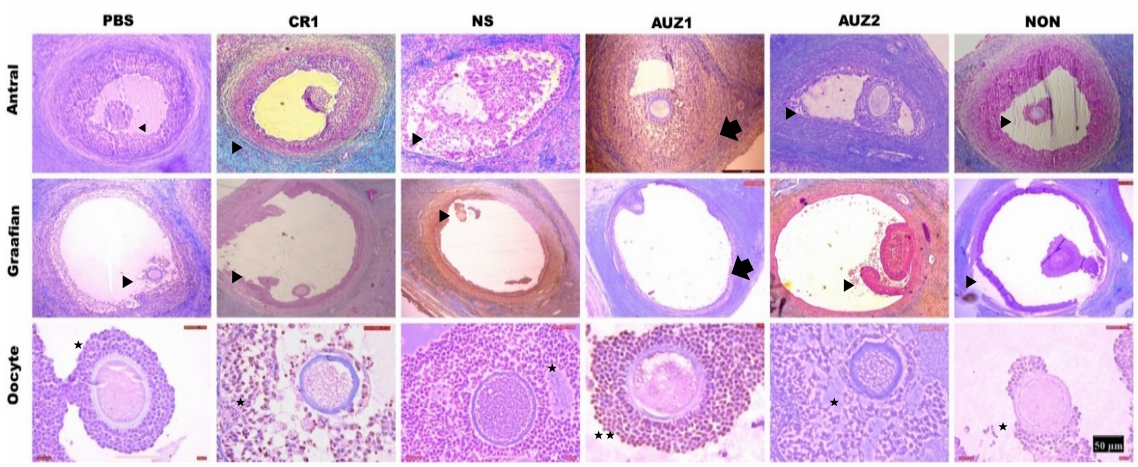

Figure 2. Histological image of antral follicles, Graafian follicles, and cumulus oophorus of ewes' ovarian tissue transported in different media. Normal follicles (arrows), degenerated follicles (arrowheads), degenerated cumulus oophorus (double asterisk), normal cumulus oophorus (asterisk).

4. Discussion

This study examined the effects of transport media on the maintenance of follicular quality after removal and during the transport of ovarian tissues, and the ability of oocytes to develop to the blastocyst stage was assessed via IVEP. To the best of our knowledge, this is the first study to verify the effects of melatonin, buffer solution, and energy substrates on the follicle quality of ewes' ovarian tissue during transportation. The main outcome of this study is that the addition of certain supplements to the AUZ1 transport medium could improve the quality of follicles and subsequent embryonic development.

The components and pH of the transport medium, as well as the period and temperature of transport, are demonstrated to affect sheep oocytes' developmental competence (Martín-Maestro et al., 2020; Sánchez-Ajofrín et al., 2020). Melatonin is an essential component in steroidogenic pathways in the follicular fluid; it is an efficient ROS scavenger that reduces oxidative stress-mediated oocyte quality deterioration and attenuates postovulatory aging oocyte abnormalities (Tamura et al., 2008; Pacchiarotti et al., 2015; Wang et al., 2017).

The data analysis showed that the addition of an antioxidant (melatonin) to the new transport medium (AUZ1) increased embryonic development rates presumably by reducing oocyte oxidative stress during transport. There are two possible mechanisms through which melatonin may have reduced oxidative stress: (i) through its direct free radical scavenging activity, and/or (ii) through the stimulation of the cellular antioxidant defense system. The transport medium (AUZ2) supplemented with Vit A and Vit E as antioxidants, had no positive effect on embryonic development rates likely because the supplements (Vit A and Vit E) had mopped up the ROS generated within the extracellular environment while melatonin scavenged the ROS produced in the COC (intracellular).

It is therefore hypothesized that transport media supplemented with biological buffers such as TRIS or HEPES are necessary for maintaining the pH levels of such media. In the present study, TRIS buffer in the AUZ1 medium also may have supported embryo development, in addition to the effects of melatonin as an antioxidant. Wongsrikeao et al. 2005 confirmed this hypothesis by suggesting that changes in pH led to a significant increase in fragmented DNA nuclei and that ZP was highly permeable to H+ ions and lacked a regulatory mechanism.

The study findings also showed that the oocytes' developmental competence to the blastocyst stage was significantly decreased during the transportation of the ovaries at temperatures less than 0 °C and between 10 °C and 35 °C. However, the AUZ1 medium significantly increased the oocyte's competence to develop into the blastocyst stage compared to the other media. Although ovaries transported in a melatonin-supplemented medium showed improvement, these beneficial effects were more pronounced at low temperatures (4 °C). Previous studies had confirmed this observation that after ovaries were transported for up to 4 h at 4 °C, the IVM rate of canine oocytes was significantly higher than that of COCs transported at 38 °C (Taş et al., 2006). Furthermore, another study confirmed that ovary transport at low temperatures positively affected the oocyte maturation rates in bovines and felines (Matsushita et al., 2004; Naoi et al., 2007). However, the data on how high temperatures affected oocyte quality during transportation is inconsistent; for instance, a study had shown that transporting bovine ovaries for 4 h at 15 °C improved embryonic development and reduced programmed cell death (Wang et al., 2011) while another study opined that ovaries' transport at high temperatures was more advantageous in terms of improving IVM and embryonic development rates in equines and porcines (Love et al., 2003; Wongsrikeao et al., 2005).

The preservation of ovarian tissue is a complicated task because the tissue contains a variety of structures and cells. According to the histology analysis in this study, the quality of the primordial, primary, and secondary follicles, as well as their morphology were not affected by the transportation of the ovarian tissue in any of the transport media at a temperature of 4 °C for up to 2 h. However, the addition of melatonin to the transport medium (AUZ1) significantly maintained the antral follicle morphology. In addition, the Graafian follicles were better maintained in AUZ1 than in the other transport media. Hence, it is hypothesized that the strong antioxidant properties of melatonin may have been responsible. Zhang et al. (2020) found that follicular fluid contains more melatonin than serum. Melatonin in follicular fluid scavenges free radicals, in addition to its steroidogenic role (Adriaens et al., 2006), and diffuses readily through tissues (Reiter et al., 2009). Melatonin improves the development of oocyte-granulosa cell complexes from porcine follicles as noted by Cao et al. (2019). Moreover, the presence of melatonin receptors in oocytes indicates the potentially important roles of this hormone in

mitochondrial characteristics, cumulus expansion, and steroidogenesis. It has been reported that melatonin supplementation in sheep ovary transport medium for 24 h led to higher rates of cumulus cell expansion. Furthermore, the addition of melatonin as a supplement increases its intra-follicular levels, thereby improving fertilization rates and embryo transfer (Tamura et al., 2008). Previous studies have also reported the diverse effects of melatonin on blood vessel proliferation even though this is not involved in IVEP (Romeu et al., 2011). The anti-apoptotic properties of melatonin have also been demonstrated; for instance, melatonin has been reported to inhibit in-vivo programmed cell death in granulosa cells of follicles (Liu et al., 2022). The histological observations in this study showed that antral follicles and Graafian exhibited disruption of the granulosa cells and cumulus cells around the oocyte after ovarian tissue transport in NS, PBS, CR1, and AUZ2 transport media. Therefore, it is hypothesized that follicles deterioration could be triggered by oxidative stress which can lead to apoptosis (Figure 2), an effect that was once again reversed by melatonin in the AUZ1 medium; this melatonin effect has also been demonstrated on different tissues in other studies (Ateşşahin et al., 2006; Mohammadghasemi et al., 2012).

Conclusion

The outcome of this study showed that melatonin, buffer solution, and energy substrates supplementation in AUZ1 transport medium maintained better follicle viability in ewe ovaries and enhanced COC survival and IVEP production. Regarding ovarian tissue damage, the lack of certain components in transport media is not considered ideal for the transportation of ovaries. This study highlights the need for the development of a standard protocol that fits the needs of ovarian tissues during transportation.

Ethical Statement

Ethical approval is not required for this study because our study was performed using only animal material collected from the slaughterhouse, there was no need to apply it to the Scientific Ethical Committee on Animal Experimentation.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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Author Contributions

The research idea was formulated by authors 1 and 3. The experiment was conducted by authors 1 and 2, with supervision from author 3. The manuscript was written by author 1

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Research Article

Effect of Winter Sowing and Different Fertilizer Sources on Physiological Parameters and Yield Components of Dragon's Head (*Lallemantia iberica* Fisch. & C.A.Mey.)

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Abstract: The effects of autumn sowing and chemical, organic, and biological fertilizer sources were explored on yield components and physiological traits of dragon's head (*Lallemantia iberica* Fisch. & C.A.Mey). The study was conducted as a factorial experiment based on a randomized complete block design with three replications in a field experiment in the 2017-2018 crop year. The fertilizer treatments included organic fertilizers (vermicompost, manure, and humic acid), biofertilizer (Thiobacillus mixed with sulfur), chemical fertilizer (macro NPK), and control (no fertilizer). The studied traits included seed yield per ha, harvest index, biological yield per ha, chlorophyll *a*, chlorophyll *b*, carotenoid, proline, and dissolved carbohydrate. The results of the comparison of the means revealed that the winter sowing outperformed the spring sowing evidently and increased traits like seed yield per ha, biological yield per ha, and harvest index significantly. The fertilization of the plants in both sowing seasons, especially in the winter sowing, increased seed yield per ha, biological yield per ha, and harvest index so that the vermicompost-fertilized winter-sown plants produced the highest seed yield per ha (0.91 g), whereas the application of manure was related to the highest harvest index in the winter sowing (27.9%). The highest biological yield (8797 kg ha^{-1}) was related to the treatment of Thiobacillus of the winter-sown plants. Proline content was higher in the spring sowing plants, and the control treatment in the spring sowing had the highest proline content (0.120 mg g^{-1}). Concerning dissolved carbohydrates, the spring sowing and the unfertilized plants had the highest content (20.3 mg g^{-1}). On the other hand, chlorophyll *a*, chlorophyll *b*, and carotenoid were higher in the treatments of Thiobacillus and vermicompost, which resulted in achieving higher yields due to the increase in photosynthesis rate. According to the results, the winter sowing of the dragon's head in the Azerbaijan region of Iran and the use of *Thiobacillus* and vermicompost could be recommended for obtaining plants with optimum quality parameters.

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1. Introduction

Medicinal plants have always been an effective treatment in history so research has revealed that plants have been a primary source of medicine for millennia. Presently, the World Health Organization reports that more than 80 percent of individuals continue to depend on traditional medicine, including the utilization of plants (Rahimi et al., 2019). The family Lamiaceae has major medicinal plant species used to treat various diseases. Some have essential oils and many are used in nutritional cases (in raw or cooked forms) or are planted for their beautiful and aromatic flowers (El-Sheshtawy et al., 2019). Dragon's head (*Lallemantia iberica* Fisch. & C.A.Mey.) is an invaluable species from this family as all of its parts have economic applications; for instance, it has curative effects as stimulator, diuretic, and expectorant and is good in the treatment of neural disorders, renal disorders, and hepatitis (Rezaei-Chiyaneh et al., 2020). The seeds of the dragon's head are dark and oval-shaped and contain oil, fiber, and protein that provide various medicinal and nutritional advantages (Amanzadeh et al., 2011; Razavi et al., 2012).

Various factors affect the medicinal plants' yield such as increasing rainfall use efficiency and proper sowing date (Talaat, 2019). Increasing plant yields were reported by Wang et al. (2019) in spring sowing, due to severe rainfalls in which case plant yields will be remarkably impaired because of adverse environmental conditions of summer such as moisture deficiency, higher temperatures, and hot winds. The results of Akhzari et al. (2018) show that yields can be considerably improved both qualitatively and quantitatively by using winter-winter sowing instead of spring sowing and taking more advantage of precipitation (Kumar et al., 2018).

A major pillar of sustainable farming involves the utilization of organic and biological fertilizers within agricultural environments to minimize or eliminate the use of chemical inputs (Rodnuch et al., 2019). Long-term studies have documented that excessive use of chemical fertilizers impairs crop yields arising from soil acidification, the decline of soil biological activities, the loss of soil physical properties, and the lack of micronutrients in macro-chemical fertilizers (Adediran et al., 2004). Organic fertilizers increase soil organic matter and improve its fertility by enhancing its chemical attributes like pH, cation exchange capacity, microorganism activity, and nutrient availability (Nejatzadeh-Barandozi and Pourmaleknejad, 2014; Wang et al., 2019). The results of Amooaghaie and Golmohammadi (2017) showed that the use of organic fertilizers, e.g. manure and vermicompost, in sustainable agriculture escalates the presence of vital nutrients like nitrogen (N), phosphorus (P), and potassium (K), in addition to increasing the population and activity of beneficial soil-borne microorganisms, and it consequently improves crop growth and yields (Abd El Ghafour et al., 2017). The results of Amooaghaie and Mardani Korrani (2018) showed that using organic fertilizers increased the phytochemical properties of medicinal plants. Similar results were found by different researchers (Amooaghaie and Golmohammadi, 2017; El Kinany et al., 2019).

In recent years, the use of biological fertilizers has drawn attention as a good alternative to chemical fertilizers in increasing soil fertility and has been interested by producers as a major nutritional approach for plants to achieve the goals of sustainable agriculture (Wang et al., 2019). The findings by Akhzari et al (2018) demonstrated that Biofertilizers are capable of converting key nutrients from unavailable to available forms in biological processes and improving root system development and seed germination, which caused to increase in medicinal and phytochemical properties of different medicinal plants (El Kinany et al., 2019). It is even known that the association of Plant Growth Promoting Rhizobacteria (PGPR) and AMF, which have the capacity to minimize the damage of abiotic stress factors especially in agricultural production, not only protects plants against stress factors but also supports growth and development (Nadeem et al., 2014; Selem et al., 2021).

Low crop efficiency and the contamination of pesticide and chemical fertilizer residues are among the major challenges of medicinal plant production, especially in Iran. The overuse of chemical fertilizers has harmful effects such as the toxicity arising from the overuse of fertilizers and the loss of crop quantity and quality. Although the application of chemical fertilizers has extensively been developed as it is the fastest way to offset soil nutrient deficiencies and improve crop yield, their application has entailed environmental pollution and ecological destructions in many cases and has pushed up production costs. Conversely, excessive utilization of N-containing fertilizers threatens human health. So, the present study aims to find the best sowing date for the dragon's head relying on an optimal organic and biological fertilization system for physiological traits and seed yield.

2. Material and Methods

The research was conducted at the research farm of the Department of Agriculture, Urmia University in Western Azerbaijan province, Iran (45°10' E., 37°44' N., 1338 m. from sea level) in the 2017-2018 crop year. Before sowing, combined soil samples were taken from five random points at a depth of 0-30 cm to analyze its Physicochemical properties and estimate the fertilizer requirements of the dragon's head. The findings of the soil examination are displayed in Table 1. Table 2, also, presents a summary of the physical and chemical characteristics of the organic fertilizers used in the study. The fertilizer requirement was estimated according to Tables 1 and 2 and was mixed into the soil before sowing.

Table 1. Some properties of soil in the study site

EC(dS m^{-1})	pH	Texture	Clay	Silt	Sand	$CaCO_3$	BC[1]
1.38	7.79	Clay loam	41%	36%	23%	15.71%	54%

N	Organic carbon	Mn	B	Zn	Fe	K	P
%		mg kg^{-1}					
0.03	1.16	11.2	0.28	1.1	8.11	282	9.02

(1) BS: base saturation.

Table 2. Some physical and chemical properties of the organic fertilizers applied in the experiment

	K (%)	P (%)	N (%)	OM[1] (%)	EC[2] (dSm^{-1})	pH
Cattle manure	1.07	1.12	1.01	61	8.87	7.49
Vermicompost	3.29	1.59	1.79	55	6.56	8.68

(1) OM = organic matter; (2) EC = electrical conductivity.

The research was designed as a factorial experiment following a randomized complete block design with three replications. The factors considered in the study were the sowing season and the type of fertilizer used. The sowing season was the primary factor with two levels: winter sowing and spring sowing. The second factor was the fertilizer source, which included manure (6.3 ton ha^{-1}), NPK fertilizer (Urea: 110 kg ha^{-1} + Triple superphosphate: 60 kg ha^{-1} + Potassium sulfate: 50 kg ha^{-1} + Micronutrients: 23 kg ha^{-1}), vermicompost (6.8 ton ha^{-1}), humic acid (400 kg ha^{-1}), and thiobacillus (2%) + granular sulfur (400 kg ha^{-1}). The experimental blocks were established on November 27, 2017, after the land was plowed and leveled in autumn. Sowing rows were then prepared, with each experimental plot covering an area of 6 m^2. Winter sowing took place on November 28, 2017, with row spacing set at 1 cm and inter-row spacing at 25 cm. Spring sowing, on the other hand, was conducted on February 27, 2018. Throughout the growing season, activities such as thinning, gap-filling, and weeding were carried out as needed. According to the sampling procedure, a total of 10 plants were selected at random from each plot to assess their morphological characteristics. To determine the yield, the two rows at the edges of the plots, as well as a 0.5 m section from both ends, were excluded to account for any potential marginal effects.

As per the sampling procedure, a total of 10 plants were selected at random from each plot to assess their morphological traits. To determine yield, the two rows at the edges of the plots, as well as a 0.5 m section from both ends, were excluded to account for any marginal effects. To find out seed yield per ha, the seeds were detached from the achene of the plants collected from an area of 1 m^2. Then, they were weighed with a digital scale and it was recorded as seed yield per m^2 and it was used to estimate seed yield per ha. To determine biological yield, three rows were harvested from an area of 1 m^2 within each plot after full maturity and the plants of different plots were placed in different packages. Then, they were oven-dried at a temperature of 39 °C for 48 hours, ensuring the complete elimination of any remaining moisture content. Following the drying procedure, they were weighed with a scale. The sum of the dry weight of aerial parts was recorded as the biological yield.

The harvest index was determined by dividing the seed yield by the biological yield. It was presented in percent.

To measure proline content, 1 mL of the alcoholic extract was mixed with 10 mL of distilled water and combined with 5 mL of ninhydrin reagent. Then, 5 mL of glacier acetic acid was introduced, followed by placing the mixture in a 100 °C water bath for 45 minutes with continuous agitation. After that, they were cooled down, added with 10 mL of benzene, and agitated so that proline could enter into the benzene phase. The samples were left undisturbed for 30 minutes Proline standards ranging from 0 to 0.1 mM/mL were prepared and finally, the absorption of the samples was measured at 515 nm with a spectrophotometer (PD-303, Japan) (Rodnuch et al., 2019). To measure dissolved carbohydrates, 0.1 mL of the alcoholic extract kept in a refrigerator was poured into a test tube with a micropipette and 3 mL of freshly prepared anthrone was introduced into the solution. The test tubes were positioned within a boiling water bath and left for 10 minutes as long as a colorful material was formed. After they were cooled down, the absorption of the samples was measured at 625 nm with the spectrophotometer. To prepare sugar standard, glucose solutions were prepared with concentrations of 0-120 ppm, and all experimental procedures were performed on them. Finally, their absorption was read at 625 nm (Abd El Ghafour et al., 2017). Also, to determine chlorophyll content, 0.25 g of fresh and fully developed leaves were harvested at the full flowering stage, crushed in a Chinese mortar, and ground with 5 mL of distilled water in a dim cool environment as long as it turned into a uniform bulk. The mixture was poured into a 25-mL volumetric flask and adjusted to the required volume. Subsequently, 0.5 mL of the solution was combined with 4.5 mL of acetone 80%, and centrifuged at 3000 rpm for 10 minutes. Afterward, the supernatant was taken and its absorption was read at 470, 646.8, and 663.2 nm with the spectrophotometer (PD-303, Japan). Chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid content were determined using the equations provided by El-Sheshtawy et al. (2019).

$$\text{Chlorophyll a (mg/ml)} = 12.25\,(A_{663.2}) - 2.79\,(A_{646.8}) \quad (1)$$

$$\text{Chlorophyll b (mg/ml)} = 21.50\,(A_{646.2}) - 5.10\,(A_{663.2}) \quad (2)$$

$$\text{Carotenoid (}\mu\mu\text{g/ml} = \frac{1000(A_{470}) - 1.8(Chl_a) - 85.2(Chl_b)}{198} \quad (3)$$

3. Results and Discussion

3.1. Seed yield

The statistical analysis of variance (ANOVA) results for the measured characteristics can be found in Table 3 Both the individual effects of sowing date and fertilizer, as well as their combined effects, were significant ($p < 0.01$) on seed yield per ha. Means comparison revealed that winter sowing in all fertilization treatments well-outperformed spring sowing. The highest seed yield (0.91 g ha^{-1}) was related to the application of vermicompost to the winter-sown plants whereas the spring-sown plants that were not fertilized exhibited the lowest seed yield of 0.19 g ha^{-1} (Figure 1). Even the fertilized spring-sown plants failed to produce yields comparable to the control winter-sown plants. It seems that the winter-sown plants produced higher seed yield because they did not meet stressful conditions, so they outperformed the spring-sown plants in most yield-related traits, which resulted in their higher seed yield per ha. So, it can be recommended to opt for winter sowing for dragon head cultivation. Similarly, Semenov et al. (2020) and Dast Borhan (2017) reported that the best yield of dragon's head and winter wheat (*Triticum aestivum* L.) was obtained from timely winter sowing. They explained that winter sowing in these regions was performed in late autumn and winter in rain-fed farming systems. Nutrition and proper sowing dates are the most effective factors that increase plant yields (Brzozowska and Brzozowski, 2020). The sowing date is a major management factor in crop production because when the sowing date is changed, the meteorological parameters are changed and this influences plant growth and production (Mazurenko et al., 2020). Nutrition can also impact the absorption and efficiency of growth-affecting environmental factors. It appears that the dragon's head plants sown in winter yielded more seeds as a result of their improved establishment, ability to withstand cold temperatures, an earlier start of spring growth, and subsequently increased vegetative growth. Brzozowska and Brzozowski (2020) attributed the higher seed yield of anise and fennel in winter sowing to these factors, especially cold hardiness and vegetation growth.

Table 3. Analysis of variance for the yield-related traits

Sources of variations	Degrees of freedom	Means of squares		
		Seed yield (kg ha^{-1})	Biological yield (kg ha^{-1})	Harvest index
Block	2	12672.8^{ns}	1210109^{ns}	0.90^{ns}
Sowing season (A)	1	2000676**	16738414**	1826**
Fertilizer (B)	5	1008159**	23120645**	38.1**
A × B	5	116117**	1764668^{ns}	17.09^{ns}
Error	22	13808.4	896857.2	7.38
Coefficient of variations		6.48	14.28	10.82

ns, *, and ** show insignificance and significance at the $p < 0.05$ and $p < 0.01$ levels, respectively.

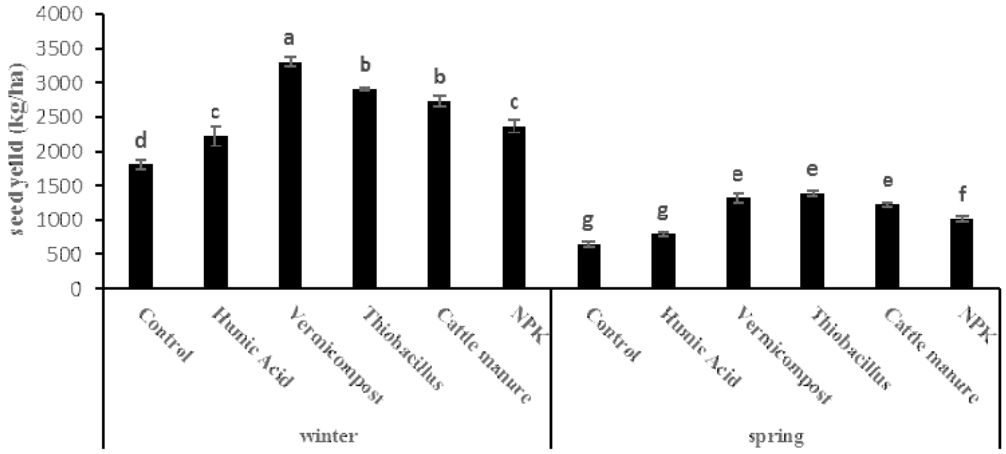

Figure 1. Means comparison of the interactive effect of sowing date × fertilizer on seed yield per ha.

3.2. Biological yield

The results of ANOVA for this trait are shown in Table 3. The sowing date × fertilizer interaction was not statistically significant for biological yield per ha, but this trait was significantly ($p < 0.01$) affected by their simple effects. According to the analysis of the simple effects of the treatments (Figure 2), it was found that the winter sowing resulted in a greater biological yield compared to the spring sowing. (7312 kg ha^{-1} versus 5948 kg ha^{-1}). Among fertilizer treatments, thiobacillus (8797 kg ha^{-1}) and vermicompost (8763 kg ha^{-1}) produced optimal biological yield, but the control no-fertilizer treatment produced a non-optimal yield (3673 kg ha^{-1}). The higher biological yields in these treatments were associated with higher root, stem, and leaf dry weight of the plants. The same result was found by Semenov et al. (2020). They reported that due to its short growth period, the plant has low nutrient requirements and does not need much N, P, and K for productivity. Haque et al. (2020) and Swathi et al., 2020 stated that the utilization of NPK at the rate of 25-50-25 kg ha^{-1} + vermicompost improved the biological yield of basils versus the control, which is in part consistent with our findings.

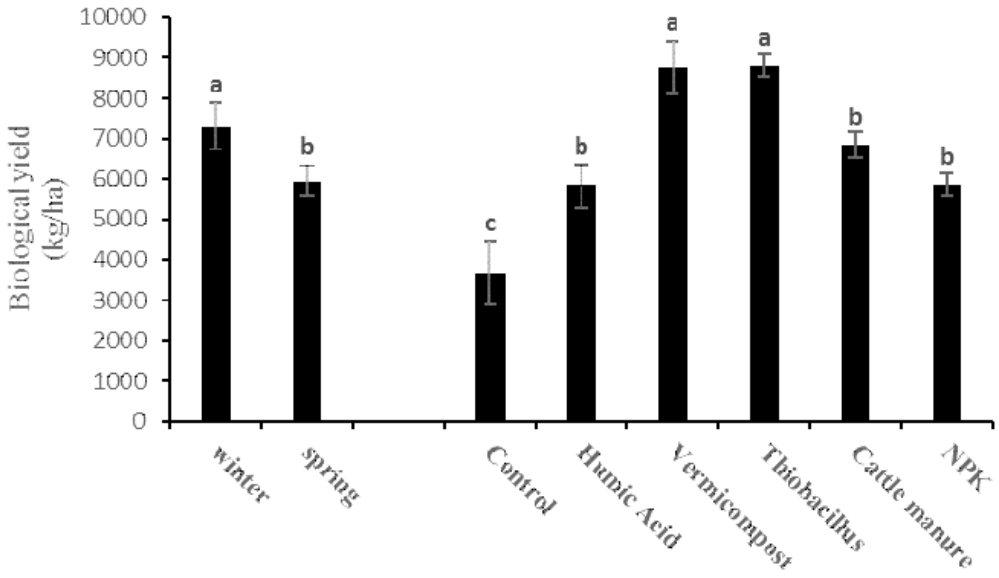

Figure 2. Means comparison of the simple effects of sowing date and fertilizer on biological yield per ha.

3.3. Harvest index

The effect of fertilizer type and sowing season was remarkable ($p < 0.01$) on the harvest index, however, the statistical insignificance of their interaction was apparent (Table 3). The insignificance of the interaction implies that different fertilization levels had a similar effect on the harvest index in both seasons. According to means comparison, the winter sowing (32.2%) had almost twice as great harvest index as the spring sowing (17.9%), and the application of manure (27.9%) and NPK (27.7%) resulted in the attainment of the greatest harvest index. and the lowest from the no-fertilization treatment (21.1%). Except for the Thiobacillus biofertilizer (24.4%), the other fertilizer treatments differed from the control significantly (Figure 3). The higher harvest index of the winter sowing may be associated with the environmental conditions of the plants during their vegetative growth period. The application of manure affects both biological and chemical processes, as well as soil P variability, thereby contributing to the better growth of the plants (Nayak et al., 2020; Haque et al., 2020).

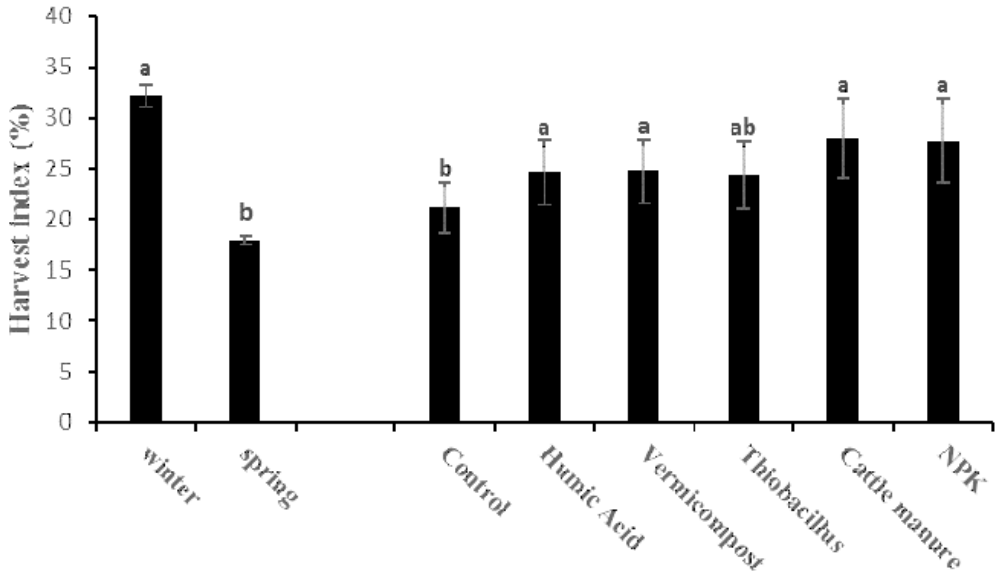

Figure 3. Means comparison of the simple effects of sowing date and fertilizer on the harvest index.

3.4. Physiological parameters

3.4.1. Chlorophyll a, chlorophyll b, and carotenoid

The results of ANOVA revealed the strongly significant effects of sowing season and fertilizer and the insignificant interactive effect of these two factors on chlorophyll *a*, chlorophyll *b*, and carotenoid (Table 4). Means comparison for the simple effects indicated higher chlorophyll *a* content of the winter sowing (0.186 mg g^{-1}) than the spring sowing (0.153 mg g^{-1}). Also, the highest chlorophyll *a* content was observed in the plants treated with Thiobacillus (0.228 mg g^{-1}) and vermicompost (0.222 mg g^{-1}) and the lowest in the control treatment (0.093 mg g^{-1}). Other fertilization treatments were ranked in the same statistical group and had a moderate content of chlorophyll *a*.

The results were similar for chlorophyll *b* and carotenoid so that, as is evident in Figure 5, the winter sowing resulted in elevated levels of chlorophyll *b* content in comparison to spring sowing (0.132 vs. 0.105 mg g^{-1}), and the plants treated with Thiobacillus and vermicompost demonstrated elevated levels of chlorophyll *b* content compared to the unfertilized plants (0.157, 0.151, and 0.079 mg g^{-1}, respectively). Brzozowska and Brzozowski (2020) reported that the winter sowing resulted in higher chlorophyll *b* content compared to the spring sowing. Furthermore, the winter sowing exhibited a higher carotenoid content (0.098 mg g^{-1}), whereas the spring sowing had a lower carotenoid content (0.070 mg g^{-1}). Among the fertilizer treatments, Thiobacillus (0.119 mg g^{-1}) and vermicompost (0.112 mg g^{-1}) produced the highest and the control treatment (0.059 mg g^{-1}) produced the lowest carotenoid contents (Figure 6). The results of Mazurenko et al. (2020) showed a higher carotenoid content in wheat with fertilizer treatments.

Vermicompost has a high water retention capacity and proper contents of available nutrients. Its microbial metabolism may, also, increase the number of chloroplasts per unit leaf area, chlorophyll density, photosynthesis rate, and finally, yield (Dao et al., 2020). In addition, the application of vermicompost contributes to maintaining soil nutrients, hindering N leaching, increasing microbial activity, and improving soil structure. Similar results have been reported (El Kinany et al., 2019; Nayak et al., 2020) so that the plants treated with vermicompost had higher total chlorophyll content than the untreated plants. By supplying the nutrient requirements of soil microorganisms, biofertilizers increase their population, thereby reducing soil pH and increasing the uptake of nutrients like Fe, Mg, and Mn that are involved in chlorophyll synthesis, so chlorophyll synthesis is enhanced (Dao et al., 2020).

Carotenoids are lipophilic compounds with low molecular weight in chloroplasts that protect plants against oxidative stresses. Carotenoids use the xanthophyll cycle to consume oxygen and protect chlorophyll against photo-oxidation. In addition to their structural role, carotenoids act as a light absorber and photosystem protectant against singlet oxygen radicals (Akhzari et al., 2018). A research study concluded that chlorophyll *a*, chlorophyll *b*, carotenoid, proline, and dissolved carbohydrate contents of dragon's head were affected by the foliar application of osmolytes and micronutrients (Swathi et al., 2020).

Table 4. Analysis of variance for the physiological parameters

S.V.	df	Means of squares				
		Chlorophyll *a*	Chlorophyll *b*	Carotenoid	Proline	Dissolved carbohydrates
Block	2	0.00097^{ns}	0.0001^{ns}	0.0001^{ns}	0.0002^{ns}	8.083^{*}
Sowing season (A)	1	0.0098^{**}	0.0063^{**}	0.0032^{**}	0.0024^{**}	74.56^{**}
Fertilizer (B)	5	0.154^{**}	0.0056^{**}	0.0032^{**}	0.0007^{**}	19.11^{**}
A × B	5	0.0011^{ns}	0.00025^{ns}	0.0002^{ns}	0.00002^{ns}	0.72^{ns}
Error	22	0.00067	0.00017	0.00016	0.00007	2.15
Coefficient of variations		15.25	11.13	14.54	8.24	8.68

ns, *, and ** show insignificance and significance at the $p < 0.05$ and $p < 0.01$ levels, respectively.

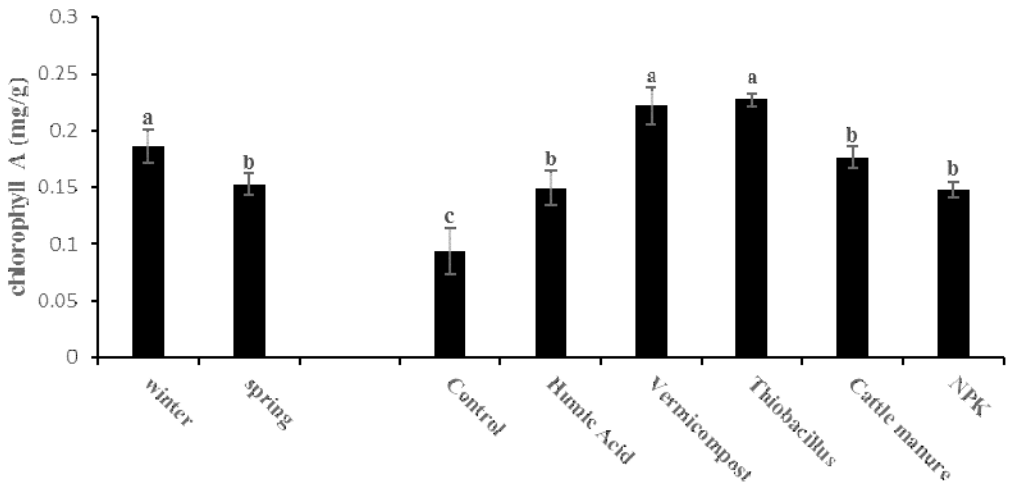

Figure 4. Means comparison of the simple effects of sowing date and fertilizer on chlorophyll *a* content.

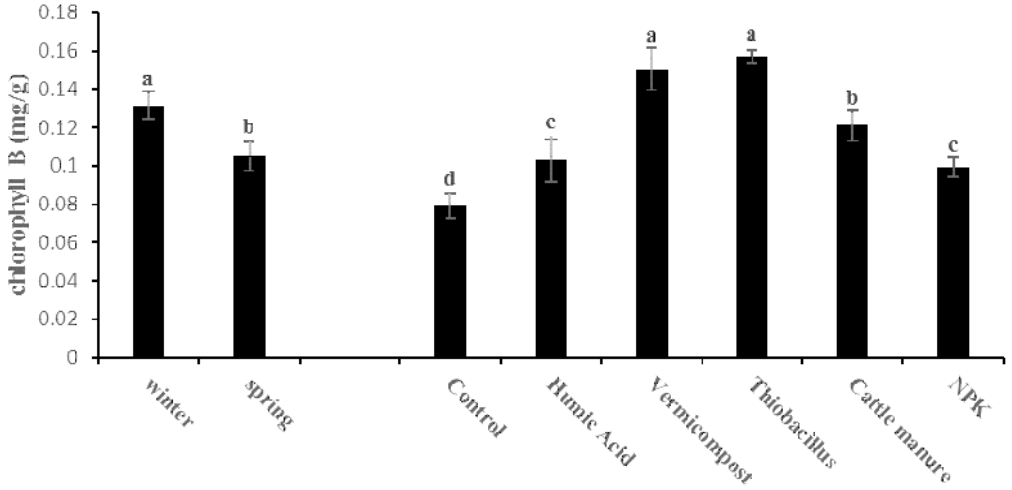

Figure 5. Means comparison of the simple effects of sowing date and fertilizer on chlorophyll *b* content.

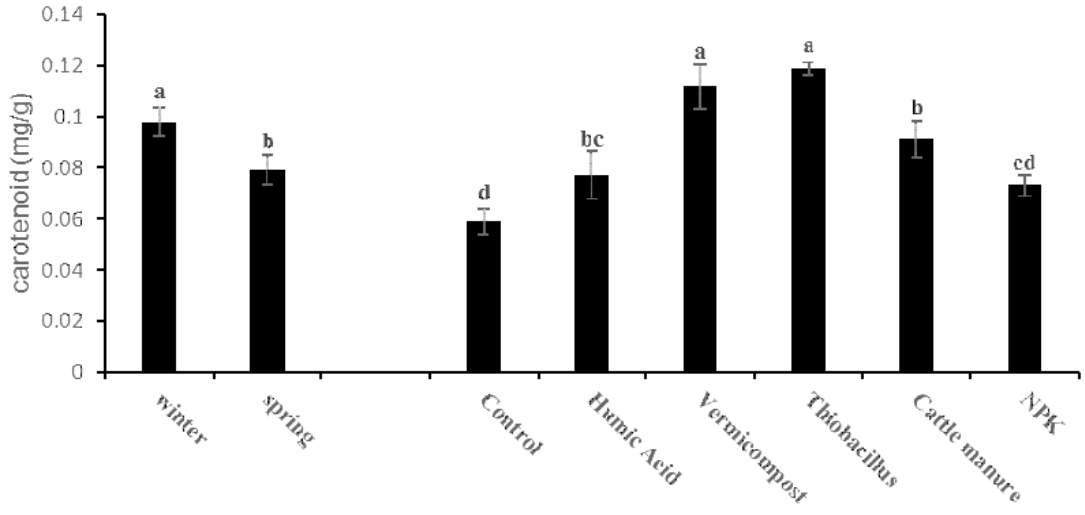

Figure 6. Means comparison of the simple effects of sowing date and fertilizer on carotenoid.

3.4.2. Proline and dissolved carbohydrates

ANOVA for proline content and dissolved carbohydrates revealed the significant effects of sowing season and fertilizer type at the $p < 0.01$ level and the insignificant interactive effect of these two factors on these two traits (Table 4). According to the findings of means comparison, the proline content of the spring-sown plants (0.108 mg g^{-1}) was higher than that of the winter-sown plants (0.091 mg g^{-1}). Also, the unfertilized plants produced the highest proline content (0.120 mg g^{-1}) followed by the NPK fertilizer (0.103 mg g^{-1}). The other fertilizers, especially vermicompost (0.090 mg g^{-1}), were related to the lowest proline content (Figure 7).

The dissolved carbohydrate content exhibited the same trend. It was higher in the spring sowing (18.3 mg g^{-1}) than in the winter sowing (13.5 mg g^{-1}). On the other hand, the unfertilized plants produced the highest dissolved carbohydrate content of 20.3 mg g^{-1}, and the different fertilization treatments, especially vermicompost (15.3 mg g^{-1}), resulted in the decline of dissolved carbohydrates in the dragon's head plants (Figure 8).

Higher plants often respond to stress by accumulating proline (Grace and Mbogue, 2020). The utilization of N-containing fertilizers has been documented to contribute to the synthesis of more proline in plants because proline is a protein compound with a nitrogenous structure (Temel and Yolcu, 2020). It has been concluded for chicory that biofertilizers have significant effects on chlorophyll *a*, chlorophyll *b*, total chlorophyll, carotenoid, dissolved carbohydrates, and proline. According to a study conducted by Nourzad et al. in 2015, it was found that the utilization of organic and inorganic fertilizers in stressed plants could effectively increase proline and carbohydrate contents. However, since the plants in the present study were not exposed to stress, the application of fertilizers failed to increase proline and dissolved carbohydrate contents. Nonetheless, the higher contents of these two traits were at a considerable level in the spring sowing.

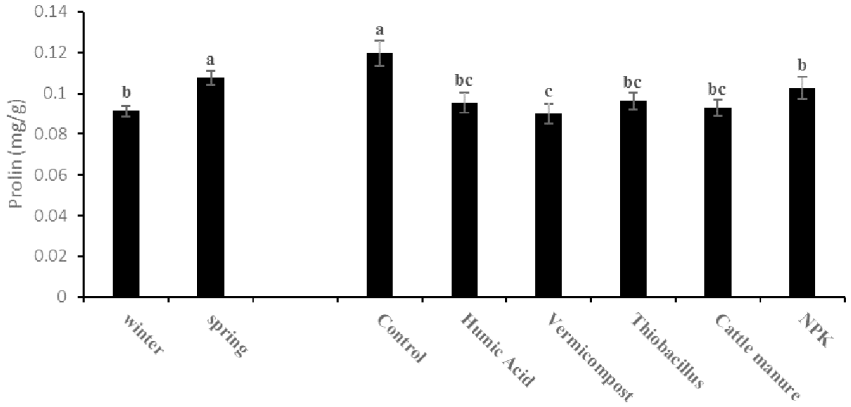

Figure 7. Means comparison of the simple effects of sowing date and fertilizer on proline content.

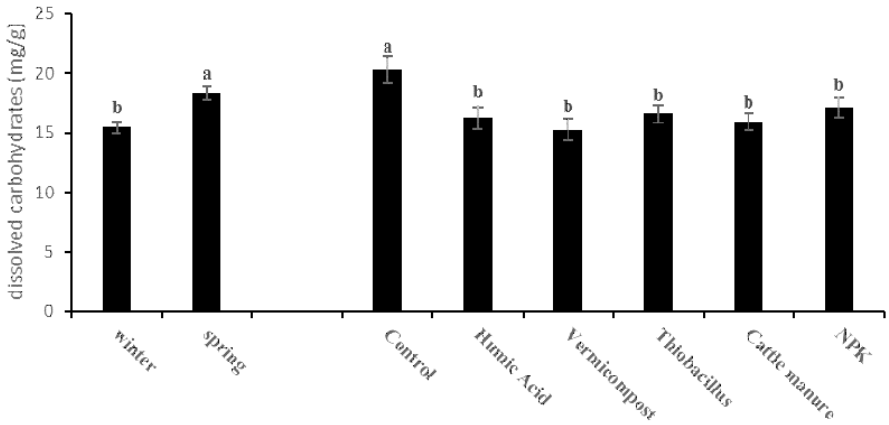

Figure 8. Means comparison of the simple effects of sowing date and fertilizer on dissolved carbohydrates.

Conclusion

The results of the present study revealed that the winter sowing of the dragon's head resulted in superior performance compared to spring sowing, leading to a significant enhancement in seed yield. Fertilization of the plants during both seasons, particularly in winter sowing, increased yield and yield components. Among the fertilizer treatments, vermicompost and Thiobacillus were found to be the most effective fertilizer treatments, while humic acid application, especially during spring sowing, was deemed the least effective in enhancing the characteristics. Unlike the other traits, proline and dissolved carbohydrates exhibited higher levels in spring sowing with no fertilizer treatment. On the other hand, chlorophyll *a*, chlorophyll *b,* and carotenoid contents were higher in the Thiobacillus and vermicompost treatments of the spring-sown plants, which could increase yield in these treatments by increasing the photosynthesis rate.

Ethical Statement

Ethical approval is not required.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Funding Statement

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Author Contributions

Authors contributed equally.

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Research Article

Discover the Most Effective Disease Management Strategies for *Fusarium* Dry Rot of Potato through Comprehensive Bio-assay of Three Techniques (Chemical, Plant extracts, and Bio-control)

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Keywords

Fungicide,
Plant extract,
Post-harvest disease,
Solanum tuberosum,
Trichoderma species

Abstract: Potato (*Solanum tuberosum*) is a multicultural staple food and cash crop. Unfortunately, production of potatoes is predominantly constrained by diseases including dry rots. To evaluate management of dry rots, three *in vitro* sub-trials were set up using the completely randomized design with each treatment replicated thrice for each trial. Firstly, synthetic fungicides were assayed against *Fusarium oxysporum* f.sp. tuberosi. Secondly, plant extracts were assessed against *F. oxysporum*. Finally, the efficacy of *Trichoderma harzianum* applied against *F. oxysporum* was evaluated. The colony radii were measured. The inhibition of *F. oxysporum* by Ketoconazole (at 100% concentration) was significantly ($p \leq 0.05$) highest, followed by Ketoconazole (50% concentration), Itraconazole (100% concentration), Itraconazole (50% concentration), Sulphur (100% concentration), Ridomil (100% concentration), Sulphur (50% concentration), and finally Ridomil (50% concentration). Percentage inhibition of the growth of the *Fusarium* species by fungicides ranged from 39.5-95.7%. Blue gum (*Eucalyptus globulus*) (at 100% concentration) gave the highest inhibition, followed by blue gum (50% concentration), Sweet alligator-pepper (*Aframomum melegueta*) at 100% concentration, locust bean (*Parkia biglobosa*) at 100% concentration, Sweet alligator-pepper (50% concentration), candle bush (*Senna alata*) (100% concentration), locust bean (50% concentration), and *Senna alata* (50% concentration) in descending order of percentage inhibition. Plant extracts caused a percentage inhibition of the fungus between 20.6-100% inhibition with time. Inhibition of *Fusarium* by *T. Harzianum* isolate BGMZ4 was significantly ($p \leq 0.05$) highest, followed by *T. Harzianum* isolate NSBM then *T. Harzianum* isolate BGMZ3. Control of *F. Oxysporum* by *T. Harzianum* ranged from 23.5-94.1% inhibition. All the methods evaluated successfully inhibited the pathogen compared to the control.

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1. Introduction

Potato (*Solanum tuberosum* L.) in the family Solanaceae, is a cosmopolitan staple food and cash tuber crop, that grows exceedingly well globally and is consumed by more than 1000 000 000 persons in 150 countries (Devaux et al., 2019; Mengui et al., 2019; Ehiobu et al., 2022; Tiwari et al., 2023). The

leading global potato-producing countries include China, India, and Russia (Muthoni et al., 2022). These countries have huge populations that will have to be properly fed and potatoes can come in very useful.

Thus, Ehiobu et al. (2022) revealed that potato cultivation has been increasing expressly because potato is one of the tuber crops that can compete with cereals in terms of productivity (potato gives 15 times more yield w/w per hectare than cereals) according to the United Nations. Potato is used mainly in the food industry (for making chips, crisps, vegetable relish/salad, canning, French fries, potato flour, potato dice, potato flakes, sauces, thickeners, and binders of soups) as well as raw material in industry (for brewing alcoholic drinks (e.g. vodka), livestock feed, in the textile industry/potato starch, as adhesives, and for making paper, boards, dextrin, and ethanol) (Horton, 1992; Gopal et al., 2006; Ogunsola and Aduramigba-Modupe, 2014; Riaz et al., 2022). Potato is a key crop that can be used to alleviate poverty and attain zero hunger globally. Potato is a complete food that contains enough nutrients, vitamins, and minerals.

However, potato production is highly constrained by the utilization of rudimentary farming practices, high incidence of pests and diseases, inefficient use of improved technologies (e.g. limited or no crop rotation and inability to take advantage of irrigation to enable year-round production), low soil fertility or declining soil fertility, high cost of inputs (like fertilizers, certified seed potato, and fungicides), poor access to credit (Ministry of Agriculture, 2006; Mengui et al., 2019; Mulema et al., 2021; Riaz et al., 2022). Production of potatoes can be given a boost if policymakers and funding agents can give it enough attention because the resource-poor farmers can barely change their status on their own.

Kankoranta (1996), Larkin and Lynch (2018), Singh and Singh (2018), and Kim et al. (2024) testified that potato yield can be negatively influenced by roughly 160 diseases. Amongst these diseases are 40 soil-borne diseases. Other constraints include post-harvest storage diseases, environmental changes, and climate change. Lee et al. (2019) lamented that 20–40% of potato yield is lost globally to pests and diseases annually despite the efforts being put in.

In India (second largest global producer), 15–22% of potato dry rot disease incidence was reported (Sagar et al., 2011). Adolf et al. (2020) reported that globally the annual potato yield loss is about €6 100 000 000. The quantitative potato yield loss ranges between 25–60% in cold storage (Masum et al., 2011; Du et al., 2012; Merlington et al., 2014; Chen et al., 2020). This yield loss could be the answer to overcoming global hunger if post-harvest pathogens are properly tackled as shown in this study.

Cullen et al. (2005), Xue and Yang (2021), Xue et al. (2023), Tiwari et al. (2023), Zongur (2024), and Kim et al. (2024) pointed out that globally, potato dry rot disease is caused by many *Fusarium* species which include *Fusarium oxysporum* f.sp. tuberosi, *Fusarium sambucinum*, *Fusarium solani*, *Fusarium graminearum*, *Fusarium coeruleum* (Libert), and *Fusarium proliferatum*. Tiwari et al. (2023) expounded that *Fusarium* species affect this crop in the field causing *Fusarium* wilt and in the store they cause dry rots.

Singh and Singh (2018) concurred that due to disease complexes, the common control measures employed worldwide (including the use of tolerant cultivars, crop rotation, and other practices; singly or collectively) have met with limited success. This is quite true disease complexes result in more damage to crops and complicate the work of breeding for resistance against most pathogens.

Fortunately, Riaz et al. (2022) reported that integrated disease management using individual or combined application of plant growth-promoting bacteria with commercial fertilizers can be effective. Proper cultural practices coupled with chemical seed-dressing (using 1200 ppm thiabendazole) can be used to manage diseases caused by *Fusarium* species (Leach and Nelson, 1975).

Singh and Singh (2018) agreed that effective disease management is essential to overcome these diseases. It is common knowledge that the principle of integrated disease management (involving chemical control in combination with bio-control agents), is the most efficient and eco-friendly way to effectively combat pathogens. The struggle against pests and pathogens is never one-off. The pests and pathogens keep evolving and adapting to the new measures being utilized to curtail their effects.

Aydın and İnal, (2018) reported that potato cultivars reacted differently to the dry rot agents *F. sambucinum* and *F. solani*, with cultivar Broke® showing more promise for selection as a potential source of resistant genes against the pathogens. With this obvious lack of highly resistant materials against this disease, an integrated approach will ultimately be our best sustainable strategy (Bojanowski et al., 2013; Xue et al., 2023).

Adolf et al. (2020), Xue and Yang (2021) and Xue et al. (2023) noted that the application of fungicides is still the most effective approach for the management of dry rots, but environmental considerations are increasing the pressure to use host resistance and other measures. Biocontrol agents (like *Trichoderma* spp. and *Pseudomonas aeruginosa*) are effective disease management agents (Gupta et al., 1999).

For instance, Aydın (2019) determined that *Trichoderma* species were effective at different levels against *F. sambucinum,* which causes potato dry rot disease. The most effective *Trichoderma* isolates were *T. viride* VG18, *T. asperellum* ÖT1, *T. harzianum* TZ16, *T. virens* KB31, and *T. inhamatum* KEB12 respectively. Additionally, commercial seed fungicides formulated using Fludioxonil (SC 100 g L^{-1}) and Azoxystrobin (SC 250 g g L^{-1}) when applied to potato tubers, revealed that Fludioxonil was more effective compared to Azoxystrobin and the biological control agents. Likewise, Orina et al. (2024) demonstrated that control of *Fusarium* species was more effective when benomyl was applied compared to Azoxystrobin.

Orina et al. (2024) reported that benomyl was the best agent against *F. sambucinum* and *F. solani* compared to the control. However, they reported that Azoxystrobin was the least inhibitory among the agents applied against dry rot agents which contradicts the findings of Aydin (2019). Zongur (2024) reported that the essential oils of *Beta vulgaris* successfully inhibited these *Fusarium* species.

Bojanowski et al. (2013) reiterated that previously thiabendazole was very effective against these fungi but resistant strains have developed against it coupled with the lack of adequate resistance in potato varieties against dry rotting. The application of different agents against these pathogens has to be carefully studied to avoid damage to the environment, and the health of man, animals, and plants. Besides, the rate of developing new effective control materials agents pathogens tends to lag behind the development of resistance against those presently being used.

This research was conceived with this foregoing information in mind. Thus, this research was carried out to put up solutions applicable against *Fusarium* dry rot of potatoes using chemical, plant extract, and biological control methods.

2. Material and Methods

2.1 Experimental site

The Faculty of Agriculture Laboratory complex (Plant Pathology Laboratory), Alex Ekwueme Federal University Ndufu-Alike, Abakaliki (6.069°N by 8.199°E) in Ebonyi State of Nigeria was used for this experiment. Many root and tuber crops (e.g. sweet potato, potato, cassava, and yams) are cultivated in this state. The area has lush vegetation and experiences high relative humidity for most of the year. The rainy season here lasts more than three-quarters of the year with high wind speeds.

2.2 Sourcing of *Fusarium* and *Trichoderma* species

Potato tubers were obtained from Bamenda, in the West Cameroons and Jos in the Plateau State of Nigeria, cleaned by washing with running tape water, dried and packaged in manila envelopes, sealed, and taken to the Laboratory for processing. Potato tubers were labeled/tagged taken from each of the samples and processed to isolate the associated fungi in the laboratory.

Potato dextrose agar (PDA) was prepared as recommended by the manufacturer (Lifesave™, USA), and then streptomycin sulphate (1 g L^{-1}) was added to it to prevent the growth of bacteria contaminants. The infected potato tubers were surface sterilized in 1% sodium hypochlorite solution for 10 minutes; based on the fact that tuber surfaces are bound to carry more pathogens than leaves and other tissues. Peeling the tubers, and surface sterilizing the peeled tubers before slicing off tissues for isolation could be very problematic. Using mild concentrations of surface disinfectants could not get rid of all the different classes of pathogens on tuber surfaces. Besides the pathogen of interest was expected to be inside the tuber. The surface sterilized tubers were then cut with infected portions and placed aseptically on each Petri dish containing PDA.

This general purpose medium usually yields many fungi isolates. The cut pieces were also placed on Acetate Differential Agar enriched with Dextrose (prepared similarly to PDA). This medium based on our experience yields many fungi isolates as well. The Actetate medium was originally meant for bacteria culture but it proved very useful for fungal isolation especially when isolating *Trichoderma*

and *Fusarium* species. The isolation of *Trichoderma* species was done using these standard media and it was similar to the isolation of any fungi agent.

Seven days were necessary for incubation of the plate at circa 29°C (Shahnaz et al., 2015; Khare et al., 2016). The emergent fungi were sub-cultured on PDA to obtain pure cultures. The fungi were identified by microscopy (ZEISS compound microscope), literature, and manuals on fungi (Barnett and Hunter, 1972). The fungal isolates were sent for confirmation of the identity of the species especially concerning the species epithet to the Crop Protection Department, Ahmadu Bello University Zaria. The identification was positively confirmed and tallied with ours.

2.3 Experimental design

2.3.1 Management of Fusarium dry rot of potato utilizing synthetic fungicides

The subtrial was conducted using a standard in the form of Ridomil (Ridomil gold plus at 2.5 g L^{-1}), Ketoconazole (2500 mg L^{-1}), Itraconazole (2500 mg L^{-1}), sulphur dust (2.5 g L^{-1}), and a Control. The synthetic fungicides utilized in this study were weighed using a mettler balance and dissolved in sterile distilled water. This rate was considered as the 100% concentration. The 50% concentration was made by diluting the 100% concentration using sterile distilled water.

The chemical agents were applied on the surface of the agar once the agar had set. The chemical agent was allowed to cover the whole surface of the plate then the excess chemical was removed with the pipette. The agar was allowed to dry in the airflow hood and no excess chemical that could be seen as runoff was permitted to remain in the agar surface.

The experiment was carried out in vitro using Petri dishes which were laid out using a completely randomized design with nine treatments that were replicated three times. Potato dextrose agar was used for this trial. The treatments included the following: Ridomil 100%, Ketoconazole 100%, Itraconazole 100%, sulphur 100%, Ridomil 50%, Ketoconazole 50%, Itraconazole 50%, sulphur 50%, and a Control. These treatments were applied once per petri dish and according to the replications of the treatments.

2.3.2 Effects of plant extract applied against Fusarium dry rot of potato

Aqueous extracts (at 100% concentration) containing candle bush (*Senna alata*) leaves (at 150.0 g L^{-1}) or locust bean (*Parkia biglobosa*) bark (at 150.0 g L^{-1}), and *Eucalyptus globulus* (i.e. blue gum) resin (at 70.0 g L^{-1}), and sweet alligator pepper (*Aframomum melegueta*) (at 70.0 g L^{-1}) were prepared per liter of distilled water. The plant tissues were collected from the university and its environs. The plant tissues were blended using a Warrington blender and the aqueous extracts were filtered using a double-layer sterile muslin cloth placed in a funnel over a beaker.

Potato dextrose agar was used for this trial. The set of treatments included 50% and 100% concentrations of the aqueous extracts of the plant materials (candle bush, locust bean, blue gum (i.e. *Eucalyptus globulus*), sweet alligator pepper, and a control (without plant extract). The nine treatments were replicated three times in the completely randomized design layout *in vitro*.

The plant extracts were applied on the surface of the agar once the agar had set. The plant extract was allowed to cover the whole surface of the plate then the excess extract was removed with the pipette. The agar was allowed to dry in the airflow hood and no excess plant extract that could be seen as runoff was permitted to remain in the agar surface.

Sweet alligator pepper (*Aframomum melegueta*) (in the ginger family Zingiberaceae) is also known as Grain of Peace. *Senna alata* is commonly called candle bush, ringworm cassia, ringworm shrub, or wild senna. *S. alata* is also called *Cassia alata. Eucalyptus globulus* (in the family Myrtaceae) is commonly known as southern blue gum or blue gum.

2.3.3 Effects of some biocontrol agents against potato dry rot pathogen

The experiment was carried out in Petri dishes which were laid out using a completely randomized design with three replications for each treatment. Thus, there were four treatments which were each replicated in triplicates. The set of treatments consisted of the following isolates: *T. harzianum* isolate BGMZ3, *T. harzianum* isolate BGMZ4, *T. harzianum* isolate NSBM, and a Control.

Potato dextrose agar was used for this trial. The isolates were applied using a cork borer. The cork borer was flamed over a bunsen burner flame and used to cut the colony which was placed at the edge of the petri dish/plate. This method ensured that fairly equal amount of propagules for the trial.

2.4 Data collection and analyses

The radius of the fungus colony was measured at 24-hour intervals from the first to the seventh day during each of the three sub-experiments. The analysis of variance (ANOVA) was utilized to determine the significance of the data and the means were ranked using Duncan's multiple range test (DMRT) ($p \leq 0.05$) as available in the Genstat 2nd Edition Discovery version. Equation (1) was employed to calculate percentage inhibition:

$$PI = [(CT-TC)/CT)]*100 \quad (1)$$

Where
PI = percentage inhibition of the pathogen (%)
CT = mean radius of the control plot (measured through the center of the plate)
TC = mean radius of the treated plot (measured through the center of the plate) (Ndifon, 2023).

3. Results and Discussion

3.1 Effects of synthetic fungicides applied against *Fusarium* species.

The results revealed that Ketoconazole (at 100% concentration) was the best fungicide which was consistently able to control ($p \leq 0.05$) dry rot agent compared to the other treatments (Figure 1). This excellent performance was followed by that of Ketoconazole-50%, Itraconazole-100%, Itraconazole-50%, Sulphur-100%, Ridomil-100%, Sulphur-50%, and finally Ridomil-50%.

All the synthetic fungicides (at both 50% and 100% concentrations) were significantly different ($p \leq 0.05$) compared to the control. The percentage inhibition of the growth of *Fusarium oxysporum* by synthetic chemicals ranged from 21.7-100% with time. All the potential chemical fungicides applied against dry rot disease agents were very effective.

These findings agree with those of Aydin (2019) who reported that Fludioxonil controlled the pathogen of dry rot more compared to Azoxystrobin and *Trichoderma* species. Likewise, Ndifon (2024) showed the efficacy of Ketoconazole when combined with *Trichoderma* species to combat the effects of *Agroathelia rolfsii* which corroborates these present findings on the excellent performance of ketoconazole.

Leach and Nelson (1975) revealed that thiabendazole was able to reduce diseases caused by *Fusarium* species which confirms the fact that chemical agents can inhibit these *Fusarium* agents effectively. Thus, Ab Rahman et al. (2017), Adolf et al. (2020), and Riaz et al. (2022) reiterated that the application of synthetic pesticides remains the main method employed to mitigate infection by almost all potato diseases. They emphasized that these pesticides significantly contribute to environmental damage and lead to pesticide resistance by pathogens when the pesticides are abused.

For example, Bojanowski et al. (2013) pointed out that sufficient resistance against potato dry rot agents is lacking coupled with the discovery that these pathogens have developed resistance against thiabendazole which was relied on for years. A need for diverse agents and methods to combat potato dry rots is prominent to prevent the development of resistance to effective agents. This current study has shown that diverse control agents can be equally effective against *F. oxysporum.*

Presently we saw that chemical fungicides caused inhibition of *Fusarium* sp. by 39.1-95.7% with time during this trial which corroborates these findings. The table for percentage inhibitions is not presented herein to avoid any implied double presentation or so. The calculation was carried out using Equation 1 for those who want to confirm the figures presented here.

Md-Mahi and Nayem (2023) utilized Mancozeb 80% WP against late blight of potato but they observed that plant infection increased to 71.7% from the prevailing 22.0% infection rate. They utilized Lycomax™ to effectively control both late blight and early blight, while Mancozeb 80% WP was potent against early blight only. This shows that the efficacy of agro-fungicides varies with the pathogen and the type of chemical as was shown in this current research.

Aydın and İnal (2018) reported the presence of some limited resistance in potato cultivar Broke against potato dry rot agent in Turkey which is a sure sign of some hope that integrated management can be carried out using such limited resistance. In the absence of full resistance against pathogens causing dry rot of potatoes, we may depend confidently on the application of the principle of integrated management.

Draper et al. (1994) reported that Chlorothalonil fungicides (i.e. an ortho multi-purpose fungicide) offer the best control of early blight, while copper fungicides are less effective, providing only fair control. Ndifon and Inyang (2022) observed that solutions of mancozeb (at 100% concentration) and mancozeb+carbendazim (at 50% and 100% concentrations) were more effective in suppressing the development of pathogenic *Lasidioplodia theobromae* compared to a combination of mancozeb+metalaxyl+copper. Thus, tank mixtures of fungicides do not always give additive effects.

Waterworth (2023) said azoxystrobin, chlorothalonil, mancozeb, pyraclostrobin, and pyraclostrobin can be applied against potato blights. These chemical fungicides are still being relied on by farmers.

3.2 Effects of plant extracts applied against potato dry rot disease agent

The results revealed that *Eucalyptus* sp. (at 100% concentration) was the best plant extract which was consistently significantly different ($p \leq 0.05$) compared to the other treatments (Figure 2). This excellent performance was followed by that of *Eucalyptus* sp.-50%, Sweet alligator pepper-100%, locust bean-100%, Sweet alligator pepper-50%, *Senna alata*-100%, locust bean-50%, and *Senna alata*-50%.

All the plant extracts (at both 50 and 100% concentrations) were significantly different ($p \leq 0.05$) compared to the control. The plant extracts caused 20.6-100% inhibition of this *Fusarium* species with time. The effective control by plant extracts lasted for 192 hours compared to the synthetic chemicals which lasted just 120 hours after inoculation.

The efficacy of plant extracts revealed that *Senna alata* was able to control the pathogenic fungus. Chatterjee (1990) reported that the oils of *Cassia* sp. and clove inhibited the growth of *Aspergillus flavus*, *Curvularia pallescens,* and *Chaetomium indicum* isolated from maize, which corroborated the findings on candle bush or *Cassia alata / S. alata* and other plant extracts.

Ehiobu et al. (2022) reported that significant antifungal activity against *Fusarium solani*, (the causal agent of potato rot disease) was obtained using *Eucalyptus camaldulensis* and five other plant extracts exhibited *in vitro* and *in vivo* which confirmed the findings on *Eucalyptus* sp. and other plant extracts tested herein.

Ndifon and Inyang (2022) inhibited the growth of *Lasidioplodia theobromae* by 70% or more using *Eucalyptus* sp. (at 100% concentration), *Ricinus* soap (at 50% and 100% concentrations), and *Guieria* sp. (at 100% concentration) which affirmed these present findings on the use of plant extracts to manage fungi infections.

Amienyo and Onuze (2015) reported that *Lantana camara* controlled the growth of *Phytophthora infestans* and *Aspergillus solani* in potatoes by 50%. Abdu et al. (2020) in Nigeria (both *in-vitro* and *in-vivo*) proved effective inhibition of potato fungal diseases (caused by *Aspergillus flavus, Thielavia terricola, Rhizopus stolonifer*, and *Scoupulariopsis brevicaulis*) using garlic oil, neem oil, and *Tridax* leaf ash.

Adolf et al. (2020) propounded the use of live garlic plants during intercropping for the management of late blight potato disease. These sources all show the potential of plant extracts, as antimycotic materials against potato diseases. Zongur (2024) revealed that *Beta vulgaris* essential oils inhibited *Fusarium* species which affirms the present findings that plant extracts were effective against *F. oxysporum*.

Özcan et al. (2024) reported that *Mentha piperita* and *Thymus vulgaris* (essential oils) completely inhibited the radial growth of *Aspergillus carbonarius*. Chatri et al. (2024) revealed that leaf extracts from *Muntingia calabura, Terminalia cattapa, Syzygium oleina, Dimocarpus longan*, and *Artocarpus altilis* effectively inhibited the growth of *S. rolfsii* compared to the control. These two trials using medicinal plant corroborated our findings on the potency of some medicinal plants against plant pathogens.

3.3 Effects of *Trichoderma* species against fungi

The results revealed that *T. Harzianum* isolates BGMZ4 was the best isolate which was significantly different in comparison with other assayed treatments (Figure 3). This excellent performance was followed by that of *T. Harzianum* isolate NSBM and then *T. harzianum* isolate BGMZ3. These *T. harzianum* isolates performed significantly better than the control. The isolates of *T. harzianum* (23.5-94.1% inhibition with time) effectively controlled the pathogen *in vitro*.

Adolf et al. (2020) reported that only a few biological control measures are used by non-organic growers of potatoes due to their slow efficacy, farmers' lack of knowledge about them, and lack of access to the most efficient biocontrol agents. These results agree with the findings of Ndifon and Inyang (2022) who reported that four isolates of *T. harzianum* inhibited the radial growth of *L. theobromae* by 8.0–100%.

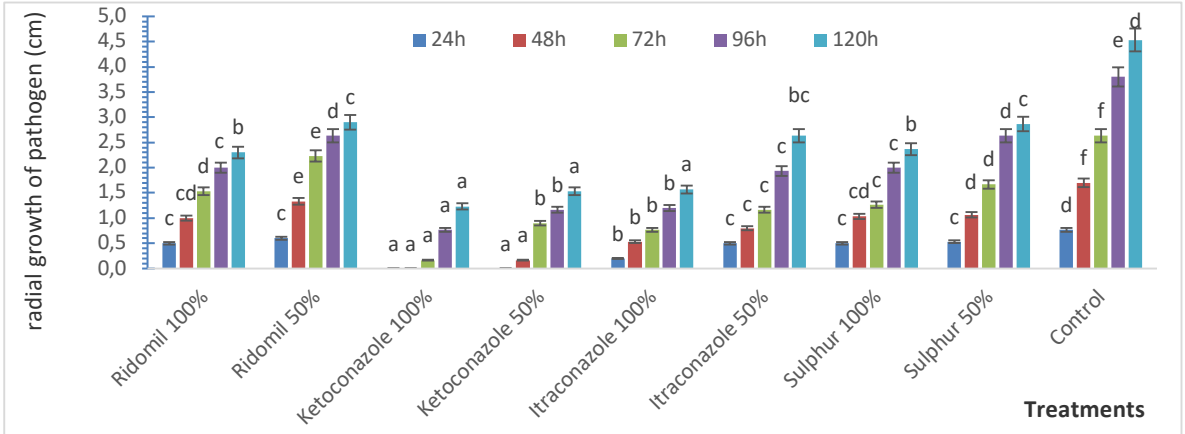
Ayed et al. (2007) and Ehiobu et al. (2022) reported that *Bacillus subtilis* var. amyloliquefaciens and *T. harzianum* inhibited the growth of *Fusarium oxysporium* from potatoes. Md-Mahi and Nayem (2023) in Bangladesh reported on the efficacy of *T. harzianum* against late blight and early blight of potatoes. These findings are in tandem with the findings of this present study.

Gupta et al. (1999) observed that *Trichoderma* spp. and *Pseudomonas aeruginosa* could successfully reduce the effects of pathogenic agents which concurs with the present findings using isolates of *Trichoderma* species. Meanwhile, Ndifon (2024) successfully utilized *T. viride, T. virens,* and *T. harzianum* isolates to inhibit *S. rolfsii* which agrees with the finding of this study herein on the use of *Trichoderma* species against pathogens.

Thus, Riaz et al. (2022) stated that the bacteria agents (like *Pseudomonas* sp., *Pantoea*, *Enterobacter*, *Bacillus cereus*, *Bacillus cepacia*, *Pseudomonas fluorescens),* and *T. harzianum*, play a major role in the mitigation of potato rot diseases. *Pseudomonas syringae* in the USA controls dry rots and silver scurf in potatoes (Al-Mughrabi et al., 2016). Seed priming with *Bacillus subtilis* formulation resulted in an approximately 56% reduction of potato common scab (Al-Mughrabi et al., 2016), which is quite promising.

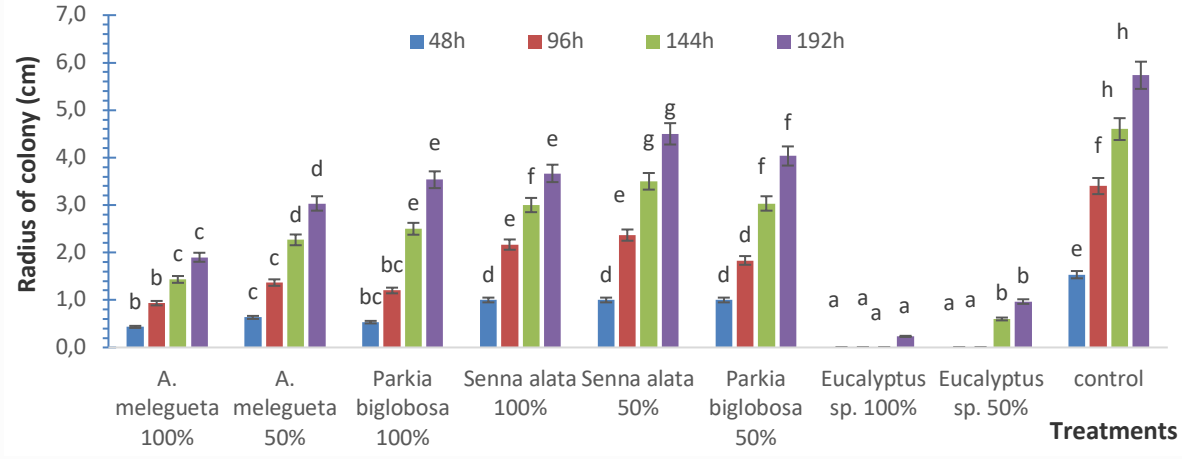
Meanwhile, Riaz et al. (2022) pointed out earlier that plant-growth-promoting bacteria are successful in reducing the growth of this fungus. In Siberia, potatoes and raspberries require maximal usage of biological agents (*Bacillus velezensis* strains) for plant protection (instead of chemicals) against *Rhizoctonia solani* (Lugtenberg, 2018; Asaturova et al., 2021).

Recently, Özakın et al. (2021) observed that eight isolates (out of 11 local isolates) belonging to Actinobacteria (mostly member of genus *Streptomyces*) exhibited antimicrobial activity against *Candida albicans* and other bacteria pathogenic species assessed. This finding is in agreement with the current study.



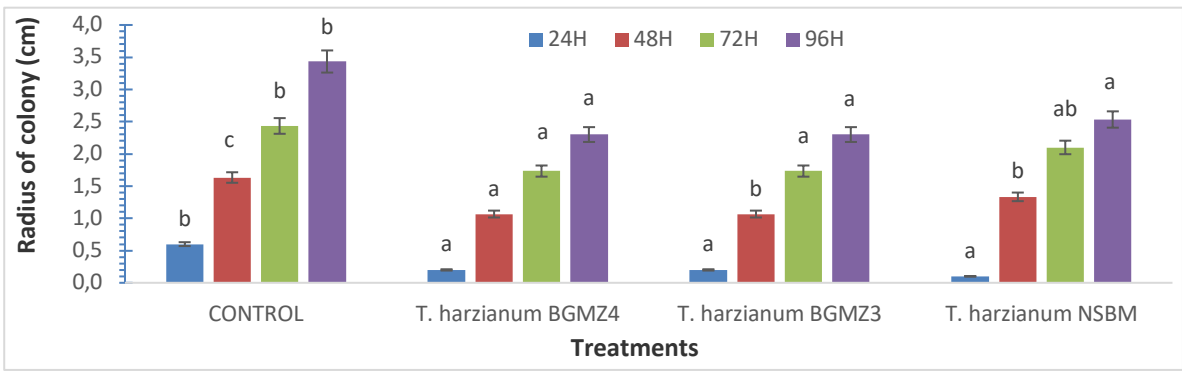
*The means of the bars in a series with similar letter(s) above them are statistically at par based on their ranking using DMRT ($p \leq 0.05$)

Figure 1. The effect of synthetic chemicals on the radial growth of *Fusarium oxysporum.*



*The means of the bars in a series with similar letter(s) above them are statistically at par based on their ranking using DMRT ($p \leq 0.05$)

Figure 2. The impact of plant extracts on the growth of *Fusarium* species.



*The means of the bars in a series with similar letter(s) above them are statistically at par based on their ranking using DMRT ($p \leq 0.05$)

Figure 3. The effect of isolates of *Trichoderma harzianum* on the radial growth of *Fusarium oxysporum*.

4. Conclusion

Potato production is a cosmopolitan activity that is carried out to obtain the tubers but this activity is consistently hindered by diseases. Three sub-trials were set up *in vitro* to determine the efficacy of different measures against potato dry rots. This study revealed that biocontrol agents (*Trichoderma harzianum* isolates), plant extracts (Blue gum, Sweet alligator pepper, and Candle bush), and synthetic chemicals (Ketoconazole, Itraconazole, Sulphur, and Ridomil) can effectively control dry rot of potato induced by *Fusarium oxysporum* f.sp. tuberosi. This study indicates that these control agents could be recommended against *Fusarium* dry rot of potatoes. Researchers should further determine the effects of these disease management materials against other fungi, bacteria, viruses, and nematodes associated with potatoes. This will help in avoiding disease replacement and the development of resistance due to suboptimal application of pesticides against these pathogens.

Ethical Statement

Ethical approval is not required for this study because the study is on plant diseases and the control agents are not poisonous to humans.

Conflict of Interest

The Author(s) declare(s) that there are no conflicts of interest.

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Author Contributions

EN: conceived the topic, designed it, carried it out, analysed and interpreted the data, wrote the article, edited it, proof read it.

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Research Article

Impact of Vermicompost and Different Plant Activators on Yield and Some Quality Parameters in Pumpkin (*Cucurbita pepo* L.)

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Abstract: In addition to being used as a snack, pumpkin seeds are an industrial product. It also has the potential to be used in the food, pharmaceutical, and cosmetic industries. Seed yield and quality are traits of economic importance. This study aims to determine the effect of vermicompost and different plant activators on the yield and some parameters of the pumpkin's quality. For this purpose, three plant activators [(ISR-2000 (I), Symbion-Vam (S), and Green-Miracle (G)] together with vermicompost (V) have been used. The experiment was conducted in the field of the Cukurova University Pozantı Agricultural Research and Application Center, Turkey. A total of 8 applications were made. Conventional fertilizer (CF) application was determined as the control group. The results showed that the applications increased the snack pumpkin's fruit, seed yield, and quality. The highest fruit and seed yield was obtained from CF (37.2 t ha^{-1}, 101.42 $g^{-1}m^2$) application, followed by V+I (27.1 t ha^{-1}, 80.09 $g^{-1}m^2$) application. Additionally, CF applications resulted in the highest fruit width (14.82 cm), length (23.31 cm), seed width (10.21 mm), and length (20.66 mm) of internal weight (74.33 %) measurements. Regarding mineral element and phenolic content, higher results were obtained when vermicompost and plant activators were combined. According to the study results, different doses of vermicompost may be recommended as an alternative to conventional fertilizer application in future studies.

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1. Introduction

Cucurbitaceae is a prominent family of vegetable and fruit crops containing approximately 125 genera and 960 species. Vegetables of the *Cucurbitaceae* family are section ancient medicine and culinary traditions (Mukherjee et al., 2022). Pumpkins (Cucurbita spp.) are a substantial economic crop in the Cucurbita. The most commonly cultivated species of the *Cucurbitaceae* family in Turkey are the *Citrullus lanatus* Thunb., *Cucumis flexuosus* L., *Cucumis sativus* L., *C. maxima* Duch., *C. moschata* Duch. and *C. pepo* L (Ermiş and Yanmaz, 2022). Pumpkin, an essential part of human nutrition, is an edible vegetable (Prommaban et al., 2021). Each piece of the pumpkin vegetable has been correlated with one or more applications in food and health (Sharma et al., 2020). Pumpkin seeds are produced from fruit processing as a by-product containing different phytochemicals such as phenolic compounds,

polyunsaturated fatty acids, vitamins, minerals, and carotenoids (Noroozi et al., 2021). Also, it is an excellent source of both oil and protein. When the amount of snack pumpkin confectionery production in Turkey between 2018-2022 is examined, there has been instability in production (TUIK, 2023). Plant nutrition and fertilization are essential in cultivating snack pumpkins to increase the amount of product to be taken from the unit area and obtain quality products. Therefore, it was stated that organic fertilizer applications should be included in addition to chemical fertilizer applications. Using organic fertilizers, like compost and vermicompost, is an efficient way to increase and protect the soil's organic matter and provide the valuable nutrients necessary for the plants (Amiri et al., 2017). Vermicompost is derived from organic wastes with the help of earthworms (Yatoo et al., 2021). Vermicompost is used in agricultural applications to increase plant yield and quality in plants (Aksu et al., 2017), suppress diseases and pests (Yatoo et al., 2021), and improve soil properties (Ding et al., 2021). Plant activators are used to increase yield and quality (Göktekin and Ünlü, 2016), increase resistance to diseases and pests (Aysan et al., 2019), and act as soil conditioners (Artyszak and Gozdowski, 2020). This study aims to determine the effect on the yield and some quality parameters of snack pumpkin fruit and seed in the case of vermicompost and plant activators.

2. Material and Methods

2.1. Trial area and climate data

The field trial was conducted at the Cukurova University Pozantı Agricultural Research and Application Center (Adana, Türkiye). Soil characteristics of the trial area: At the beginning of the study, soil samples were collected in April 2019 and analyzed in the Cukurova University Faculty of Agriculture, Department of Soil Science and Plant Nutrition laboratory. It was determined that the soil used in the field contained a salt-free organic matter content of 1.8%, a pH of 7.16, and some macro and microelements in specific amounts (Table 1). After analysis of the soil samples taken from the field, the content and quantity of conventional fertilizer application were determined.

Table 1. Physical and chemical properties of soil used in the study

Soil Characteristics	Value
K (kg ha^{-1})	4770 ± 1.7
P (kg ha^{-1})	18.3 ± 0.13
Fe (mg kg^{-1})	10.61 ± 0.38
Mn (mg kg^{-1})	16.41 ± 0.86
Zn (mg kg^{-1})	1.47 ± 0.13
Cu (mg kg^{-1})	3.91 ± 0.01
Organic Matter (%)	1.8
pH	7.16 ± 0.04
EC (mS cm^{-1})	0.18 ± 0.00
CaCO3 (%)	0.70 ± 0.01
Sand (%)	18.1
Clay (%)	31.9
Silt (%)	63.0

Climate data for the experimental field are presented in Table 2. The highest average temperature in 2019 was recorded in August, and the highest in 2020 was recorded in July. The average humidity was highest in April "(67.84) in 2019 and March (65.60%) in 2020. "Total precipitation was highest in March in both years" (Table 2).

Table 2. Climatic characteristics of the experiment area for the year 2019-2020

Year	Month	Minimum Temperature (^{0}C)	Maximum Temperature (°C)	Average Temperature (°C)	Average Relative Humidity (%)	Total Precipitation (mm)
2019	March	1.92	12.89	6.80	63.58	2.56
	April	5.17	15.66	9.66	67.84	2.27
	May	11.09	25.26	17.93	52.14	0.57
	June	15.75	29.24	21.62	59.20	1.77
	July	17.72	31.27	24.13	44.14	0.02
	August	18.83	32.65	25.11	44.32	0.03
	September	14.16	28.28	20.62	46.38	0.64
2020	March	2.63	13.95	8.09	65.60	3.50
	April	5.67	18.51	11.67	60.17	1.69
	May	10.93	23.44	16.77	54.26	1.30
	June	14.45	27.61	19.93	57.32	1.59
	July	18.42	34.16	26.36	42.75	0.01
	August	18.77	33.15	25.54	31.74	0.32
	September	15.53	33.08	24.32	44.45	0.01

2.2. Plant materials and experimental treatments

The current study used the Çağlayan pumpkin cultivar (Obtained from Ahmet Erkan Company located in Turkey; http://erkantarim.com/) as the plant material. ISR-2000 (I), Green Miracle (G), and mycorrhiza (Symbion-Vam) (S) were used as plant activators. In addition, vermicompost (V) and conventional fertilizer applications (CF) were included within the scope of this study.

2.3. Vermicompost and plant activator description

The vermicompost used in this experiment was made of organic plant materials, changing the physical and chemical structures of *Lumbricus rubellis* and *Eisenia foetida* earthworm species. Some chemical properties of vermicompost were determined: humidity, 72.05 (%); organic matter, 61.11 (%); nitrogen, 1.95 (%); C/N, 18.18 (%); lime, 8.7 (%); pH, 7.54; Electrical conductivity, 3.29 (mS cm^{-1}).

I: Contains *Lactobacillus acidophilus*, yeast extract, Yucca plant extract, and benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH). When ISR-2000 is applied, the receptors on the plant send a signal as if they have detected the presence of a pathogen, activating the defense system to resist attacks. It is a plant activator that provides natural resistance against disease factors and stress conditions in herbal production, stimulates and activates the natural defense system of the plant with its components, and is commercially produced (Çıngı Tarım, Türkiye).

G: Contains 80% vegetable fatty acids and is commercially available as an emulsion concentration (EC) (Agrobest, Türkiye). It is a highly effective plant activator in liquid form. It is a reflection-type antitranspirant and a surface coating agent that prevents sweating, heating, and water loss in plants. It is a balanced combination of vegetable fatty acids, amino acids, surface tension reducers (surfactants), and organic biological stimulants.

S: Include *Glomus fasciculatum*, *Glomus intraradices*, *Glomus mosses*, nitrogen bacteria (*Azotabacter*), phosphorus bacteria (*Bacillus megaterium*), and potassium bacteria (*Frateuria aurantia*), which are sold commercially. They play essential roles in plant root growth (Agrobest, Türkiye). It eliminates the problems caused by factors that limit plant nutrition in the soil, such as high pH and excessive salinity. It increases the plant's defense mechanism and resistance against the adverse effects that undesirable climate conditions, such as excessive heat and excessive cold, will create on the plant. It stores additional water and nutrients for the plant. Application doses of vermicompost and plant activators were made according to the recommendations of the companies.

CF: NPK (N:100 kg ha^{-1} P_2O_5: 50 kg ha^{-1} K_2O: 200 kg ha^{-1}) fertilized the plants. CF application was determined as the control group.

Vermicompost and plant activator in their combination as follows:

V+I: 100 g plant^{-1} +1 L / 100 L

V+S: 100 g plant^{-1}+ 10 kg ha^{-1}

V+G: 100 g plant^{-1} + 200 ml/100 L

V+G+S: 100 g plant^{-1} + 200 ml/100 L+10 kg ha^{-1}

V+G+I: 100 g plant^{-1} +200 ml/100 L+ 1 L/100 L

V+I+S: 100 g plant^{-1} + 1 L / 100 L +10 kg ha^{-1}

V+I+S+G: 100 g plant^{-1} +1 L / 100 L+10 kg ha^{-1} +200 ml/100L

V: 100 g plant^{-1}

CF: N:100 kg ha^{-1} P_2O_5: 50 kg ha^{-1} K_2O: 200 kg ha^{-1}

The experiment was conducted in a randomized complete block design with four replications of each treatment. Çağlayan pumpkin cultivar was sown the second week of May with plant-plant and row-row distances of 70 cm and 70 cm, respectively. The size of each treatment was 6.86 m^2. Seedling planting was carried out on 22 May 2019 and 29 April 2020.

2.4. Morphological measurements

The fruit was harvested on 15 August 2019 and 13 August 2020. After harvesting, their weights were taken, and then morphological measurements were made. After completing the measurements on the fruit, the seeds were removed from the fruit and dried (for approximately ten days), and both morphological measurements were made on the seeds. Finally, biochemical analyses were carried out on the seeds.

Fruit and yield per treatment: The data was recorded in grams with the help of scale and then converted into kg per hectare. Fruit weight, width, and length as "cm" were inscribed. For measurement purposes, five fruits were randomly selected from each application. Seed width and length were recorded as "mm." For fruit measurement purposes, five fruits were randomly selected from each application. Seed width and length were recorded as "mm." For seed measurement, fifty seeds from four replications were chosen randomly. The seed's internal weight was measured as %.

2.5. Chemical analysis in seed

Seed samples were dried in an oven at 50 - 55 °C for approximately a week. After drying, the samples were ground to powder. The samples were kept in the muffle furnace at 550 °C for approximately 7-8 hours. According to Kacar (1972), solutions were obtained from the samples taken from the furnace. Macroelements (Potassium (K), Phosphorus (P), Magnesium (Mg), and Calcium (Ca) and microelements (Zinc (Zn), Manganese (Mn), Iron (Fe), and Copper (Cu) concentrations were measured using Inductively Coupled Plasma (ICP) devices. Total phenolic content: First, fat was extracted from the seeds. The phenolic content was determined using a spectrophotometer according to the Folin-Ciocalteu method made by (Ayaz et al., 2017).

Statistical Analysis: The data obtained were analyzed using the JMP package program. They were subjected to variance analysis, and the statistical differences between the averages were grouped at a 5% significance level by the LSD test, and correlation analysis was performed.

3. Results

According to the statistical analysis, the averages of 8 different applications made within the trial were to be significant. However, the applications increased both fruit and seed yields in snack pumpkins. The highest fruit and seed yield was obtained from CF application (37.2 t ha^{-1}, 101.42 g^{-1}m^2). This application was followed by the V+ I application (27.1 t ha^{-1}, 80.09 g^{-1}m^2). The highest measurement in fruit width, length, seed width, and length of internal weight measurements was taken from CF applications (Table 3).

Table 3. Findings of total fruit yield, fruit weight, fruit width (cm), fruit length (cm) and fruit circumference (cm), total seed yield ($g^{-1}m^2$), seed internal weight (%), seed width, seed length as a result of vermicompost and some plant activator applications regarding pumpkin

Applications	Total fruit yield (t ha $^{-1}$)	Fruit width (cm)	Fruit length (cm)	Fruit diameter (cm)	Total seed yield ($g^{-1}m^2$)	Seed internal weight (%)	Seed width (mm)	Seed length (mm)
V+I	27.1 b[1]	13.21 bc	20.87 b	42.9 bc	80.09 b[1]	73.15 ab	9.76 ab	19.97 ab
V+S	22.2 bcd	12.42 cd	19.47 bc	40.49 cd	52.28 c	70.78 ab	9.32 bc	18.54 cd
V+G	26.0 bc	13.73 b	19.85 bc	44.47 b	73.99 b	73.66 ab	9.21 c	19.76 abc
V+G+S	21.8 bcd	12.51 cd	19.11 c	40.55 cd	53.06 c	74.27 a	9.35 bc	19.01 bcd
V+G+I	20.7 cd	12.24 d	18.71 c	39.35 d	42.21 c	66.70 ab	9.03 c	18.30 d
V+I+S	22.9 bcd	12.73 cd	19.61 bc	41.29 bcd	53.61 c	66.35 b	9.18 c	18.69 bcd
V+I+S+G	19.3 d	11.89 d	18.71 c	38.39 d	40.61 c	73.61 ab	8.97 c	18.10 d
V	22.8 bcd	12.41 cd	19.28 c	40.45 cd	53.17 c	70.05 ab	9.31 bc	18.67 bcd
CF	37.2 a	14.82 a	23.31 a	48.92 a	101.42 a	74.33 a	10.21 a	20.66 a
$LSD_{0.05}$	0.22***	0.65***	1.74***	7.88***	256.48***	41.46*	0.19**	1.19*

V; Vermicompost, I; ISR-2000, S: Symbion-Vam, G; Green miracle, CF; Conventional fertilizer application.
Statistical differences between means shown with separate letters in the same column are significant.***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$.

The difference between vermicompost and plant activator application averages was statistically significant regarding nutrient content in snack pumpkins. K, P, Mg, and Ca were dominant in nutrient element analyses. It was determined that the seed's K, P, and Mg contents were more effective in V+S application. The V+I application was more effective in Ca content. The lowest measurements of macro and microelements were generally taken from CF applications. Microelements have been dominant in different applications. As a result of the applications, the total phenol content in the confectionary pumpkin seeds showed a distribution between 20.08 and 31.65 (mg GAE g^{-1}). This distribution obtained the maximum total phenol content from the V+G+I application. The lowest total phenol content was obtained from the V+I+S application (Table 4).

Table 4. Macro and microelement, phenolic content (mg GAE g^{-1}) content obtained from the seed as a result of vermicompost and some plant activator applications (g mL^{-1})

App.	K	P	Ca	Mg	Cu	Fe	Mn	Zn	Total phenolic
V+I	75.44 c[1]	44.90 b	9.76 a	30.55 b	0.18 bc	1.07 a	0.60 b	1.24 ab	24.17 de[1]
V+S	84.90 a	52.05 a	8.44 b	32.77 a	0.12 c	0.94 bcd	0.70 a	1.25 ab	23.70 e
V+G	74.31 c	43.57 bc	7.82 bc	24.59 cd	0.15 c	0.95 bcd	0.54 bcd	1.17 b	25.04 cd
V+G+S	83.82 ab	47.05 b	8.21 b	26.40 c	0.25 ab	0.97 bc	0.52 bcd	1.25 ab	23.03 e
V+G+I	79.50 abc	43.41 bc	8.29 b	25.26 cd	0.32 a	0.94 bcd	0.51 cd	1.23 ab	31.65 a
V+I+S	83.65 ab	39.14 cd	6.86 c	23.98 d	0.32 a	1.00 ab	0.54 bcd	1.27 ab	20.08 f
V+I+S+G	76.22 c	37.62 d	7.25 bc	23.46 d	0.33 a	0.86 de	0.57 bc	1.34 a	28.97 b
V	77.80 bc	35.10 d	8.42 b	21.17 e	0.26 ab	0.90 cd	0.54 bcd	1.33 a	27.82 b
CF	56.51 d	18.35 e	7.95 bc	13.47 f	0.21 bc	0.81 e	0.47 d	1.19 b	25.45 c
$LSD_{0.05}$	26.96***	16.44***	2.59***	2.05***	0.00***	0.00***	0.00*	0.01***	1.04***

Statistical differences between means shown with separate letters in the same column are significant. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$.

When the correlation between the yield and the quality parameters was evaluated ($P < 0.01$), it showed that total fruit yield (t ha^{-1}) had a significantly positive correlation with fruit width (cm) ($r=0.96$; $P<0.0001$), fruit length (cm) ($r=0.85$; $P<0.0001$), fruit diameter (cm) ($r= 0.89$; $P< 0.0001$), total seed yield ($g^{-1}m^2$) ($r=0.70$; $P<0.0001$), seed internal weight (%) ($r=0.20$; $P=0.08$), seed width (mm) ($r=0.48$; $P<0.0001$) and seed length (mm) ($r=0.38$; $P<0.0007$). However, a negative correlation was observed between total fruit yield and a negative correlation with some macro and microelements. Correlation and significance values between nutrients and total yield; K, ($r=-0.14$; $P=0.21$), Ca, ($r=-0.07$; P=0,54), P ($r=-0.19$; $P=0.10$), Mg ($r=-0.15$; $P=0.18$) Cu ($r= -0.14$; $P=0.23$), Fe ($r= -0.06$; $P=0.58$), Mn ($r=-0.13$; $P=0.24$).

Total seed yield was not correlated with K and Mg, but there was a positive correlation with Ca ($r=0.16$; $P=0.15$) and a negative correlation with P ($r=-0.04$; $P=0.72$). While total seed yield showed a positive correlation with Zn ($r=0.12$; $P=0.50$), Mn ($r=0.06$; $P=0.60$), and Fe ($r=0.16$, $P=0.17$), there was a negative correlation with Cu ($r=-0.19$; $P=0.10$). When the correlation relationship between the nutritional elements was evaluated, there was a positive and strong correlation between all elements except Cu. Total Phenolic content was negatively correlated with total fruit yield ($r=-0.10$; $P=0.39$) and positively correlated with total seed yield ($r=0.04$; $P=0.69$). The correlation with the nutrient elements

was positive and strong with all the elements except Cu. This study observed significant positive correlations between total phenolic content and other mineral elements [K (r=0.74; P<0.0001), Ca (r=0.68; P<0.0001), P (r=0.73; P<0.0001), Mg (r=0.72; P<0.0001), Fe (r=0.79; P<0.0001), Mn (r=0.73; P<0.0001), Zn (r=0.83; P<0.0001)] except the Cu element (r=-0.11; P=0.34).

4. Discussion

As a result of the applications, there has been an increase in fruit and seed yield in snack pumpkins. The data obtained regarding fruit yield are consistent with those reported in previous studies (Altun, 2017; Günhan, 2020). However, these results differ from previous studies (Durukan et al., 2019; Rahimi et al., 2019; Üçok et al., 2019). The reason for this can be attributed to the plant material used, climatic conditions, different dosages and contents of vermicompost (Dayan and Sarı 2019), and the activities of microorganisms in the plant activators used in the study (Basu et al., 2022). The findings of some researchers (Saket et al., 2014; Santos et al., 2018; Sadegh et al., 2020) regarding seed yield support the conclusions of this study. However, other studies have obtained different results Ceritoglu and Erman (2020). This difference may be because the amount of vermicompost used in the current study was low, resulting in less translocation and accumulation of yield components such as proteins and carbohydrates in the reproductive organs (Saket et al., 2014). Also, vermicompost doses have a significant relationship with plant growth (Singh et al., 2012; Blouin et al., 2019).

The K, P, Mg, and Ca contents were higher according to the data obtained from macro and micronutrient element analyses. The data obtained were compatible with the results of some researchers (Erdinç et al., 2018; Martinec et al., 2019). However, different results have been obtained in some studies regarding the amounts of these elements (Seymen et al., 2016; Devi et al., 2018). Different ecological conditions, genotypes, and soil and seed maturation periods are possible reasons for these different results. Studies have emphasized that leaf senescence plays a role in transferring macro- and micronutrients to seeds (Gregersen et al., 2013; Dass et al., 2022). While the result obtained was similar to the result of Meru et al. (2018), it was different from the findings of Seymen et al. (2016). The phenolic content obtained in this study was higher than those reported in other studies. Saavedra et al. (2015), 0.95 – 3.43 mg GAE g^{-1}; Boujemaa et al. (2020) total phenol content, 13.70 mg EAG g^{-1}; Peng et al. (2021), 2.44–3.82 mg GAE g^{-1}. In terms of phenolic content, the differences between the findings of the current research and the literature can be attributed to the climatic factors (Kabtni et al., 2020), extraction methods (Saavedra et al., 2015), and cultivar (Seymen et al., 2016).

Correlation analysis was performed to determine the relationship between the investigated parameters. Total fruit yield was positively correlated with fruit width, fruit length, fruit diameter, total seed yield, seed internal weight, seed width, and seed length. These findings from the current study were consistent with previous studies (Nagar et al., 2017; Seymen et al., 2019; Yetişir and Aydın 2019). Total seed yield analysis correlates positively with K, Mg, and Ca. But negative correlation with P. While total seed yield showed a positive correlation with Zn, Mn, and Fe, there was a negative correlation with Cu. These findings may be related to the ability to chelate ion metals by polyphenol compounds (Rehecho et al., 2011). The balance between the nutritional content of the applications made causes these applications. However, the concentration of nutrients can also be effective (Karaköy et al., 2012). It is necessary to conduct detailed research on the correlation between the elements and the yield of snack pumpkins. Total phenolic content was negatively correlated with total fruit yield and positively correlated with total seed yield, with the nutrient elements being positive and strong with all the elements except Cu. Present results demonstrated that current levels of mineral elements except Cu affected the total phenolic content as the correlations were primarily positive. These findings agree with our previously published result (Aras, 2022). However, these results differ from previous studies (Sulaiman et al., 2011; Ngamdee et al., 2016). It can be explained by the gene expression between nutrients and antioxidant compounds (Lillo et al., 2007).

Conclusion

Under the scope of the trial, the Çağlayan pumpkin cultivar was subjected to vermicompost and different plant activators. The applications made were practical in the snack pumpkin, and there was an increase in fruit and seed yield and quality. The highest measurement in fruit width, length, seed width,

and length of internal weight measurements was taken from CF applications. The V+ I application followed this application. Better results were obtained from conventional fertilizer application than vermicompost application, possibly because the vermicompost dose used in the study was insufficient. K, P, Mg, and Ca were dominant nutrients in snack pumpkins. V+S application is more effective in increasing the seed's K, P, and Mg contents, while V+I application is more effective in boosting Ca content. On the other hand, different applications were practical in microelements. In general, CF applications produce the lowest measurements of macro and microelements the maximum total phenol content obtained from the V+G+I application.

Correlation studies indicated that yield was significantly and positively correlated with all the fruit and seed characters under study. However, there were both positive and negative correlations between total fruit yield, total seed yield, and total phenolic compounds and nutrients. From this study, depending on the producer's preference, the vermicompost and plant activator (I) combination can be used within the scope of sustainable agriculture as an alternative to conventional agriculture. According to the study results, the application of different doses of vermicompost may be recommended as an alternative to conventional fertilizer application in future studies.

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

All contributions to this article were made by the corresponding author.

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Research Article

Evaluation of Sesame (*Sesamum indicum* L.) Lines Under Salt Stress for Seed Yield Using SSR Markers

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Abstract: Salinity has undesirable effects on sesame yield. In order to reduce salt's harmful effects, sesame tolerance needs to be increased. Twenty-three lines of sesame were irrigated with saline water (70 and 90 mM NaCl) and evaluated based on seed yield over two seasons (2019–2020). Genotypes were evaluated in a randomized complete block design (RCBD) with three replications. Ten SSR molecular markers were used to evaluate these lines for salt tolerance. Genotypes showed significant differences ($p < 0.05$) and recorded a wide range of seed yields under optimum and salinity conditions. Four lines (C1.5, C2.2, C8.4, and C9.15) achieved the highest average performance for seed yield compared to other lines under salinity conditions. Ten SSR markers revealed 15 alleles, ranging from 1 to 4 alleles. The polymorphism information content (PIC) ranged from 0.00 to 0.44. The range of expected heterozygosity (He) was 0.00 to 0.444. The UPGMA dendrogram analysis divided all sesame genotypes into two main clusters. In addition, SSR 3 and SSR 6 markers elucidated the possibility of using them in breeding programs for enhancing salt tolerances in sesame cultivars. These lines may be used as a salt-tolerant source in future breeding to create new sesame cultivars.

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1. Introduction

The sesame (*Sesamum indicum* L.) crop has many advantages, including stress tolerance, its composition of amino acids matches animal protein, thus its seed protein is outstanding to other oil crops (Boureima et al., 2011). Sesame seeds are contained protein, vitamin B1, manganese, phosphorous, copper, calcium, manganese, iron and zinc, and fiber, so their seed is a high nutritional value. The oil contains sesaminol and sesamin lignans that play an important role in the activity of tocopherols and other antioxidants (Lee et al., 2008).

Sesame is more adaptable to a broad range of soil types (Islam et al., 2016). This quality raised sesame as an attractive crop specially designed for challenging climatic changes (Li et al., 2018). Although

salt stress is a serious factor affecting productivity (Bahrami et al., 2016; Zhang et al., 2020). Meanwhile, the shortage of freshwater resources in Egypt poses a major threat to agricultural production in the present and the near future. Moreover, Egypt imports most of its vegetable oils. In general, cultivated sesame in Egypt encounters several stress factors including salinity and drought, which limited its productivity. So, growing sesame cultivars that can withstand salinity is a very significant option to fix this problem and reduce oil imports. Moreover, increasing the area planted for sesame crops to contribute to covering the need for edible oil (El-Hamidi and Zaher, 2018). Also, the development of plants that can withstand salt supports sustainable agriculture and offers a longer strategy to manage salt-affected soils and less impact on seed quality and yield becoming a hard mission for breeders (Qin et al., 2020).

To assess sesame genetic variability and identify new genetic sources for biotic and abiotic stress tolerance, phenotypic and molecular analyzes are combined (Bekele et al., 2017; Bose et al., 2017). There are genetic factors that control the diversity in sesame responses to salt stress. Consequently**,** the detection of QTLs and candidate genes associated with these characters will be important to speed the development of abiotic tolerance breeding in sesame. (Dossa et al., 2017). Therefore, many various molecular markers were used to estimate the genetic diversity of sesame and to detect associated genetic markers with salt-tolerance traits (Dossa et al., 2016; Wei et al., 2016; Asekova et al., 2018; de Sousa Araújo et al., 2019; Stavridou et al., 2021).

Among molecular markers, SSR markers are characterized by multi-allele nature, co-dominant inheritance, distribution in the genome, and reproducibility (Baruah et al., 2019). In comparison to other genetic markers, SSR markers provide more information about genetic diversity (Wei et al., 2014; Baruah et al., 2019). Dossa et al*.* (2016) identified 91 SSR markers related to the AP2/ERF genes in sesame. These SSR markers are useful for marker-assisted selection (MAS) to improve sesame toward abiotic stresses.

Our study aimed to evaluate new lines of sesame based on seed yield under salt stress and using SSR markers to confirm the salinity tolerance of these lines.

2. Material and Methods

2.1. Description of the study area

Genotypes were evaluated based on seed yield ha^{-1} under two concentrations of sodium chloride (70 and 90 mM) in an open field in sandy conditions, and we used two tanks (1 m^3) for irrigation and we used drip irrigation. Irrigation was weekly. The experiments were applied for two years (2019 and 2020) at the Research and Production Station, National Research Centre, Al-Nubarya, El-Behira Governorate (latitude 30° 30\ N, and longitude 30° 19\ E, and mean altitude 21 m above sea level).

2.2. Breeding materials

The twenty-three new lines of sesame were obtained from the Department of Agronomy, Faculty of Agriculture, Cairo University, Egypt (Table 1.). C1.3, C1.5, C1.6, C1.8, C1.9, C1.10, C2.2, C2.3, C2.6, C3.4, C3.8, C5.7, C6.3, C6.5, C6.7, C6.9, C8.4, C8.8, C8.11, C9.6, C9.7, C9.15, and C9.20 are the names of the lines. And two check cultivars (cv. Shandweel and Sohag) were obtained from the Ministry of Agriculture and Land Reclamation, Egypt.

2.3. Design of an experiment

Randomized complete block design (RCBD) with 3 replicates was utilized for each concentration. Plots are composed of 1 row that is 1.5 m long, spaced 70 cm apart, and planted at a distance of 10 cm. Reservoirs were supplemented with NaCl (10 m^3) to irrigate the field's rows. The recommendation of the Ministry of Agriculture was used. Seed yield ha^1 of the samples from the three replications' net areas (1.04 m^2) was collected. Recommended agricultural practices were used to cultivate the genotypes. According to Saber (2015), Table 1 displayed the parents' descriptions. Variance analysis was calculated by the computer program MSTAT-C (MSTAT-C program, 1991).

Table 1. Source of new sesame lines according to their breeding status and parents' traits

Parents	Breeding status	Source of seeds *	Specific traits
P1 (HM19)	F_8-hybrid pop	Cairo Univ.*	Early maturity, three capsules per axil, first capsule set low, non-branching, resistance to *Fusarium Oxysporum is very high.*
P2 (EUL90)	Mutant line	Cairo Univ.*	Non-branching, premature, the base of first capsule low, three capsules per axil, moderately resistant to *Fusarium Oxysporum.*
P3 (Mutant48)	Mutant line	Cairo Univ.*	Branching, high susceptibility to *Fusarium oxysporum,* number capsules per axil three
P4 (Giza 32)	Local variety	Ministry of Agric.& Land Reclamation, Egypt	Heavy seed weight, medium branching, one capsule/axil, long capsule, late maturity, resistance of *Fusarium Oxysporum* is moderate
P5 (NM59)	Exotic line	India through IAEA**	*Fusarium oxysporum*-resistant, rigid stem, late maturity, one capsule/axil
P6 (Babil)	Exotic variety	Iraq through IAEA**	Decreased branching, semi- shattering capsules, 3 capsules/axil, resistant to *Fusarium Oxysporum*

*Advanced breeding materials resulted from the breeding program conducted at Agron. Dept. Fac. Of Agric. Cairo Univ.
**Inter. Atomic Energy Agency.

2.4. Genotypic analysis

Nine lines from 23 were selected for SSR analysis according to mean performance for seed yield ha^{-1} under salt stress. The following nine lines: C1.5, C1.6, C2.2, C3.8, C6.3, C6.5, C8.4, C8.8, and C9.15 to be compared with two check cultivars (Shandweel and Sohag) of sesame. Utilizing the DNeasy Plant Mini Kit and the manufacturer's recommendations, DNA was extracted from young fresh leaves (Qiagen). Genomic DNA was loaded in 0.8% agarose gel and separated by electrophoresis for 60 min at 100 volts.

2.4.1. SSR-PCR analysis

Ten SSR primers (Table 2.) were used for the amplification among eleven sesame genotypes to be utilized as markers for screening sesame lines differing in salinity response. In this study were identified four SSR primers (from SSR 1 to SSR 4) based on the salt-responsive candidate gene (cg-SSR) which was published by Li et al. (2018). Using an online SisatBase database, (http://www.sesame-bioinfo.org/SisatBase/) and (http://www.sesame-bioinfo.org/PMDBase) following BLAST (https://blast.ncbi.nlm.nih.gov/). While the other 6 primers (from SSR 5 to SSR 10) were selected from 91 SSR markers from a published source (Dossa et al., 2016).

Each 10µL of PCR mixture for the amplification of SSR bands consisted of 5 µL (2X) of KAPA2G Fast Ready Mix[2] (KK5101), a 0.5µL of forward primer, a 0.5µL of reverse primer, a 1µL of DNA template and H2O up to 10 µL. Amplification was performed on a Primus thermal cycler, programmed for 37 cycles as follows; Initial denaturation, 95°C/4 min (one cycle), denaturation 94°C/1 min, annealing, 58°C /45 sec, extension 72°C/ 1.5 min (35 cycles), final extension, 72°C/10 min (one cycle), then kept at 4°C until use. The amplification product was separated on agarose (3%) by electrophoresis. The UV-transilluminator filter was used to see the DNA bands in the gel. A digital imaging device was used to take pictures of the bands. Solis BioDyne 100 bp DNA Ladder (07-11-00050) was employed as a size marker. M (100 bp Ladder DNA), 1 (C1= Shandweel), 2 (C2 = Sohag), 3 (C1.5), 4 (C1.6), 5 (C2.2), 6 (C3.8), 7 (C6.3), 8 (C6.5), 9 (C8.4), 10 (C8.8) and 11 (C9.15) respectively.

2.4.2. Analysis of gel images

Gel images were analyzed using Total lab TL 120 to determine the molecular size of amplified fragments. Amplified fragments were classified as present (1) or absent (0). Polymorphic Information Content (PIC), Expected Heterozygosity (He), and Effective Multiplex Ratio (EMR) values were determined using the online program (https://irscope.shinyapps.io/iMEC/) according to Amiryousefi et al. (2018). The NTSYS program was used to construct the dendrogram (Rohlf, 2000).

Table 2. SSR primers, gene name, candidate gene ID, forward sequence, and reverse sequence

SSR no.	Gene Name	Candidate Gene ID	Forward sequence	Reverse sequence
SSR 1	SiGPAT3	SIN_1007701	ACAAAGCTCACGAGGAAGGA	CATGCACTTTTACCGCAGTG
SSR 2	SiMLP31	SIN_1021337	CCAACTCGTCCGCACATAAT	ATGCCACCCAAGAAATTGAG
SSR 3	SiGRV2	SIN_1001572	CGTCGAATCATATTGGAGCA	GTGAACTTGAAGGCCTCTGC
SSR 4	SiGRF5	SIN_1024695	TACAGGCACACCAGAAACCA	ATGAGTGGTGGTGGGAGAAG
SSR 5	AP2si2	SIN_1009557	CCGTCGTGCTCGTCTTCT	CGGATTCAGCCACCCTTC
SSR 6	AP2si11	SIN_1013899	CTCCTCATCGGACTCTTC	GCGTCTTCATTCCCACT
SSR 7	AP2si16	SIN_1017978	TCTTGCGAATTAGAAGGC	ACTCACATTTATTACCACCATC
SSR 8	AP2si90	SIN_1010530	TCCATCGTCCTCCCATCA	AAACATCGCCTCCTCGTC
SSR 9	AP2si106	SIN_1008520	CTCCACCTCTTCGCCGTCTG	CGCCCTTATCATCTTCTTCTGC
SSR 10	AP2si116	SIN_1003959	CACAGCCGTGTACTACCTCC	TGCCGCCTTCTTCCTTAT

3. Results and Discussion

3.1. Mean performance and variance

Table 3 shows the results of a statistical analysis of the sesame genotypes for seed yield under various conditions. Sesame genotypes varied significantly ($p > 0.05$) in terms of seed productivity. And they showed a wide range of seed yield under different conditions, suggesting that some of these genotypes may be tolerant to salt conditions, which reflected positively on selection for salinity tolerance. Similar results were reported by Bahrami et al. (2016), Anter and El-Sayed (2020), and Dangue et al. (2022). The variable performance of genotypes was due to their genetic make-up, which caused the lines to respond differently when exposed to salt levels, similar results were noted by Suassuna et al. (2017).

In this study, line C5.7 achieved the highest seed yield (1171.8 kg ha^{-1}) followed by C1 (1079.7 kg ha^{-1}) and C2.6 (1004.4 kg ha^{-1}) under normal conditions.

On the other hand, we found that the four lines C1.5, C2.2, C8.4, and C9.15 performed better than the control cultivars (C1 and C2) and other lines in terms of seed yield in salt conditions. These lines may sense the expression of salt-stress-responsive genes, which regulate processes including detoxification, ion transport, and osmotic balance. Numerous regulatory elements, including phytohormones, lipids, the cell wall, and the cytoskeleton, are used by these mechanisms (Van et al., 2020; Gong, 2021).

According to CR% values (rate decrease in seed yield under salinity conditions compared to seed yield under normal conditions), fives lines C8.4, C8.8 C3.8, C6.3, and C8.11 were less affected by salinity conditions compared to check cultivars despite of were less seed productive under normal condition.

In general, the seed yield of all genotypes was affected by salinity conditions. The adverse effects of poor irrigation water quality on genotypes are evident may be due to the inhibition some of biochemical, and physiological processes, and ion imbalance (Dias et al., 2017; Shahid et al., 2020). In addition, the line's ability to absorb nitrogen is reduced under salinity conditions (Saha et al., 2015).

Table 3. Seeds yield ha^{-1} of sesame genotypes under normal and salinity conditions

Genotypes	70mM NaCl	90mM NaCl	Mean seed yield under salinity conditions ($\bar{X}$)	Seed yield under normal condition	Change rate in seed yield under salinity conditions (CR %)
C1.3	160.5±2.21	130.5±1.83	145.5	948.6±12.6	84.7
C1.5	187.0±2.65	152.0±2.13	169.5	788.6±10.5	78.5
C1.6	177.5±2.49	144.4±2.03	160.9	669.6±8.9	76.0
C1.8	151.1±1.14	123.1±0.93	137.1	558.5±7.4	75.5
C1.9	134.4±1.89	109.7±1.54	122.0	651.0±8.6	81.3
C1.10	158.9±1.2	129.5±0.98	144.2	684.4±9.1	78.9
C2.2	192.2±2.7	156.2±2.19	174.2	892.8±11.8	80.5
C2.3	167.0±2.34	136.0±1.91	151.5	688.2±9.1	78.0
C2.6	161.6±2.77	131.2±2.25	146.4	1004.4±13.3	85.4
C3.4	122.9±0.93	99.9±0.75	111.4	703.0±9.3	84.2
C3.8	171.2±2.4	138.8±1.95	155.0	587.7±7.8	73.6
C5.7	113.1±0.85	92.2±0.7	102.6	1171.8±15.5	91.2
C6.3	176.4±2.47	143.7±2.02	160.0	613.8±8.1	73.9
C6.5	171.6±1.3	139.2±1.05	155.4	892.8±11.8	82.6
C6.6	150.2±1.13	122.5±0.93	136.3	613.8±8.1	77.8
C6.7	127.7±0.96	103.8±0.78	115.7	628.6±8.3	81.6

Table 3. Seeds yield ha^{-1} of sesame genotypes under normal and salinity conditions (continued)

Genotypes	70mM NaCl	90mM NaCl	Mean seed yield under salinity conditions ($\bar{X}$)	Seed yield under normal condition	Change rate in seed yield under salinity conditions (CR %)
C8.4	191.8±3.29	156.2±2.68	174.0	591.4±7.8	70.6
C8.8	178.6±3.06	144.9±2.48	161.8	610.0±8.1	73.5
C8.11	170.1±2.92	138.7±2.38	154.4	598.9±7.9	74.2
C9.6	160.7±2.75	130.6±2.24	145.6	747.7±9.9	80.5
C9.7	149.3±2.56	121.2±2.08	135.2	788.6±10.5	82.9
C9.15	182.3±1.38	148.2±1.12	165.8	967.2±12.8	82.9
C9.20	120.0±2.06	97.4±1.67	108.7	788.6±10.5	86.2
C1	120.5±3.0	124.5±4.5	122.7	1079.7±14.1	88.6
C2	100.0±2.7	104.1±1.17	102.1	900.0±12.0	88.7
Significant level **(P<0.05)**	88.0	63.1	-	490.0	-
Coefficient of variation (CV%)	5.3	3.0	-	14.8	-

±: Stander error, C1: Shandweel, C2: Sohag.

3.2. Rank genotypes

To make a good judgment on the extent to which the current study materials are affected by environmental conditions, we ranked genotypes based on mean performance for seed yield, ranks mean, and stander deviation under different conditions (Table 4.). Lines with low overall rankings ($\bar{X}$) were regarded as generally adaptive to salinity conditions and distinguished from others. Abate (2015) pointed out that.

Table 4. The rank of sesame genotypes under normal and salt conditions

Genotypes	70mM NaCl	90mM NaCl	Seed yield ha^{-1} (kg) under normal condition	Ranks mean ($\bar{X}$)	Standard deviations (Sd)
C1.3	14.0	14.0	5.0	11.0	5.2
C1.5	3.0	4.0	8.0	5.0	2.6
C1.6	6.0	6.0	15.0	9.0	5.2
C1.8	5.0	17.0	25.0	15.7	10.1
C1.9	19.0	20.0	17.0	18.7	1.5
C1.10	15.0	16.0	15.0	15.3	0.6
C2.2	1.0	1.0	7.0	3.0	3.5
C2.3	11.0	11.0	14.0	12.0	1.7
C2.6	12.0	12.0	3.0	9.0	5.2
C3.4	21.0	23.0	13.0	19.0	5.3
C3.8	9.0	9.0	25.0	14.3	9.2
C5.7	24.0	25.0	1.0	16.7	13.6
C6.3	7.0	7.0	19.0	11.0	6.9
C6.5	8.0	8.0	9.0	8.3	1.0
C6.6	17.0	18.0	20.0	18.3	1.5
C6.7	20.0	22.0	18.0	20.0	2.0
C8.4	2.0	2.0	23.0	9.0	12.1
C8.8	5.0	5.0	21.0	10.3	9.2
C8.11	10.0	10.0	22.0	14.0	6.9
C9.6	13.0	13.0	12.0	12.7	0.6
C9.7	18.0	19.0	10.0	15.7	4.9
C9.15	4.0	3.0	4.0	3.7	0.6
C9.20	23.0	24.0	11.0	19.3	7.2
C1	22.0	16.0	2.0	13.3	10.3
C2	25.0	21.0	6.0	17.3	10.0

C1: control 1 (Shandweel cultivar), C2: control 2 (Sohag, cultivar).

Line C2.2 achieved a low-rank mean (3.0), and a low value of standard deviation (3.5), and was ranked first under salinity conditions while being categorized as seventh under normal conditions. As shown by rank mean and low standard deviation, it also showed little variation in relative performance across environments. Line C9.15 was ranked fourth at 70 mM, and third at 90 mM. It has been categorized as fourth under normal conditions. It also achieved a low-rank mean (3.7) and a low value of standard

deviation (0.6). Line C1.5 is ranked third at 70 mM and fourth at 90 mM and it achieved a low-rank mean of 5.0 and a middle value of the standard deviation of 2.6 and it was ranked eighth under normal conditions.

The results highlighted the ability of these lines to adapt to salinity conditions. These lines may possess useful genetic factors to increase salt tolerance, as suggested by Zhang et al. (2019) in rice crops. Additionally, these lines may be able to resist water stress and/or be tolerant to ion toxicity as indicated by Shrivastava and Kumar (2015), and/or they may produce osmols such as organic acids, soluble sugars, free amino acids and increased accumulation of potassium ions (Parvaiz et al., 2012). Here appear the role of the crops breeder, through the combination between salt tolerance and a high seed yield potential as bio-ameliorators.

3.3. Genetic polymorphism of the SSR markers

Molecular markers are used in breeding programs to improve their efficiency and effectiveness. Simple sequence repeats (SSRs) are a considerably effective technique for identifying crop varieties (Raghunath, 2022). Numerous studies were performed on the tolerance of drought and salt stresses in sesame including those by Bazrafshan and Ehsanzadeh, (2014), (2016) and Dossa et al. (2016). In addition, several studies indicate the important role of the AP2/ERF family of transcription factors (TFs) in plant biotic/abiotic stress tolerance (Akhtar et al., 2012; Mizoi et al., 2012; Chen et al., 2022). Furthermore, Dossa et al. (2016) determined 91 SSR markers related to the AP2/ERF genes in sesame. Li et al. (2018) found 27 candidate genes for salt responses helpful for enhancing salt tolerance in sesame cultivars.

Therefore, we used 10 SSR markers, from SSR 1 to SSR 4 were identified based on the salt-responsive candidate gene (Li et al., 2018), these SSRs were distributed on four chromosomes (chr5, chr2, chr4, and chr11), respectively. This in harmony with those obtained by Sharma et al. (2021) used the wheat genome to generate 177 heat-responsive gene-based SSRs for heat tolerance. The other six markers used in this study were selected according to Dossa et al. (2016) for evaluating the genetic variability of new lines for salinity tolerance.

The results revealed amplified fragments ranging from 66 bp to 1250 bp through the 10 SSR markers among 9 lines and two cultivars of sesame (Table 5.). Out of 10 SSRs screened, only two SSRs were polymorphic (SSR 3 and SSR 6) primers (20% polymorphism) as shown in Figure 1. This indicates that the number of polymorphic SSR primers was very low in this study. This point is agreed with and supported by Pandey et al. (2015) reported that only eight primers from thirty-six East-SSR were used to identify the accessions. Likewise, Ramprasad et al. (2017) used 75 SSR primer pairs, only 20 were polymorphic (29.4% polymorphism). The level of polymorphism was higher in our study compared to an earlier report by Yepuri et al. (2013) they found only 12 % of 156 primers polymorphic in a set of 49 sesame accessions consisting of germplasm.

A total of 15 alleles among the 11 sesame sample were observed (Table 5.). Each marker produced 1 to 4 alleles, with an average of 1.5 alleles per locus. The highest alleles number per locus was observed for SSR 3 (4 alleles) followed by SSR 6 (3 alleles). These results were lower than those reported by Pandey et al. (2015) reported that the number of alleles ranged from 2 to 6 alleles, with an average of 3.37 alleles per locus.

The PIC value varied from 0.00 to 0.35 with an average of 0.039. Similar results were also reported by Teklu et al. (2021) who found that the highest value of PIC in sesame was 0.37. The average of PIC (0.039) is lower than the values of 0.25 and 0.82 revealed by Teklu et al. (2021) and Stavridou et al. (2021) used 27 SSR markers and 28 EST-SSR markers to assess 100 and 35 sesame genotypes, respectively.

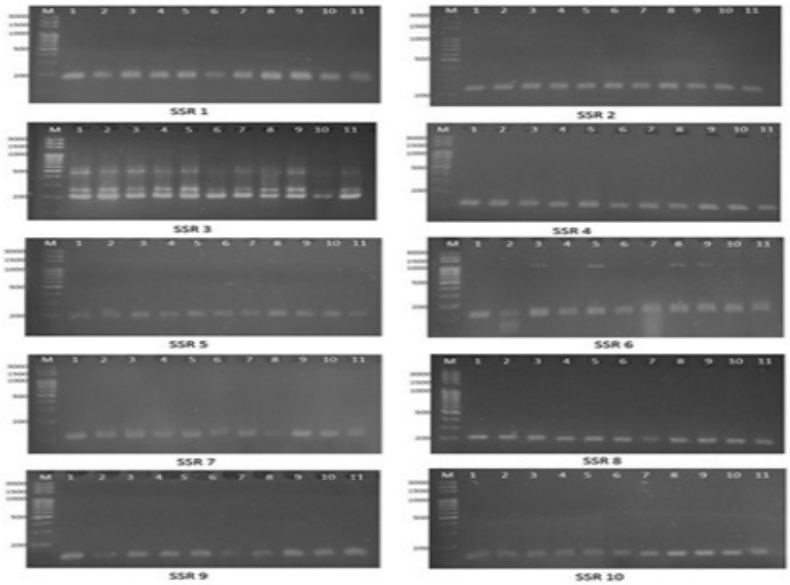

Figure 1. Amplification profile of SSR markers for eleven sesame genotypes.

To describe genetic diversity, expected heterozygosity (He) is usually used (Chesnokov and Artemyeva, 2015). In our study, the (He) ranged from 0 to 0.444 with an average of 0.049. This value was higher than reported by Ramprasad et al. (2017) who found the (He) ranged from 0.00 to 0.2162, with a mean of 0.0465 in 41 sesame genotypes. The mean expected heterozygosity (0.049) was lower than the value of 0.30, 0.34, and 0.72 reported by Teklu et al. (2021), Asekova et al. (2018), de Sousa Araújo et al. (2019) when evaluating 100, 129 and 36 sesame accessions using 27, 23 and 10 SSR markers, respectively.

Effective multiplex ratio (EMR) equals the total number of polymorphic loci for each primer multiplied by the rate of polymorphic loci from the total number (Nagaraju et al., 2001). The effective multiplex ratio varied from 1(SSRs 1, 2, 4, 5, 7, 8, 9, and 10) to 3.9 (SSR 3) with an average of 1.39.

Table 5. The results obtained from amplification with SSR markers

SSR no.	No. of alleles	He	PIC	EMR	Product size bp
SSR 1	1	0	0	1	175
SSR 2	1	0	0	1	234
SSR 3	4	0.044	0.043	3.9	223-614
SSR 4	1	0	0	1	126
SSR 5	1	0	0	1	207
SSR 6	3	0.444	0.346	2.0	66-1250
SSR 7	1	0	0	1	111
SSR 8	1	0	0	1	196
SSR 9	1	0	0	1	116
SSR 10	1	0	0	1	147
Total	15	0.488	0.389	13.9	
Average	1.5	0.049	0.039	1.39	

He: expected heterozygosity, PIC: Polymorphic Information Content, EMR: Effective multiplex ratio.

In the above, the details of the ten SSR primers were mentioned. But when excluding the monomorphic primers, we found the average values of PIC, He, and EMR for only two polymorphic primers (SSR3 and SSR 6) were 0.195, 0.244, and 2.95 respectively. In addition, the average number of alleles was 3.5 alleles per locus. SSR 3 showed the highest number of total bands (4) and the effective multiplex ratio (3.9), whereas SSR 6 gives the highest value of expected heterozygosity (0.444) and PIC values (0.346). This indicates that SSR 6 is more informative because the higher values of expected heterozygosity (He = 0.444) evidence that there is more allelic variation (Gaballah et al., 2021). And the PIC values (0.346) between 0.25 and 0.5 imply moderate levels of polymorphism for SSR 6 (Botstein et

al., 1980). The obtained results imply that SSR6 followed by SSR3 was more efficient in evaluating the new line for salinity stress in sesame.

Generally, these results show low genetic variation among genotypes because of the use of less number of primers. Or due to the selection of the SSRs linked to salinity tolerance, not random SSRs. A similar finding was also reported by Mir et al. (2012) and Shafi et al. (2021) detected less diversity by trait-specific SSRs compared to random genomic SSR markers in wheat.

3.3.1. Cluster analysis of genotypes

The dendrogram was created based on the binary data obtained from the SSR marker-based DNA profiles of the genotypes examined (Figure 2.). Among 10 SSR markers, SSRs 3 and 6 were able to distinguish the genotypes into two main clusters based on their salinity tolerance. The first cluster involved C8.8 only. The second cluster was split into two sub-clusters, the first of which had Shandweel, C1.6, and C6.3, whereas the other of which had two sub-sub-clusters. Sub-sub-clusters I included only C2. On the other hand, sub-sub-clusters II consisted of the last six lines C1.5, C2.2, C9.15, C3.8, C8.4, and C6.5.

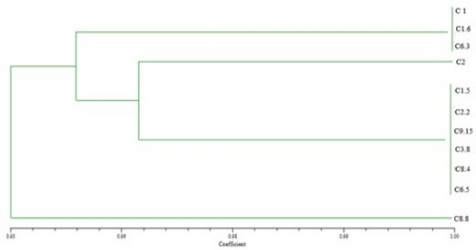

Figure 2. UPGMA dendrogram of the eleven genotypes based on the genetic similarity matrix.

Conclusion

The study showed sesame lines differed significantly in terms of seed yield ha^{-1} under different conditions, which indicated the possibility of obtaining genotypes that are tolerant to salinity conditions. Four lines C1.5, C2.2, C8.4, and C9.15 recorded the higher seed yield ha-1 under salinity conditions. Two lines C5.7 and C2.6 recorded the higher seed yield under normal conditions. SSR markers especially SSR 3 and SSR 6 were effective in screening salt tolerances in sesame cultivars. These markers would be useful in sesame breeding towards abiotic stresses. Finally, if we want to combine the results from field and genetic analysis there was an obvious similarity between the four lines C1.5 C2.2, C8.4, and C9.15 which reflect more tolerance to salinity and clustered in one group according to the UPGMA dendrogram.

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